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Anbu Poosakkannu

Endosphere Microbial Community Assemblage of an Inland Sand Dune Colonizing Plant

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ABSTRACT

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Plant-associated microbes could play a role in plant colonization of sand dune ecosystems, but microbes associated with plants colonizing those ecosystems in the arctic are poorly known. I characterized *Deschampsia flexuosa*-associated microbiomes in two successional stages (early and late) of arctic inland sand dune differ in their plant species richness and soil physiochemical properties. The work based on culturable microbes showed that different plant parts harbour generalist and specific groups of endosphere microbes and most of the endosphere bacteria were closely related to other cold habitat microbes. Also, most of the endosphere bacteria possessed an important plant growth promoting property of solubilizing organic phosphate. Next generation sequencing methods showed that endosphere microbial species richness was determined by soil characteristics (succession) and plant compartment. Successional stage strongly affected the microbial community composition. Further, reciprocal transplantation experiment showed that endosphere microbial species richness was determined by successional stage rather than transplantation type (self or reciprocal). Irrespective of successional stage, after reciprocal transplantation microbial community compositions in most of the leaf and root compartments differed from local non-transplanted control. In contrast, the microbial community composition only in few root compartments was affected by self-transplantation. Further, leaf endosphere bacterial community composition was significantly affected by arbuscular mycorrhizal fungal (AMF) inoculation under greenhouse conditions. Overall, my work provided data from poorly characterized arctic biota and novel insight into endosphere bacterial and fungal community assemblage in arctic inland sand dune ecosystem. These results could be utilized when restoring vegetation in sand dunes and similar extreme ecosystems.

Keywords: Succession; transplantation; endosphere microbes; arbuscular mycorrhiza; sand dunes; arctic.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their roman numerals I-IV.

I Poosakkannu, A., Nissinen R. & Kytöviita, M.-M 2015. Culturable endophytic microbial communities in the circumpolar grass, *Deschampsia flexuosa* in a sub-Arctic inland primary succession are habitat and growth stage specific. *Environmental Microbiology Reports* 7: 111–122.

II Poosakkannu, A., Nissinen R. Männistö, M. & Kytöviita, M.-M 2016. Microbial community composition but not diversity changes along succession in arctic sand dunes. *Environmental Microbiology*, in press.

III Poosakkannu A., & Kytöviita M.-M 2016. Endosphere microbial legacy after plant transplantation in arctic sand dunes. Manuscript.

IV Poosakkannu, A., Nissinen R. & Kytöviita, M.-M 2016. Native arbuscular mycorrhizal symbiosis changes leaf endosphere bacterial community composition in *Deschampsia flexuosa*. Manuscript.

The table shows the contributions to the original papers.

	I	II	III	IV
Planning	AP, RN, MMK	AP, RN, MM, MMK	AP, MMK	AP, RN, MMK
Data	AP, RN, MMK	AP, RN, MMK	AP, MMK	AP, MMK
Analyses	AP, RN, MMK	AP, RN, MMK	AP, MMK	AP, MMK
Writing	AP, RN, MMK	AP, RN, MM, MMK	AP, MMK	AP, RN, MMK

AP = Anbu Poosakkannu, RN = Riitta Nissinen, MM = Minna Männistö, and MMK = Minna-Maarit Kytöviita

1 INTRODUCTION

1.1 Plant-associated microbes

The microbes that live in close association with plants are known as plant-associated microbes. They are mainly divided into phyllosphere (leaf surface), rhizosphere (root surface) and endosphere (inside plant) microbes. The plant-associated microbes mainly include bacteria and fungi. Many of these microbes have neutral or positive impact on host plants such as improved growth and protection from different biotic and abiotic stresses (Haney et al. 2015, Ludwig-Müller 2015, Panke-Buisse et al. 2015, Hansen and Moran 2014, Karasov et al. 2014). Recent advances in next generation sequencing techniques enhanced understanding the plant-associated microbes at comprehensive community level (Gottel et al. 2011, Bulgarelli et al. 2012, Peiffer et al. 2013, Bulgarelli et al. 2015, Edwards et al. 2015, Coleman-Derr et al. 2016). These studies mainly focused on tropical and temperate agricultural ecosystems and show that rhizosphere microbes are much more diverse than endosphere microbes. However, comprehensive microbial community level studies on arctic plant species are still in its infancy.

1.2 Endosphere microbes

Endosphere microbes are taxonomically diverse and mixture of bacterial and fungal communities lives inside the asymptomatic host plants (van Overbeek and Saikkonen 2016). The endosphere microbes interact with each other and also interact with their host (van Overbeek and Saikkonen 2016). Many of the endosphere microbes are known as commensals and their function in plants yet unknown (Hardoim et al. 2015). Some of the endosphere microbes are known to have beneficial effect on plants such as increasing the plant growth and development by biological nitrogen fixation, phosphate solubilisation and indole acetic acid production (Compant et al. 2010). They also protect the plants

from different biotic and abiotic stresses (Compant et al. 2010). A very few endosphere microbes are known to have negative effect on plants (Hardoim et al. 2015).

The major source of the rhizosphere and phyllosphere microbes is soil (Berg and Smalla 2009, Normander and Prosser 2000, Philippot et al. 2013, Singh et al. 2007, Zarraonaindia et al. 2015) and air (Maignien et al. 2014). This means that rhizosphere and phyllosphere act as major sources of endosphere microbes (Bulgarelli et al. 2012, Edwards et al. 2015, Long et al. 2008, Lundberg et al. 2012). Recent evidence suggests that invertebrates such as insects could act as vectors of microbial transmission in to the plants (Pažoutová et al. 2013). Vertical transmission via seeds is also one of the significant sources of the endosphere microbes (Johnston-Monje et al. 2014, Johnston-Monje and Raizada 2011, Hardoim et al. 2012, Puente et al. 2009). The soil, microbial and plant factors determine the endosphere microbe survival, colonization, compatibility, and competing ability within the plant (Gaiero et al. 2013).

1.3 Succession

1.3.1 Ecological succession

Ecological succession is the sequence of changes in structural and functional properties of species community composition over a period of time (Drury and Nisbet 1973). The change in species composition in a newly formed land such as lava flows, sand dunes, landslides, and glacial till is known as primary succession (Nemergut *et al*. 2007). In contrast, secondary succession includes the changes in already established ecosystem after disturbance (e.g., forest fire, and cyclone).

The conceptual understanding of the ecological succession was initiated from the study of vegetation succession (Clements 1916). It is a general assumption that the plant and animal species richness increase along the progressive ecosystem succession (Bazzaz 1975, Tews *et al*. 2004). Also, soil organic matter increases towards climax stage (Berendse *et al*. 1998, Walker and del Moral 2003, Chapin *et al*. 1994). Similar understanding of succession in microbial system is poor (Schmid *et al*. 2014). Recent evidence suggests that soil microbial species richness increases along the successional trajectory in primary successional sites (Brown and Jumpponen 2014, Brown and Jumpponen 2015). These studies are mainly focused on vegetated or non-vegetated soil microbial succession. However, the microbes have the ability to colonize different plant parts or different soil compartments for the nutritional (Belnap and Lange 2003) or non-nutritional (Diaz *et al*. 1993) purposes. Nevertheless, comparisons of plant-associated microbe successional dynamics are rare (Blaalid *et al*. 2012) and could give us an idea of whether general concept of plant successional trajectory is also applicable to microbial communities.

1.3.2 Sand dune primary successional habitats

Sand dunes are considered as severe environments which are characterized by low available nutrients, low water holding capacity and persistent strong winds. In arctic sand dune habitat, above factors combined with cold and highly fluctuating temperature make it even harder for plant survival and establishment. Sand dunes are dynamic ecosystems presenting a sequence of vegetation spanning from barren sand to climax vegetation and provide different successional stages with distinct plant species richness (Cowles 1899, Olson 1958, Lichter 1998, Maun 2009). It is possible to study the primary successional patterns using the spatial gradient as a substitute for long time observations.

Inland sand dunes are expanding in some parts of the world, which reduces the forest and arable land area. It could be possible to stabilize the sand dunes by successful establishment of vegetation. There is some evidence that microbial aggregates and especially mycorrhizal fungi play a role in establishment of plant species in sand dune areas (Sutton and Sheppard 1976, Koske and Poison 1984, Forster 1990, Miller and Jastrow 1992, Belnap and Lange 2003). However, there are no studies examining the possible role of endosphere microbes in plant establishment in sand dunes. A comprehensive community level study of endosphere microbes related to sand dune colonizing plant species along the succession could give us an idea of their potential role in sand dune primary successional sites.

1.4 *Deschampsia flexuosa*

Deschampsia flexuosa is commonly known as wavy hair grass, a cosmopolitan species of temperate and subarctic regions (Scurfield 1954). The optimum pH for *D. flexuosa* growth is 5.5 to 6. However, the plant has the ability to grow also in more acidic soils (Hackett 1965). Further, *D. flexuosa* is one of the early colonizers of sand dunes (Borgegård *et al*. 1990, Ujházy *et al*. 2011).

A few studies have characterized the plant-associated microbes in seeds and roots of *D. flexuosa*. Studies using the staining techniques show that seeds and roots of *D. flexuosa* harbour mutualistic and endophytic fungi (Saikkonen *et al*. 2000, Pietikäinen *et al*. 2005, Ruotsalainen *et al*. 2007). A study using culturable and clone library methods shows that *D. flexuosa* root endosphere mainly comprises dark septate endophytic fungi (*Phialocephala fortinii*), the abundance of which increases along the coastal sand dune succession (Tejesvi *et al*. 2010). Further, *D. flexuosa* is known to host root endosphere fungal communities dissimilar to those in neighbouring plant species (Tejesvi *et al*. 2013).

1.5 Reciprocal transplantation

Reciprocal transplantation is one of the oldest approaches in macro-ecology to study local adaptation of an organism in to the new environment (Ross *et al*. 2009, Scheepens and Stöcklin 2013). In reciprocal transplantation experiments, plants are reciprocally transplanted from local (home) environment to a novel (away) environment which can be compared with self-transplanted from local to local (Ågren and Schemske 2012).

Recently, soil microbiologists adopted the reciprocal transplantation strategy to tests for effects of both environment and microbial community composition (and their interactions) on the functioning of microorganism to multiple environments from their original environments (Reed and Martiny 2007, Rawls *et al*. 2006). After transplantation to new environment, microbial communities seem resistant to the new environmental conditions for long time (Bottomley *et al*. 2006, Liu *et al*. 2015, Zhao *et al*. 2014). In contrast, planting soil back to home system seems not to affect the soil microbiota (Lazzaro *et al*. 2011). It is unknown that whether endosphere microbes which depend on both soil and plant characteristics follow the same resistant behaviour as their soil microbial counterparts.

It could be possible to study phenotypic plasticity of a plant species in a new environment using reciprocal transplantation and correlate it with change or no change in endosphere microbial community status of the transplanted plants. It has been shown that a specific endosymbiotic microbial association is one of the factors affecting the phenotypic plasticity of the plants in new environment (Sultan 1995, Rodriguez et al., 2008, Rodriguez et al., 2010). However, it is not known how the endosphere microbial community shift is reflected in plant adaptation in new environment.

1.6 Arbuscular mycorrhiza fungi interaction with other microbes

Arbuscular mycorrhizal fungi (AMF) live inside the plant root as well as in the soil connecting the inside of the host plant to the outer soil (Miller *et al*. 1995). AMF increase the host plant nutrient and water uptake from soil (Finlay 2008) and utilize photosynthetic carbohydrates of host plants. The positive effect of AMF on plant growth and development is well documented.

It has been shown that AMF interact with other soil microbes (Frey-Klett *et al*. 2007, Bonfante and Anca 2009, Scervino *et al*. 2009). They are known to affect the physiology and functioning of AMF. Also, AMF could affect the soil bacterial and fungal populations (Andrade *et al*. 1997). AMF interaction with other plant-associated microbes are rarely studied and restricted to root-associated microbes, mainly to the rhizosphere. AMF could affect the soil and plant physio-chemical properties, for example, by altering the root exudate composition which in turn affects the root-associated microbes (Wamberg *et al*.

2003, Gryndler 2000, Jeffries *et al*. 2003, Scheffknecht *et al*. 2006, Gupta 2003, Vigo *et al*. 2000, Marschner *et al*. 2001). However, all these studies used culturable microbes or molecular finger-printing methods. There are no studies specifically targeted to endosphere microbes. Considering the role of AMF in assisting the plants in nutrient poor and harsh environments such as sand dunes (Sutton and Sheppard 1976), the study of AMF interaction with other endosphere microbes could give a new dimension in ecological relevance of their role in plant physiology.

1.7 Aim of the study

In this PhD project, I focused on the grass *Deschampsia flexuosa* that has circumpolar distribution and is a major component of the northern ecosystems. *Deschampsia flexuosa* has a wide ecological tolerance and is commonly found in early successional ecosystems to late, climax stage ecosystems. Especially *D. flexuosa* has an important role as reindeer fodder and as one of the first plant species colonizing eroded arctic soil and thus enabling ecosystem restoration. The long-term goal of this project is to identify the potential microbes that could be used in soil ecosystem restoration and in enhancing plant survival and well-being in high stress environments in general. It would be possible only if we know the different microbial species present and the factors responsible for their assemblage.

Considering that plant-associated microbes in arctic sand dune habitats are yet unknown, this thesis mainly aimed at exploring the sand dune colonizing grass plant, *Deschampsia flexuosa* associated microbes and their ecological relevance to possible extent. I characterized in-depth composition of endosphere microbes in two successional stages of arctic inland sand dune primary successional sites. In this thesis, I investigated the effect of different factors such as succession, transplantation and AMF inoculation on endosphere microbial assemblage. In the studies I, II and III, I investigated the changes in endosphere microbial community in two different successional stages located closely to each other in natural sand dune ecosystem. I adopted greenhouse condition for study IV. It helped me to control the AMF infection and study changes in endosphere microbial community.

In study I, microbes were isolated and grown in pure culture to identify the differences at species level and answer the following specific questions: (i) Does the culturable endophyte community composition reflect the cold climate of their host plants? (ii) Do different plant parts (leaf, root and seed) and plants in different successional stages have distinct culturable endophyte communities? and (iii) Do endophytic bacterial isolates solubilize organic phosphate rather than mineral phosphate?

In study II, I used high throughput sequencing techniques to compare the total microbial communities to answer the following specific questions: (i) Does late succession host more numerous microbial species than early succession? (ii)

Does successional stage of the ecosystem affect microbial community composition significantly? and (iii) Are co-occurring microbes succession stage specific?

In study III, I used reciprocal transplantation strategy to elucidate the following questions: i) will original endosphere community displaced by destination habitat microbes result in marked community shift or change in species richness? and (ii) is plant performance lower in the novel environment when compared to home environment?

In study IV, plants inoculated with AMF were used to answer the question whether AMF colonization affects microbial species richness and microbial community composition inside plant tissues.

2 MATERIALS AND METHODS

2.1 The field experiments

2.1.1 Study site

Aeolian inland sand dunes are common in northern Finnish Lapland. The present study site is located in 68° 29' 16" N, 24° 42' 13" E and falls under the sub-arctic region. The 1981-2000 average annual temperature in the region was -1.3 °C, with extreme temperatures between -51.5 °C to +30.2 °C (Pirinen *et al*. 2012). Sand drifting has been occurring at least for the 700 years in this region (Seppälä 1995). Thus, the ecosystem has eroded to such an extent that large areas are devoid of vegetation and the present plant cover consists of a mosaic of different successional stages.

The studies (I, II, III) were carried out in two successional stages i.e., early and late. The early successional stage comprised of a grass species (wavy hair grass) *Deschampsia flexuosa* in exposed sand surfaces (here after referred to as "early"). In contrast, late successional stage comprised of *D. flexuosa* in continuous ground cover with other 10 plant species such as *Empetrum nigrum* (black crowberry), the moss *Pleurozium schreberi* and sparse mountain birch (*Betula pubescens ssp. czerepanovii*) trees (here after referred to as "late"). Apart from the difference in plant species richness, the successional stages also significantly differ in physio-chemical properties (Table 1).

TABLE 1 Physiochemical properties of bulk soil samples from early and late successional stages.

Successional stage	C %	N %	P mg/kg	Moisture %	Organic matter %	pH
Early	0.05	0.02	1.43	0.15	0.17	6.10
Late	0.41	0.04	9.56	0.24	1.08	5.10

2.1.2 Experiments and the sampling scheme

Plant and soil samples from two successional stages (early and late) were collected in four different areas between 150 and 2250 meters distance apart. These areas are referred to as 'block' hereafter. Within block, the distance between early and late succession stage was 10-20 meters.

2.1.3 Study I

This experiment included five different *Deschampsia flexuosa* plant parts (108 samples): seeds, seedlings of less than 3cm in height collected from the field (hereafter referred to as "field seedlings"), and seedlings germinated from surface sterilized seeds in green house (hereafter referred to as "experimental seedlings"), and matured plant leaves and roots. In field, a total of 84 samples of seeds (early -12 and late - 12), field seedlings (Only early - 12), and matured plant leaves (early -12 and late - 12) and roots (early -12 and late - 12) were collected. A total of 24 experimental seedlings were germinated in greenhouse using the seeds collected from two successional stages i.e., 12 seedlings per succession. *Deschampsia flexuosa* field seedlings and leaves and roots of matured plants were collected in 24th July 2011 and seeds were collected 29th August 2011.

2.1.4 Study II

This experiment included two soil categories and two plant parts: bulk soils, rhizosphere soils, and *D. flexuosa* leaves and roots (the same leaf and root samples were used in study I and II). These different sample types are collectively referred to as "compartment" hereafter. Each of these compartment samples comprised of 24 replicates, i.e. there were 12 replicates per each succession. Successional stage samples were collected in four blocks, i.e., there were 3 replicates per block, per succession and per compartment. The samples were collected in 24th July 2011.

2.1.5 Study III

Deschampsia flexuosa plants naturally growing in the two successional stages were transplanted in 27 and 28th August 2011 within successional stage (self-transplantation) and between successional stages (reciprocal transplantation) in four different blocks. The transplanted plants were allowed to grow for two years. The transplanted (both self and reciprocal) plant leaf and root samples and control (non-transplanted plant samples) were collected in 19th August 2013. The plant biomass before and after transplantation were measured.

2.2 Greenhouse experiment (Study IV)

The experiment was carried out in greenhouse because it is possible to control the AMF colonization in greenhouse. *Deschampsia flexuosa* associated AMF (*Claroideoglomus etunicatum*) isolated from our study site was used in this experiment. The AMF inoculation experiment was started in 1st May 2014. A total of six plants for each control (no AMF inoculation) and AMF inoculated treatments were maintained in greenhouse. The harvesting was done 15th October 2014. The start and end plant biomasses were measured.

2.3 Plant tissue surface sterilization

The plant samples from all the studies (I, II, III and IV) were surface sterilized using the following method. Pre-weighed tissues of seeds, seedlings, leaves and roots were soaked in 70% ethanol for 1 min, 3% sodium hypochlorite for 3 minutes (except seeds, 6 minutes), 1% sodium thiosulphate for 3 minutes, and washed three times with sterile deionized water for 3 minutes.

2.4 Endophyte isolation, identification and characterization

The bacteria were isolated using the sterilized and homogenized plant tissues. The seedlings, leaves and roots were homogenized in 50 mM potassium phosphate buffer (pH 6.5) and plated in serial dilutions on the R2A media. The seed sterilization was carried out as described by Nissinen *et al*. (2012). In brief, the seeds were homogenized in BSE buffer (50 mM Tris–HCl [pH7.5], 1% Triton X-100 and 2 mM 2-mercaptoethanol) and centrifuged at 300×g for 5 min (at 15°C) to get the supernatant. The second centrifugation was done at 12 000 ×g for 15 min (at 10°C) to get the pellets and they were suspended in 50 mM potassium phosphate buffer (pH 6.5). Serial dilutions of suspended pellets were prepared and plated on the R2A media (pH 6.5). Plates were incubated at room temperature for a week and single colonies of bacteria were transferred to new plates to obtain pure cultures.

The fungi were isolated using the sterilized tissues cut into 1 cm pieces and plated directly into malt extract fungal media (Zijlstra *et al*. 2005). Plates were incubated at room temperature for a month or more. Hyphal tips of the developing fungal colonies were transferred to fresh malt extract agar plates.

The 16S rRNA gene of bacterial isolates were sequenced using PCR products amplified with 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5′-GGYTACTTGTTACGACTT-3′) primer pairs. The ITS region of fungal isolates was sequenced using PCR products amplified with ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-

TCCTCCGCTTATTGATATGC-3′) primer pairs. These isolates sequence data have been submitted to the GenBank databases under accession number KJ528986-KJ529110. The close phylogenetic relatives of bacterial and fungal isolates were identified by NCBI BLAST analysis.

I characterized all bacterial strains for their ability to solubilize mineral as well as organic forms of phosphate using National Botanical Research Institute's phosphate growth medium (NBRIPM; Nautiyal 1999) and phytase screening medium (PSM; Jorquera *et al*. 2011). The ability to utilize tri-calcium phosphate and phytate on NBRIPM and PSM agar was examined after incubation for 4 days at room temperature. The development of clearing zone around the colonies was used as an indicator of phosphate solubilization by the isolates.

2.5 Molecular analyses of microbes in plants and soil

2.5.1 DNA isolation, PCR and Ion torrent sequencing

Microbial DNA was extracted from bulk and rhizosphere soil using the PowerSoil DNA isolation kit (MoBio, Carlsbad, CA, USA) and from plant samples using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) or Invisorb Spin Plant Mini Kit (Stratec Biomedical AG, Germany) following manufacturer's instructions.

I used nested PCR approach for bacterial 16S rRNA region amplification, first round of 16S rRNA PCR was performed with 799f/ 1492R primer pairs to exclude plastid DNA amplification (Chelius and Triplett 2001). The second round of 16S rRNA PCR was performed with 1062F/1390R primer pairs. I used fITS7/ITS4 primer pairs for the amplification of fungal ITS regions (Ihrmark *et al*. 2012). I used M13 system for library preparation as described by Mäki et al., (2016). The sequencing was carried out using the Ion PGM Sequencing 400 Kit (Ion 314 chips; Life Technologies, Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions.

2.5.2 Bioinformatics

The bacterial 16S rRNA and fungal ITS sequences were reassigned to their respective samples and quality filtered using the Mothur v.1.35.0 (parameters: minlength = 200; maxambigs = 0; maxhomop = 8; qwindowaverage = 25; qwindowsize = 50; and bdiffs = 1). Further processing of bacterial 16S rRNA gene sequences in Mothur was performed following a standard procedure (Schloss *et al*. 2011). Fungal ITS sequences were processed as described in Tedersoo *et al*. (2014). The OTUs were clustered at 97% similarity level. The rare OTUs with five or less than five sequences across the samples were excluded from downstream analyses. The OTU abundance tables were rarefied to their minimum sequencing depth. The raw sequence data are available in National

Center for Biotechnology Information Sequence Read Archive under accession number SRP063711, SRP087752, SRP087758. The observed species richness, estimated species richness (Chao 1) and Shannon diversity indices were estimated using Mothur v.1.35.0.

2.5.3 Statistical analyses

To visualize shifts in the microbial community composition, principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities and Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson, 2001) were used. The PCoAs and PERMANOVA were performed in PRIMER software v6 (http://www.primer-e.com, Clark and Warwick 2001).

Linear statistics were applied to find out significant differences in observed species richness, estimated species richness (Chao 1) and Shannon diversity indices between different samples. I performed Kruskal Wallis test with the log transformed (log [X+1]) relative abundance data to identify the taxa (phyla/class/OTUs) that are responsible for community separation between different samples. I carried out co-occurrence network analyses as described by Williams *et al*. (2014) to identify modules of co-occurring OTUs within communities. Linear statistics, co-occurrence analyses and Kruskal Wallis test were performed in R statistical software (version 3.3.0).

3 RESULTS AND DISCUSSION

3.1 Cold habitat specificity of culturable endophytic microbes (I)

In our study, 52% of the total endophytic bacterial isolate 16S rRNA sequences were highly similar to bacterial sequences from cold environments, including arctic, antarctic and high alpine soils, snow, and in glacier or arctic-alpine plants. In the study by Nissinen *et al*. (2012) focusing on three arctic plant species, 40% of the bacterial endophytic isolates were similar to bacteria from cold climates. Sheng *et al*. (2011) reported that 46% of the endophytic isolates from subnival plants were highly similar to bacteria from other cold climates. Furthermore, 58-100% of the isolates in the vegetative tissues in *D. flexuosa* were very closely related (99-100% sequence identity) to endophytes from arctic plants from fell tundra in Lapland (Nissinen *et al*. 2012) and a great portion of these were also closely related to soil bacteria in Lapland, with greatest relative abundance of close relatives of arctic soil bacteria (Männistö and Häggblom 2006) found in the root tissues, indicating horizontal, but selective acquisition of endophytes. Taken together, these results suggest that cold climate bacteria are to large extent habitat specific. In contrast, only 13% of the endophytic fungal isolates were similar to fungi isolated from other cold environments. This indicates that fungal communities in cold environments are poorly studied when compared to their bacterial counterpart, or that unlike fungi, bacteria have developed lineages endemic to cold climates.

3.2 Seed endophytes able to mobilize organic phosphate (I)

Phosphorus is one of the main nutrients limiting plant growth worldwide. Soil mineral phosphorus is often bound to phosphates inaccessible to plants and in plants phosphorus is stored as phytate. Conversion of phytate into inorganic phosphate by the phytase enzyme is one of the important steps in seed germination (Scott and Loewus 1986). I compared the ability of endophytic

bacterial isolates for the solubilisation of mineral (tricalcium phosphate) and organic phosphate (phytate). A greater portion of the endophytes were able to mobilize organic (74% of the isolates) than mineral (57%) phosphate. In particular, great majority of seed endophytes were able to solubilize organic phosphate (92%). The seed isolates in the genus *Pseudomonas* were able to solubilize organic phosphate (100%) and mineral phosphate (87%). It seems likely that seed endophytic bacteria have a significant role in resource mobilization from stored reserves in the plant. Abundance of phosphate solubilizing bacteria was higher in the early than late successional experimental seedlings, which may be linked to the difference in phosphorus availability in these two successions.

3.3 Succession affects the microbial communities (I and II)

Effect of plant succession on microbial species richness was compartment dependent. In our arctic sand dune ecosystem bulk soil bacterial richness increased across the succession. However, succession did not affect soil fungal richness. Similar observation is reported in the primary succession of glacier forefront (Brown and Jumpponen 2014, Brown and Jumpponen 2015). Apparently, more energy was available for microbes in late successional stage with more organic matter and higher carbon content in the soil than in early successional stage in our ecosystem. The results of this study violated the general assumptions that organic matter quantity and quality as well as amount of energy in ecosystems drive fungal species richness (Read 1989, Evans *et al*. 2005, Pennanen *et al*. 2001, van der Wal *et al*. 2013).

Increased plant species richness leads to higher root exudates and available nutrients (de Ridder-Duine *et al*. 2005, Compant *et al*. 2010). In our ecosystem, both bacterial and fungal species richness of rhizosphere compartment didn't increase across the succession. In contrast, bacterial species richness in rhizosphere compartment has been shown to increase along the succession in salt marsh chronosequence (Wang *et al*. 2015). In the present study, rhizosphere microbial species richness was similar across the succession suggesting that host plant species specific selection rather than bulk soil characteristics determined microbial species richness in rhizosphere. In contrast to rhizosphere, I found that endosphere microbes in leaf (bacteria) and root (fungi) follow the traditional successional trajectory in the arctic inland sand dune ecosystem.

Succession had significant effect on microbial community composition (i.e., both bacteria and fungi) in all compartments. The relative abundance of different microbial taxa (at phyla/class/OTU level) in rhizosphere was the most affected by succession followed by bulk soil, root and leaf endosphere. Both culturable and non-culturable methods showed that Actinobacteria and Acidobacteria were dominant in the early and late successional stages, respectively. Actinobacteria are known to be oligotrophic and successful in

nutrient deficient and dry environments (Dion and Nautiyal 2008). Some of them are able to fix atmospheric nitrogen, which could explain their prevalence in the early successional stage with low plant cover. Late successional stage soils in our system had clearly higher organic matter content and lower soil pH in comparison to the early successional stage, which could explain increased relative abundance of Acidobacteria. It is known that Acidobacteria dominate acidic, high organic matter content soils in arctic tundra and to correlate negatively with pH (Jones *et al*. 2009, Männistö *et al*. 2013).

Ubiquitous and specific microbial groups were identified from different plant parts and two successional stages. Both root and leaf isolates of the fungus *Phialocephala fortinii* were confined to the late successional stage in line with the general notion that *P. fortinii* along with other dark septate endophytes favour habitats with high soil organic matter content (Caldwell *et al*. 2000; Tejesvi *et al*. 2010). Also members of the genus *Hymenoscyphus* isolated in this study in root samples of late successional stage are common endophytes in roots of ericaceous species (Zijlstra *et al*. 2005) and in other woody species such as pines (Villarreal-Ruiz *et al*. 2004).

Among the different *Pseudomonas* sp. identified, a tightly clustering group of isolates closely related to *P. fluorescens* were isolated from the root, leaf and experimental seedlings in both successional stages. This suggests that this group of endophytes is tightly associated with *D. flexuosa*, and that these *P. fluorescens* strains may be vertically transmitted. *Pseudomonas fluorescens* is a well-known plant growth promoting bacterium that has been isolated, among others, in many grasses previously and has been shown to affect plant growth and development, but also reduce seedling disease incidence in rice (Adhikari *et al*. 2001, Mercado-Blanco and Bakker 2007). In contrast to *P. fluorescens*, the isolates closely related to *P. graminis* and *P. chlororaphis* were more abundant in *D. flexuosa* in the early succession. *Pseudomonas graminis* has been reported previously in temperate sand dune plants (Park *et al*. 2005). Colonization of plants by *P. chlororaphis* has been proven to be effective in increasing drought tolerance as well as directly inhibiting the growth of fungal pathogens (Cho *et al*. 2008). All the isolates of *P. graminis* and *P. chlororaphis* have the ability to solubilize organic phosphate suggests that they may have prominent role in establishment of *D. flexuosa* in early succession.

3.4 Transplantation affects endosphere microbial communities (III)

In the transplantation experiment, effect of transplantation (self and reciprocal) on microbial species richness and microbial community composition was successional stage dependent and independent, respectively. Transplantation affected marginally the microbial species richness in some of the early successional stage samples, mainly leaf, but it did not affect late successional

stage sample richness. It is often assumed that change in species richness due to disturbance is a "lottery effect" which creates random opportunities for the species living in a similar niche to be nearby the resources after disturbance (Chesson and Warner 1981). It could be possible that nutrient availability for different microbial species due to disturbance was higher in early successional stage which was characterized by low and uneven resource availability to start with.

In this study, after reciprocal transplantation microbial community compositions in most of the leaf and root compartments were different from local non-transplanted control. In contrast, self-transplantation affected the microbial community composition only in few root compartments. Further, most of the differentially expressed OTUs in self-transplantation were also differentially expressed in reciprocal transplantation, but reciprocal transplantation possessed more unique differentially expressed OTUs. This could be due to the marginal effect of self-transplantation in comparison to reciprocal transplantation. Also, pairwise comparison of reciprocal transplantation community compositions to their original successional stage (before transplantation) un-transplanted control community composition shows that two of the root endophytic community compositions still resembled their origin. In previous experiments, it has taken at least two years (Waldrop and Firestone 2006, Liang *et al*. 2015, Sun *et al*. 2014) for the soil microbial community composition in transplanted soil samples to resemble the destination soil. These results together with mine indicate that soil and endosphere microbial community compositional changes in a new environmental condition are a slow process.

3.5 Mycorrhiza affects leaf endosphere bacterial composition (IV)

Genetically modified plants that are able to avoid mycorrhizal colonization in field harbour similar root-associated fungal species richness and bacterial richness (except one transgenic line) to mycorrhiza-colonized plants (Groten *et al*. 2015). In our study, arbuscular mycorrhizal fungi (AMF) inoculated and non-inoculated plants harboured similar bacterial and fungal species richness in both leaf and root endosphere. The difference in bacterial richness of the one transgenic line in Groten *et al*. (2015) could be due to lack of CCaMK expression which influences the bacterial colonization of roots (Sanchez *et al*. 2005). Also, I used clonally propagated initial plant materials which most likely contained same initial microbial community and my study exclusively targeted endosphere microbes.

In my study, AMF inoculation affected the leaf endosphere bacterial community composition. It is possible that changes in nutrient and carbon availability of AMF treated plants lead to change in bacterial populations (Snellgrove *et al*. 1982). In my study, AMF inoculation had positive effect on the abundance of the representative of the phylum Firmicutes and negative effect

on the representatives of the phyla Proteobacteria (Alpha, Beta and Gamma) and Bacteroidetes in leaf endosphere. The positive or negative impact of AMF on microbes could be due to simulation or repression of those bacteria (Wamberg *et al*. 2003). However, more investigations are needed to understand the interactions between AMF and leaf endosphere microbes.

4 CONCLUSIONS

My results suggest that endosphere bacteria in *Deschampsia flexuosa* growing in an arctic habitat are to large extent cold habitat specific and most of them are able to solubilize organic and inorganic phosphate. Overall, my research shows that succession, transplantation and AMF colonization can affect the endosphere microbial assemblage in a sand dune colonizing plant species (Table 2). In my work, microbial diversity (Shannon) was not affected by succession, which runs counter the general conception that species diversity is increases along succession. Species diversity is expected to be linked with the amount of energy in the ecosystem. In my work, I could not link the microbial diversity with the amount of energy in the soil ecosystem. Moreover, my results show that plant succession affects endosphere microbial species richness in compartment dependent manner and community composition in compartment independent manner. Root but not leaf fungal endophyte richness increased across succession. In contrast, leaf but not root bacterial endophyte richness increased across succession. Based on this limited evidence, it seems that the aboveground endophytic richness responds to succession in terms of bacteria and belowground in terms of fungi. This pattern was also supported by the transplantation experiment. Furhermore, the transplantion experiment shows that endosphere microbial community changes after transplantation to novel conditions are a slow process. Further research is needed including different plant species in different ecosystems to fully understand the successional differences between prokaryotic and eukaryotic microbes.

In my work, I discovered microbes that are sensitive to disturbance. Changes in microbial communities due to disturbance may directly affect ecosystem processes and are therefore of specific interest. In previous studies related to endosphere microbial community assemblages the arbuscular mycorrhiza was not included as a potential factor that affects the other endosphere microbial community. My experiment showed that mycorrhizal inoculum may affect plant leaf bacterial endophyte community composition, but whether these changes are related to mycorrhizal effect on plant physiology need to be studied in targeted experiments.

All of the factors I studied mainly affected the microbial community composition. Bacteria and fungi responded differently either in terms of microbial species richness (in case of succession) or microbial community composition (in case of reciprocal transplantation and AMF inoculation). All through my thesis work, bacteria and fungi responded differently to manipulations (Table 2). Although commonly used, it may be more appropriate to use the word 'bacteria' when only bacteria have been studied in stead of 'micro-organisms'. Similarly, it may be more correct to limit the generalization to fungi when only fungi have been studied as it seems that the two kingdoms do not respond similarly to environment.

TABLE 2 Summary of the effect of the different factors (A) succession (B) reciprocal transplantation (C) arbuscular mycorrhizal fungi inoculation on bacterial and fungal communities in *Deschampsia flexuosa* leaf and root endosphere compartments. The red colour letters indicate statistically significant effects of the respective factors. Roman letters I, II, III and IV indicate the respective manuscript numbers. The detailed descriptions for the codes in the table are as follows: Early = Early successional stage (monospecific *D. flexuosa* stands), Late = Late successional stage (*D. flexuosa* stands with other plant species). Early control = Non-transplanted control from early successional stage, Late control = Non-transplanted control from late successional stage, Late to early = reciprocally transplanted from late to early successional stage, early to late = reciprocally transplanted from early to late successional stage.

(A) Effect of succession on microbial richness and composition (I, II)

Tissue	Succession	Bacterial richness	Difference in bacterial composition	Fungal richness	Difference in fungal composition
Leaf	Early Vs late	Increase from early to late	Yes	Similar in early and late	Yes
Root	Early Vs late	Similar in early and late	Yes	Increase from early to late	Yes

(B) Effect of reciprocal transplantation on microbial composition (III)

Tissue	Reciprocal transplantation	Difference in bacterial composition	Difference in fungal composition
Leaf	Early control Vs late to early	No	Yes
	Late control Vs early to late	Yes	Yes
Root	Early control Vs late to early	Yes	No
	Late control Vs early to late	Yes	No

(C) Effect of arbuscular mycorrhizal fungi (AMF) on microbial composition (IV)

Tissue	AMF treatment	Difference in bacterial composition	Difference in fungal composition
Leaf	Control (no AMF) Vs AMF	Yes	No
Root	Control (no AMF) Vs AMF	No	No

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YHTEENVETO (RÉSUMÉ IN FINNISH) TRANSLATED BY MINNA-MAARIT KYTÖVIITA

Metsälauhan endofyyttisten mikrobien yhteisökoostumus lentohiekka-alueiden sukkessiossa

Kasvien kanssa elää suuri joukko mikrobeja enemmän tai vähemmän kiinteästi. Nämä liittolaiset ovat tärkeitä kasvien menestymiselle erilaisilla kasvupaikoilla. Erityisen tärkeitä kiinteästi kasveihin liittyneet mikrobit ovat kasvipeitteen muodostumisessa alkujaan kasvittomilla alueilla kuten hiekkadyyneillä. Tietoa kasvien kanssa liittoutuneiden mikrobien merkityksestä arktisilla alueilla yleisesti ja erityisesti lentohiekka-alueilla ja hiekkadyyneillä on hyvin vähän. Tässä väitöskirjatyössä selvitin metsälauhan (*Deschampsia flexuosa*) kanssa liittoutuneiden mikrobien yhteisöjä kahdessa eri sukkession vaiheessa Enontekiön Lapissa. Ensimmäinen vaihe edusti sukkession alkuvaihetta, jossa kasvipeite oli hyvin niukka ja suurin osa maan pinnasta on liikkuvan lentohiekan peittämää. Vain muutama kasvilaji kasvaa arktisilla lentohiekka-alueilla ja tutkimukseeni valitsin alueita, joilla kasvoi metsälauhaa harvakseltaan puhtaina kasvustoina. Toinen tutkimuksieni sukkession vaihe edusti myöhäistä vaihetta, joka syntyy pitkän ajan kuluessa kasvillisuuden kehittyessä ilman suuria häiriöitä. Sukkession päätevaihe tutkimusalueillani on harva tunturikoivikko, jonka aluskasvillisuudessa kasvaa varpuja (*Vaccinium uliginosum, Empetrum nigrum*), ruohovartisia kasveja kuten metsälauhaa, kultapiiskua (*Solidago virgaurea*), lampaannataa (*Festuca ovina*) ja kissankäpälää (*Antennaria dioica*), pohjakerroksen muodostaa yhtenäinen seinäsammalkerros (*Pleurozium schreberi*). Sukkession myötä lentohiekan pinnalle muodostuu loppuvaiheseen mennessä paksu kerros humusta, mikä muuttaa maan kemiallista koostumusta huomattavasti.

Ensimmäisessä työssäni eristin puhdasviljelmäkasvatuksiin metsälauhan lehtien ja juurten sisällä kasvavia sieniä ja bakteereita. Osa metsälauhan lehtien ja juurten sisältä eristetyistä mikrobeista oli sellaisia, joita tapaa myös maaperässä. Osa eristetyistä mikrobeista oli kuitenkin aiemmin löydetty ainoastaan endofyyttisinä eli kasvin sisäisinä. Suurin osa eristetyistä mikrobeista oli arktisia, eli nykytietämyksen mukaan niiden elinalue rajoittuu maapallon kylmiin osiin. Suurin osa eristetyistä endofyyttisistä mikrobeista kykeni liuottamaan eloperäistä fosfaattia, mikä on kasvien kasvun kannalta erittäin tärkeä ominaisuus.

Kolmessa seuraavassa työssäni käytin nykyaikaisia sekvensointimenetelmiä, joiden avulla pystyin kuvaamaan mikrobiyhteisöt kattavasti. Yhdessä osatyössä kasvatin metsälauhaa kasvihuoneessa keräsienen (*Claroideoglomus etunicatum*) kanssa tai ilman sienijuurisientä. Kasvien inokulointi keräsienellä muutti lehtien endofyyttistä bakteeriyhteisöä, mutta ei vaikuttanut kasvin juuren sisällä kasvavien sienten tai bakteerien yhteisöihin. Maastokokeiden tulosten mukaan sukkession vaihe ja kasvin osa (lehti, juuri) vaikutti merkitsevästi metsälauhan endofyyttisten mikrobien lajirunsauteen. Sukkession vaihe vaikutti myös erittäin voimakkaasti mikrobien yhteisökostumukseen. Tein siirtoistutus-

kokeen, jossa metsälauhaa siirtoistutettiin kasvupaikallaan tai sukkessiovaiheesta toiseen. Myös tämän tämän tutkimuksen perusteella kasvupaikan sukkession vaihe vaikutti endofyyttisten mikrobien lajirunsauteen. Kasvupaikan sukkession vaiheesta riippumatta siirtositutettujen kasvien endofyyttisten mikrobien yhteisökoostumus erosi useimmissa tapauksissa (juuri, verso, sienet, bakteerit, alkuvaiheesta loppuvaiheeseen siirretyt kasvit, loppuvaiheesta alkuvaiheeseen siirretyt kasvit) kasvupaikalla kasvavien siirtämättömien kasvien mikrobiyhteisöistä. Sen sijaan kasvupaikallaan siirtoistutettujen kasvien kohdalla vain endofyyttisten bakteerien yhteisöt erosivat muutamassa tapauksessa.

Yleisesti ottaen ekologisen teorian mukaan lajistollinen monimuotoisuus lisääntyy sukkession edetessä. Syyksi monimuotoisuuden kasvuun on esitetty ekosysteemin sisältämän energian määrää. Vastoin näitä yleisiä käsityksiä, sukkession vaihe ei vaikuttanut mikrobien lajistolliseen monimuotoisuuteen omissa tutkimuksissani. Sen sijaan sukkessio vaikutti mikrobien lajirunsauteen riippuen tutkitusta kasvin osasta ja mikrobiyhteisöjen koostumukseen tutkitusta kasvin osasta riippumatta. Juurten sisällä kasvavien sienten lajirunsaus lisääntyi sukkession myötä, mutta bakteerien lajirunsaus ei. Sen sijaan lehden sisällä kasvavien bakteerien lajirunsaus lisääntyi sukkession myötä, mutta sienten ei. Tämä tulos saatiin myös kokeessa, jossa siirtoistutettiin kasveja sukkession myöhäisestä vaiheesta alkuvaiheeseen ja päinvastoin. Näiden rajallisten tutkimusten valossa näyttää siltä, että kasvin maanpäällisissä osissa sukkession etenemiseen reagoivat bakteerit, maanalaisissa osissa sienet. Lisäksi siirtoistutuskoe osoitti, että endofyyttisten mikrobien yhteisökoostumuksen sopeutuminen uuteen kasvupaikkaan on hidas prosessi.

Koska endofyyttisten sieni- ja bakteeriyhteisöjen koostumuksen, lajirunsauden ja monimuotoisuuden suhdetta sukkession etenemiseen ei ole aiemmin tutkittu, omien tulosteni yleispätevyys jää tulevien tutkimusten selvitettäväksi.

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ORIGINAL PAPERS

I

CULTURABLE ENDOPHYTIC MICROBIAL COMMUNITIES IN THE CIRCUMPOLAR GRASS, *DESCHAMPSIA FLEXUOSA* IN A SUB-ARCTIC INLAND PRIMARY SUCCESSION ARE HABITAT AND GROWTH STAGE SPECIFIC

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Culturable endophytic microbial communities in the circumpolar grass, *Deschampsia flexuosa* in a sub-Arctic inland primary succession are habitat and growth stage specific

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Summary

Little is known about endophytic microbes in cold climate plants and how their communities are formed. We compared culturable putative endophytic bacteria and fungi in the ecologically important circumpolar grass, *Deschampsia flexuosa* growing in two successional stages of subarctic sand dune (68°29′N). Sequence analyses of partial 16S rRNA and internal transcribed spacer (ITS) sequences of culturable endophytes showed that diverse bacteria and fungi inhabit different tissues of *D. flexuosa*. A total of 178 bacterial isolates representing seven taxonomic divisions, *Alpha*, *Beta* and *Gammaproteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Acidobacteria*, and 30 fungal isolates representing the phylum *Ascomycota* were identified. Several endophytes were affiliated with specific plant tissues or successional stages. This first report of bacterial endophytes in *D. flexuosa* revealed that the genus *Pseudomonas* is tightly associated with *D. flexuosa*, and encompassed 39% of the bacterial isolates, and 58% of seed isolates. Based on 16S rRNA and ITS sequence data, most of the *D. flexuosa* endophytes were closely related to microbes from other cold environments. The majority of seed endophytic bacterial isolates were able to solubilize organic form of phosphate suggesting that these endophytes could play a role in resource mobilization in germinating seeds in nutrient-poor habitat.

Introduction

Ever since their emergence, plants have lived in constant interaction with different bacteria, fungi and viruses (Sessitsch *et al*., 2004; Aly *et al*., 2011; Reinhold-Hurek and Hurek, 2011). Microbial association with plants can be external as is the case of the rhizosphere or phyllosphere microbes and internal as for the endophytic microbes. Endophytic microbes are ubiquitous in plants, and they can provide plants with mineral nutrients or fixed nitrogen in exchange for carbon (Pillay and Nowak, 1997; Compant *et al*., 2010; Reinhold-Hurek and Hurek, 2011). Endophytes can also help their hosts to overcome environmental stresses by producing or modifying phytohormones or secondary metabolites (Sturz *et al*., 1998; Siciliano *et al*., 2001; Barac *et al*., 2004; Ma *et al*., 2011a,b; Ardanov *et al*., 2012). Most of endophytes are thought to be acquired horizontally, mainly from the rhizosphere (Hardoim *et al*., 2008), but endophytes can also be transmitted vertically from parent to offspring (Ferreira *et al*., 2008; Hardoim *et al*., 2012). Further, different plant organs and tissues have been shown to harbour different microbial communities (Wearn *et al*., 2012; Bodenhausen *et al*., 2013).

Bacterial and fungal endophytes are known to have wide range of hosts (Cannon and Simmons, 2002; Sheng *et al*., 2011) or show host specificity (Arnold *et al*., 2001; Long *et al*., 2008; Nissinen *et al*., 2012). Most of the reports on endophytic diversity concern tropical or temperate agriculture and forest systems, and there is very little data on endophytic microbial diversity in cold environments, especially information from the arctic is lacking. Recently, the first reports on arctic plant endophytic bacteria (Nissinen *et al*., 2012) and endophytic fungi (Higgins *et al*., 2007; Bjorbækmo *et al*., 2010; Walker *et al*., 2011) have emerged. These studies were conducted on different plant species, and currently, there is no knowledge of the culturable microbial community composed of both bacteria and fungi in any arctic plant species.

In this study, we focused on the microbial endophytes in the grass *Deschampsia flexuosa* growing in subarctic Aeolian sand dune area. Aeolian sand dunes were formed in the northern hemisphere during glacial retreat when strong winds resulted in the formation of large areas covered by sand dunes (Koster, 1988). These Aeolian sand dunes were later stabilized by vegetation, but erosion has created large deflation areas without

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vegetation relatively recently (Seppälä, 1995). Plant colonization in the Aeolian sand dunes is very slow because of extreme environmental conditions such as highly fluctuating temperatures, short growing season, water and nutrient limitation and unstable soil structure (Hodkinson *et al.*, 2003). Together these result in primary successional gradient (Seppälä, 1995) characterized by different successional stages starting from no vegetation to change in plant species composition from pioneer plant species to later successional plant species (Svoboda and Henry, 1987; Hodkinson *et al.*, 2003). The role of soil microbes in plant development in primary successional areas (Grootjans *et al.*, 1997) and in arctic primary successional areas (Borin *et al.*, 2010) have been recognized, but the importance of plant endophytic microbial communities remains unexplored.

The vegetation in our experimental area has been destroyed in several occasions by fire during the last 800 years (Seppälä, 1995). The fires together with wind effect and intensive grazing by herbivores, particularly by domestic reindeer, have resulted in the reversion of succession in some areas. As a result, the current vegetation is patchy and large areas without any vegetation cover exist next to relatively undisturbed mountain birch forest stands and other successional stages. *Deschampsia flexuosa* is one of the few pioneer plants that are able to colonize bare sand under arctic conditions (Polunin, 1938).

We compared the putative endophytic microbial community in *D. flexuosa* growing in two successional stages, one that consists of the grass *D. flexuosa* as the only plant species (early successional) and one that represents the vegetation cover that will develop without extreme disturbance (late successional) in a subarctic inland sand dune area. We focused on culturable endophytes because of their feasibility for future bioassays. We isolated both fungi and bacteria in leaves, roots, seeds and seedlings. Although usually explored separately, we analysed both fungi and bacteria in order to tentatively explore their interactive roles in plant life in cold climate. We characterized the bacterial endophytes for their ability to solubilize mineral phosphate which is considered an important trait in plant growth promoting bacteria (de Freitas *et al.*, 1997; Richardson, 2001). Phytate is the main storage form of phosphorus seeds in grass species in particular and up to 80% of the phosphorus is in the form of phytate in plants (Scott and Loewus, 1986). Conversion of phytate into inorganic phosphate by the phytase enzyme is one of the important steps in seed germination (Scott and Loewus, 1986). Phytate degradation by soil microbes and their role in plant growth in phosphate-poor environment has been reported (Idriss *et al.*, 2002). However, the role of endophytes in phytate degradation is poorly understood, and might be relevant in arctic habitats with low levels of soluble nutrients.

We asked the following specific questions: (i) Does the culturable endophyte community composition reflect the cold climate of their host plants? (ii) Do different plant parts (shoot, root and seed) and plants in different successional stages have distinct culturable endophyte communities? and (iii) Do endophytic bacterial isolates solubilize of organic phosphate rather than mineral phosphate?

Results and discussion

Culturable bacterial endophytes

A total of 178 putative endophytic bacterial strains were isolated from *D. flexuosa* plants collected from the two successional stages. Based on genomic fingerprinting (BOX PCR, for details, see Appendix S1), 101 unique isolates were detected and tentatively identified by 16S rRNA gene sequencing and comparison to reference sequences in public databases. Overall, the endophytic bacterial isolates from *D. flexuosa* represented seven taxonomic divisions: *Alpha*, *Beta* and *Gammaproteobacteria* (10%, 11% and 60% of total community respectively), *Actinobacteria* (8%), *Bacteroidetes* (8%), *Firmicutes* (2%) and *Acidobacteria* (1%). The relative abundance of several phyla between samples collected from the different successional stages differed. *Actinobacteria* and *Alphaproteobacteria* were more abundant in plants growing in sand, whereas Bacteroidetes and *Gammaproteobacteria* were relatively more abundant in forest (Fig. S1). The isolates representing *Firmicutes* and *Acidobacteria* (4 and 2 isolates respectively) were all isolated from forest samples. Actinobacteria are known to be oligotrophic and successful in nutrient-deficient and dry environments (Dion, 2008), and this could explain their prevalence in the sand samples.

Likewise, several phyla were unevenly detected in different tissues of *D. flexuosa*; *Gammaproteobacteria* were predominant in seed and experimental seedling samples, with 95% and 80% of isolates respectively. In contrast, *Actinobacteria*, *Beta* and *Alphaproteobacteria* were found in leaf, root and field seedling samples, but not in seeds or experimental seedlings, suggesting their acquisition from environment during plant development.

The isolates represented in total 32 bacterial genera, with the highest taxonomic diversity detected in root (16 genera) and leaf (15) tissues, with 7, 6 and 8 genera in field seedlings, seeds and in experimental seedlings respectively. *Pseudomonas* was the most prevalent genus in our isolate collection, accounting for 39% of all (total isolate collection) isolates, followed by *Burkholderia* (5%), *Pedobacter* (5%), *Rahnella* (5%) and *Pantoea* (4%). *Pseudomonas* was consistently present in all *D. flexuosa* tissues and in both successional stages studied.

However, several other genera showed preference to specific tissue or successional stage (Figs 1 and 2). For example, *Alphaproteobacterial* genera *Sphingomonas*, *Rhizobium* and *Mesorhizobium* were almost exclusively detected in samples from the sand (Fig. 2). This could be related to their function in nitrogen acquisiton in a nutrient-poor sand habitat; *Rhizobium* and *Mesorhizobium* are well-known nitrogen-fixing genera (Franche *et al*., 2009). Endophytic bacteria belonging to the genera *Pseudomonas*, *Erwinia*, *Paenibacillus*, *Flavobacterium* and *Stenotrophomonas* were identified in seed material. These same genera have been discovered previously in seed material in temperate (Ferreira *et al*., 2008; Hardoim *et al*., 2012) as well as arctic (Nissinen *et al*., 2012) plant species. Within endophytic genera, tissue or habitat specific groups were detected (Fig. 2 and Table 1). For example, isolates SL56, SR-A19, FL97-1 and FR302, closely related to *Burkholderia sordidicola* type strain SNU 020123, were present in leaf and root samples of both successional stages. In contrast, isolates SL28-1 and SR306, closely related to *Curtobacterium flaccumfaciens*, were present only in the leaf and root samples from the sand.

Pseudomonas *is the dominant genus in* D. flexuosa *endophytic bacterial communities*

Pseudomonas spp. isolates dominated the endophyte community of *D. flexuosa* in both sand and forest samples, and were present in all the tissues and growth stages of *D. flexuosa* investigated in this study. These results agree well with previous reports: temperate sand dune plant species as well as *Pennisetum glaucum* (a grass family plant) grown on low-nutrient soil have been shown to be dominated by *Gammaproteobacteria*, in particular *Pseudomonas* (Park *et al*., 2005; Gupta *et al*., 2013). *Pseudomonas* isolates were most abundant in seed tissues, with 58% relative abundance, followed by 44% relative abundance in field seedlings, 35% in leaves, 34% in experimental seedlings and 27% in root. In order to gain better insight into this genus, we performed a separate phylogenetic analysis of partial 16S rRNA gene sequences of all *Pseudomonas* isolates in our collection (Fig. 3). This enabled detection of several distinct tightly clustered groups within the genus *Pseudomonas*. For example, a group of isolates (SL10, SR310-1, FL97-2, FR129, SGS1 and FGS11), closely related to *P.fluorescens* type strain IAM12022 and glacier isolate KOPRI 25853, were present in all *D. flexuosa* tissues and both successional stages (Fig. 3). This suggests that this group of endophytes is tightly associated with *D. flexuosa* and might be vertically transmitted. *Pseudomonas fluorescens* is a well-known plant growth promoting bacterium that has been isolated, among others, in many grasses previously and has been shown to affect plant growth and development, but also reduce seedling disease incidence in rice (Adhikari *et al*., 2001; Mercado-Blanco and Bakker, 2007). In contrast, another group of isolates (SL11, SR12, SS4, SS8 and SS12) was mainly detected in the sand samples (seeds, leaves and roots). These isolates were closely related to *Pseudomonas graminis* type strain DSM11363 and to a group of strains isolated previously from seeds of arctic plants (Fig. 3). *Pseudomonas graminis* has been previously detected in temperate sand dune plants (Park *et al*., 2005), and also in seeds of *Juncus trifidus* and *Diapensia lapponica* collected in low-nutrient, high-stress habitats in fell tundra in northwestern Finland (Nissinen *et al*., 2012).

Phosphate solubilization ability in endophytic bacteria

All the putative endophytic bacterial isolates were qualitatively analysed for their *in vitro* mineral as well as organic phosphate solubilizing ability in order to evaluate the potential of the isolates for plant growth promotion (for details, see Appendix S1). In total, 57% of the endophytic bacterial isolates were able to solubilize the tricalcium (mineral) phosphate (TCP) and 74% were able to degrade phytate, the organic form of phosphate, common phosphate storage form in plant seeds. All TCP solubilizing isolates were also able to degrade organic phosphate. Proportionally most phosphate solubilizing isolates were detected in forest root samples, with 73% (TCP) and 80% (phytate) in the forest and 39% (TCP) and 57% (phytate) in the sand respectively. In contrast, relatively higher proportion of phytate solubilizing isolates was detected in experimental seedlings from sand samples, with 100% in the sand samples and 70% in the forest samples respectively (Table S1).

Soil mineral phosphorus is often bound to phosphates inaccessible to plants. In plants, phosphorus is stored as phytate. Conversion of phytate into inorganic phosphate by the phytase enzyme is one of the important steps in seed germination (Scott and Loewus, 1986). We hypothesized that endophytic bacterial isolates solubilize the organic phosphate rather than inorganic phosphate. The results supported our hypothesis and greater portion of the endophytes was able to mobilize organic (74%) than mineral (57%) phosphate. In particular, the great majority of seed endophytes was able to solubilize organic phosphate (92%). The seed isolates in the genus *Pseudomonas* were able to solubilize organic phosphate (100%) and mineral phosphate (87%). It seems likely that seed endophytic bacteria have a significant role in resource mobilization from stored reserves in the plant. Abundance of organic phosphate solubilizing bacteria was relatively higher in the early than late successional

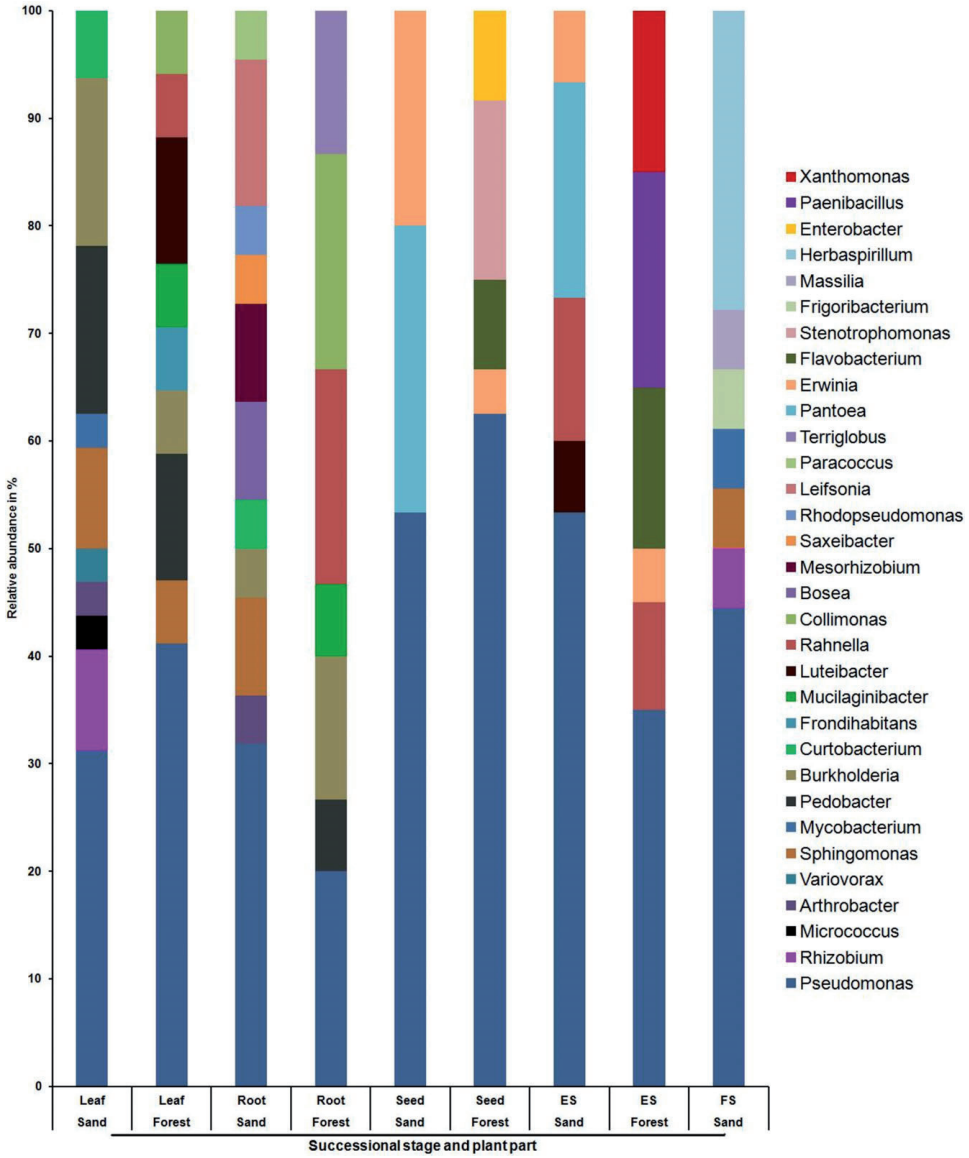

Fig. 1. Distribution of different endophytic bacterial genera from *Deschampsia flexuosa* plant parts expressed as relative abundance in early (sand) and late (forest) successional stages. Bacteria were identified and classified using Ribosomal database (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

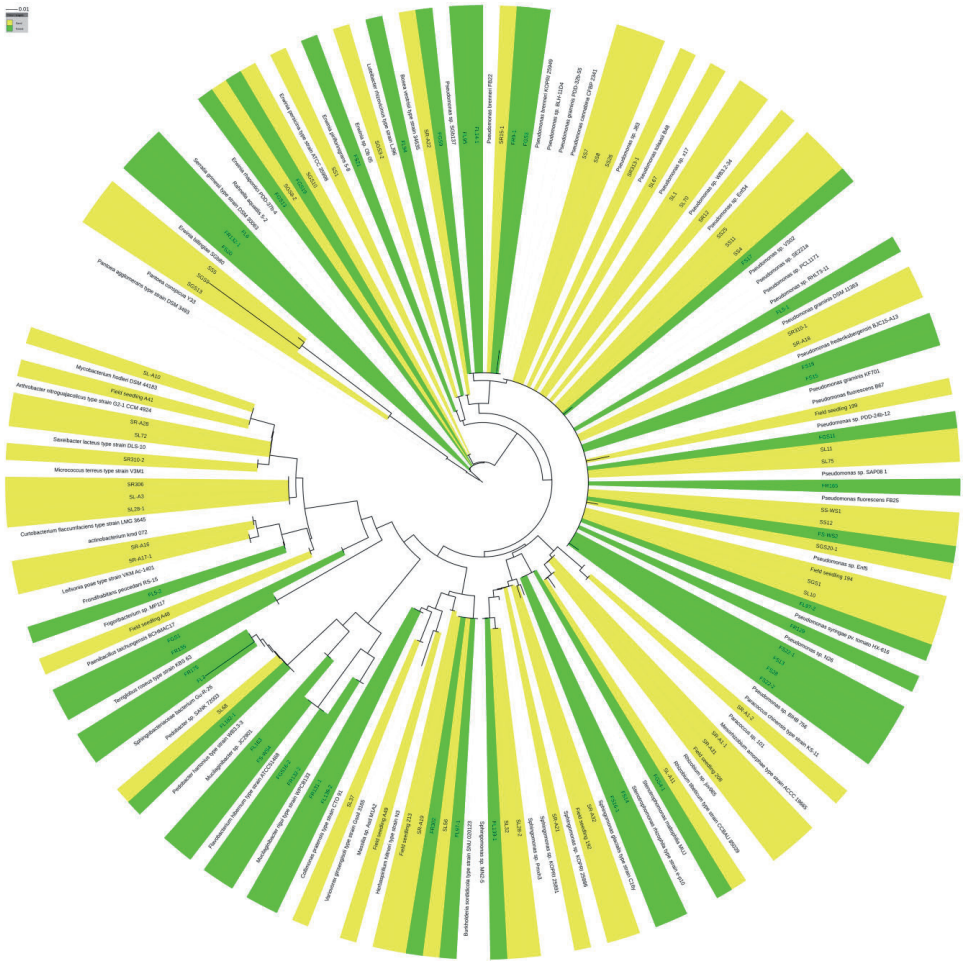

Fig. 2. A phylogenetic tree based on the partial 16S rRNA of endophytic bacterial isolates (unique isolates) associated with *Deschampsia flexuosa* and their closest related matches was constructed using the neighbor-joining method (MEGA 5 software). The analysis involved 176 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + non-coding. All positions containing gaps and missing data were eliminated. There were a total of 229 positions in the final dataset. Isolate samples are coded with tripartite code indicating sampling site (S = sand and F = forest), plant parts (L = leaf, R = root, S = seed and GS = experimental seedlings), followed by isolate number. (Exception-field seedlings followed by isolate number only). The phylogenetic trees were visualized and annotated with iTOL tool at http://itol.embl.de/.

experimental seedlings, which may be linked to the difference in phosphorus availability in these habitats.

Isolates representing genera *Pseudomonas*, *Sphingomonas*, *Rhizobium*, *Burkholderia*, *Frondihabitans*, *Pedobacter*, *Rahnella*, *Collimonas*, *Mesorhizobium*, *Arthrobacter*, *Leifsonia*, *Erwinia*, *Enterobacter*, *Stenotrophomonas*, *Luteibacter* and *Paenibacillus* were able to solubilize either mineral or organic forms of phosphate. In particular, most of *Pseudomonas* sp. isolates in our collection were able to solubilize the both forms of the phosphate. In particular, all the isolates of *P. graminis* group, present mainly in sand samples (Fig. 3), had the ability to

Table 1. Successional or tissue stage specific/dominant culturable bacterial isolates (53 isolates out of 178 total isolates) from different parts (leaf, root, seed and seedlings) of *Deschampsia flexuosa* in early (sand) and late (forest) successional stage.

Isolate name	Closest sequence match in RDB[a] database	No. of isolates	Similarity score in RDB	Specificity/dominance[b]	
				Tissue	Successional stage
SL11	*Pseudomonas graminis* DQ339600	1	1	Seed	Sand
SS26	*Pseudomonas graminis* DQ339586	1	0.973		
SS8	*Pseudomonas graminis* HQ256853	1	0.98		
FS18	Pseudomonas graminis HQ256858	4	0.983		
SS4	*Pseudomonas graminis*	1	0.987		
SR12	*Pseudomonas graminis* DQ339614	1	1		
SS11	*Pseudomonas graminis* HQ256853	1	0.978		
SR306	*Curtobacterium flaccumfaciens* JN378724	2	1		Sand
SL28-1	*Curtobacterium flaccumfaciens* JQ977194	2	0.992		
Field seedling 199	*Pseudomonas chlororaphis* JX081311	6	0.975	Field seedling	Sand
Field seedling 213	*Herbaspirillum hiltneri* DQ150565	5	1		
Field seedling 208	*Rhizobium* sp. JN590331	1	0.999		Sand
SL-A11	*Rhizobium* sp. JN590331	3	1		
FGS16-2	*Flavobacterium* sp. FJ889628	3	0.975	Seed and field seedlings	Forest
FS-WS4	*Flavobacterium* sp. FN397666	2	0.964		
FL139-2	*Collimonas pratensis* AY281143	1	1		Forest
FR131-1	*Collimonas pratensis* AY281143	3	1		
FGS4-1	*Stenotrophomonas maltophilia* AB661774	3	0.983	Seed and field seedlings	Forest
FS16-1	*Stenotrophomonas rhizophila* JQ977663	1	0.988		
FL182-1	*Pedobacter cryoconitis* EU169155	1	0.997	Leaf	
SL68	*Pedobacter cryoconitis* HQ824869	5	0.992		
FS15	*Pseudomonas* sp. HQ260323	1	0.971	Seed	
FS28	*Pseudomonas alcaligenes* HQ224627	2	0.975		
SS25	*Pseudomonas alcaligenes* HQ224627	1	0.97		
SS7	*Pseudomonas* sp. HQ260323	1	0.97		

a. RDP: Ribosomal Database Project, Release 11.
b. Only species with more than three isolates were considered as dominant or specific to tissue/successional stage.
Bacteria were identified and classified using analysis tools and reference sequence databases at the Ribosomal Database Project, Release 11 (http://rdp.cme.msu.edu).

solubilize organic phosphate, suggesting that they may have a prominent role in the establishment of early stages of *D. flexuosa* in sand.

Culturable fungal endophytes

A total of 30 putative endophytic fungi were isolated from *D. flexuosa* tissues. Based on the phenotype and partial ITS sequencing, 24 unique isolates were detected. Of these, 11 isolates (leaf – 2, root – 4 and seed – 5) were from the sand samples and 13 isolates (leaf – 5, root – 6 and seed – 2) were from the forest. Based on the ITS sequence analysis, all the fungal isolates belonged to the phylum *Ascomycota* and clustered into groups corresponding five classes, *Sordariomycetes*, *Dothideomycetes*, *Leotiomycetes*, *Eurotiomycetes* and *Saccharomycetes*, and seven orders, *Helotiales*, *Eurotiales*, *Capnodiales*, *Hypocreales*, *Sordiariales*, *Pleosporales* and *Saccharomycetales* (Fig. 4).

A total of 17 different fungal genera were identified, of which 10, 7 and 5 genera were found in root, leaf and seed material respectively (Fig. S2). Several of these

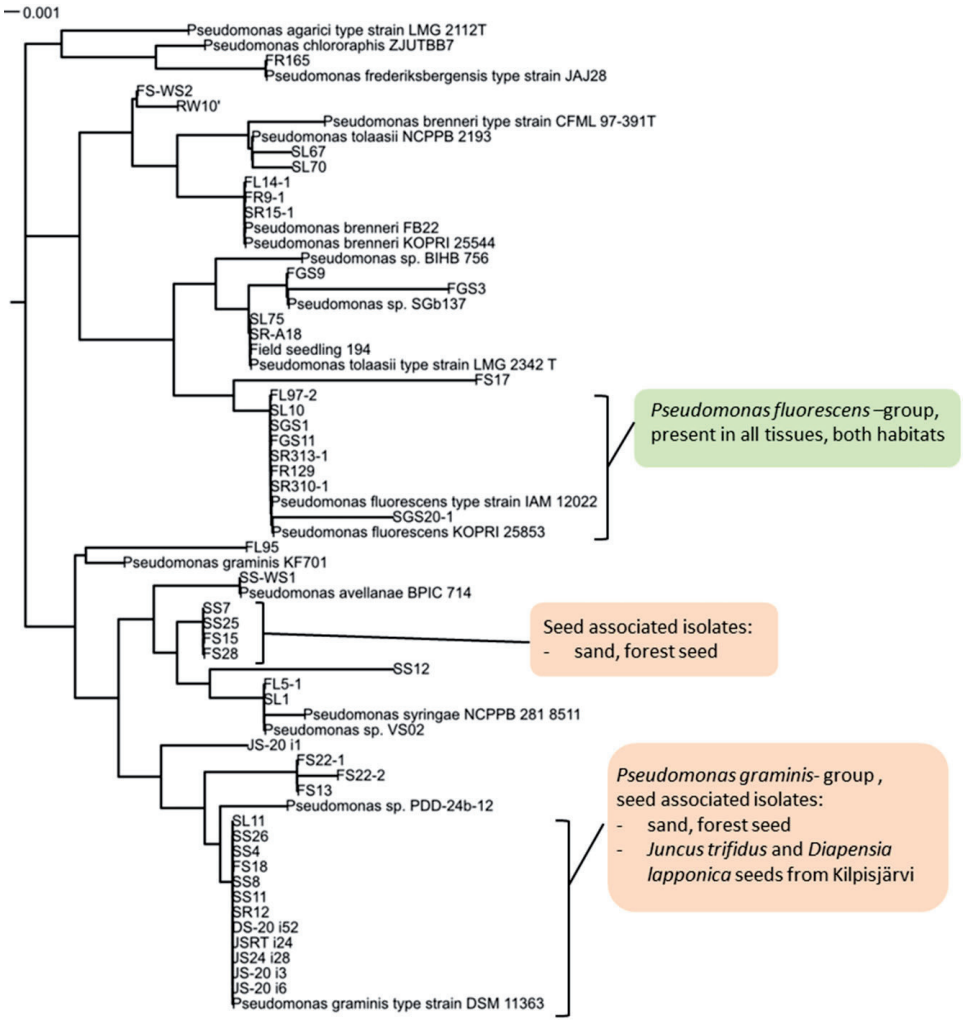

Fig. 3. Phylogenetic tree of partial 16S rRNA gene sequences of *Pseudomonas* spp. isolates from *Deschampsia flexuosa* (representative sequences from different plant parts), seed isolates from Nissinen and colleagues 2012 (*Juncus trifidus* and *Diapensia lapponica*) and their closest relatives from public databases was constructed using the neighbor-joining method (MEGA 5 software). The analysis involved 66 nucleotide sequences. All positions containing gaps and missing data were eliminated, and there were a total of 424 positions in the final dataset. Isolate sequences are coded with tripartite code indicating successional stage (S = sand and F = forest), plant tissue (L = leaf, R = root, S = seed and GS = experimental seedlings), followed by isolate number. (Exception: field seedlings are followed by isolate number.) *Diapensia lapponica* seed isolates = DS and *J. trifidus* seed isolates = JS, followed by isolate number only. The phylogenetic tree was visualized and annotated with iTOL tool at http://itol.embl.de/.

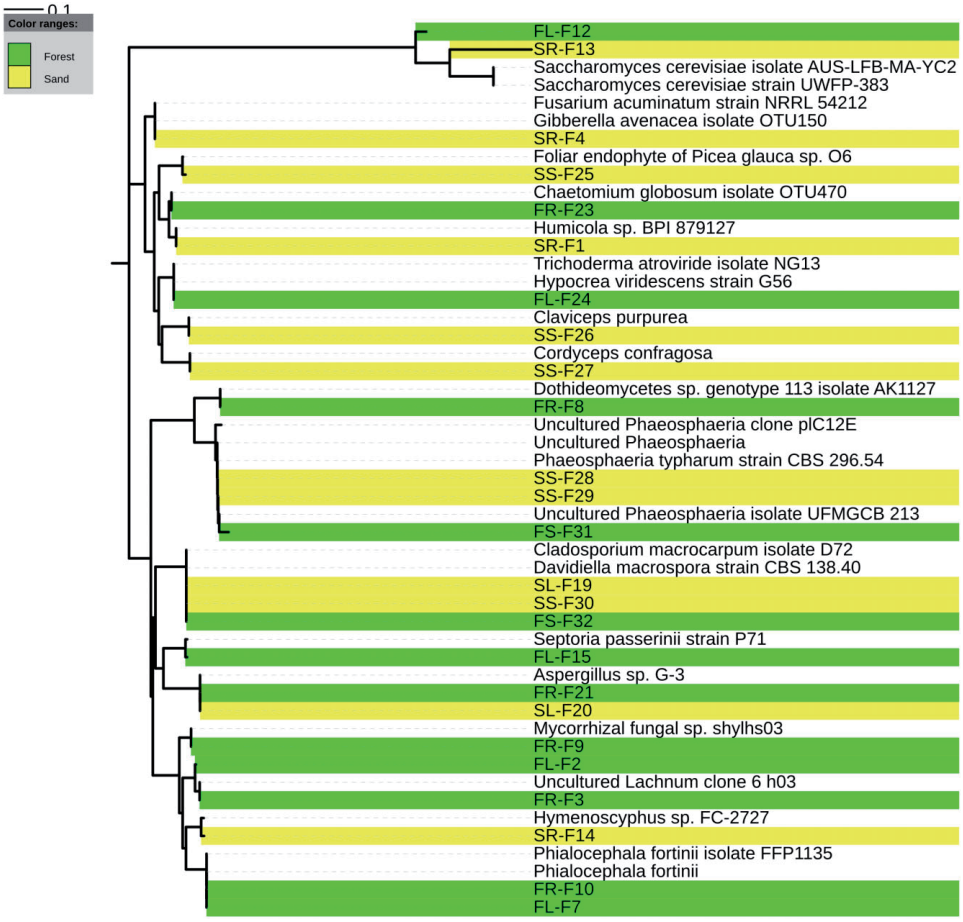

Fig. 4. A phylogenetic tree based on the partial ITS rDNA of endophytic fungal isolates (unique isolates) associated with *Deschampsia flexuosa* and their closest related matches was constructed using the neighbor-joining method (MEGA 5 software). The analysis involved 49 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + non-coding. All positions containing gaps and missing data were eliminated. There were a total of 206 positions in the final dataset. Isolate samples are coded with tripartite code indicating sampling site (S = sand and F = forest), plant parts (L = leaf, R = root, S = seed), followed by (F = fungi) and isolate number only. The phylogenetic tree was visualized and annotated with iTOL tool at http://itol.embl.de/.

genera showed affiliation with habitat or plant tissues. For example, isolates FL-F7 and FR-F10, closely related to *Phialocephala fortinii* (AB671499.2 and JQ711965.1), were found in forest leaf and root samples. Isolates FL-F15 and FL-F16, closely related to *Septoria passerinii* (AF181701), were discovered only in forest leaf samples. In contrast, isolate FR-F4, closely related to *Gibberella avenacea* (GU934531), was found only in sand root samples.

Both root and leaf isolates of the fungus *P. fortinii* were confined to the late successional habitat in line with the general notion that *P. fortinii*, along with other dark septate endophytes, favour habitats with high soil organic matter content (Caldwell *et al.*, 2000). Also members of the genus *Hymenoscyphus* isolated in this study in root samples of the early successional stage are common endophytes in roots of ericaceous species (Zijlstra *et al.*, 2005). In comparison with reports from other ecosystems

(Zijlstra *et al*., 2005; Tejesvi *et al*., 2010; 2013), it seems that so far, there is no common fungal endophyte species identified in *D. flexuosa* except *P. fortinii. Phialocephala fortinii* is commonly isolated from roots of different plant species (Menkis *et al*., 2004; Addy *et al*., 2005). It has been described as an important fungus in plant roots forming the so-called 'dark-septate endophyte' colonization (Jumpponen and Trappe, 1998). This is the first report of *P. fortinii* from the plant leaves, which likely reflects the relatively low number of studies on leaf fungal endophytes in comparison with root fungal endophytes.

It is highly likely that endophytic bacteria and fungi may also form functional associations with each other. An endophytic fungus carrying *Luteibacter* sp. as endohyphal bacteria produced more of the plant hormone auxin than fungus alone (Hoffman *et al*., 2013). In our study, the bacterial isolate FR131-1 (*Collimonas* sp.) and the fungal isolate FR-F23 (*Chaetomium globosum*) were specific to the late successional stage and plant tissue type, suggesting potential cohabitation. In earlier studies, *Collimonas* sp. (AJ496444) was previously isolated from living fungal hyphae of *C. globosum*, *Fusarium culmorum* and *Mucor hiemalis* from sand dune soils in the Netherlands (de Boer *et al*., 2001; 2004). However, it will require targeted studies to reveal whether the bacteria and fungi isolated from the same tissues are functionally linked.

Plant association and cold habitat specificity of endophytic isolates

Sequence alignment analysis revealed that 71% of the closest matches in public data banks for our putative bacterial endophyte isolate 16S rRNA genes sequences were from plant-associated bacteria and 25% of the best hits originated from grass family plants (Fig. 2). Interestingly, the closest relatives of 52% of our endophytic bacterial isolates had been isolated from cold environments, including arctic, antarctic and high alpine soils, snow, glacier or arcto-alpine plants. For example, endophytic bacterial isolates SL11, SL32 and SL28-1 were closely related to *P. graminis* (DQ339600), *Sphingomonas* sp. (JQ977308) and *C. flaccumfaciens* (JQ977194) from subnival plants of high-elevation habitats in Tibet and China (Sheng *et al*., 2011) respectively. Nissinen and colleagues (2012) and Sheng and colleagues (2011), focusing on arctic and alpine plant species, reported that 40–46% of the bacterial endophytic isolates were similar to bacteria from cold climates.

We also performed a targeted screen of the isolate sequences with a collection of endophyte sequences from *J. trifidus*, *D. lapponica* and *Oxyria digyna* isolated in northwestern Lapland (Nissinen *et al*., 2012; 325 strains, NCBI accession HE814625–HE815460). Intriguingly, 65% of the endophytes from *D. flexuosa* vegetative tissues (roots, leaves, field seedlings) showed 99–100% sequence homology to the endophytic isolates reported from Lapland (Nissinen *et al*., 2012), whereas only 10% of the seed or experimental seedling isolates had 99–100% match, and all of these were *P. graminis* isolates from seeds of mainly *J. trifidus*, also a monocot plant species. To test whether our endophytes are related to cold climate, we performed another targeted screen against collection of over 600 bacterial isolates from soils in Lapland (Männistö and Häggblom, 2006). Of the *D. flexuosa* isolates, 40% from vegetative tissue (99% or higher sequence homology), but none of the seed and seedling isolates, were closely related to the soil bacteria from subarctic Lapland (Table 2). The root tissues had highest relative abundance of close relatives of arctic soil bacteria. Taken together, these findings suggest a presence of vertically transmitted (seed) core microbiota, and horizontal, but selective acquisition of endophytes from soil in later growth stages.

Like the bacterial endophytes, the great majority (83% of closest relatives based on ITS sequence alignment) of the endophytic fungal isolates were plant-associated fungi previously isolated from phyllo-, endo- or rhizospheres of different plant species. For example, the fungal isolates SS-F25 is a close relative of the foliar endophyte (AY561215) of *Picea glauca*. A total of 17% of isolates were closely related to fungi isolated from grass family plants. For example, the fungal isolate FL-F15 is a close relative of *Septoria passerinii* (AF181701) of barley plants. However, unlike our bacterial isolates, only 13% of closest relatives of our endophytic fungal isolates were similar to fungi isolated from cold environments. This could suggest that fungal communities from cold environments are poorly studied when compared with their

Table 2. Percentage of bacterial isolate partial 16S rRNA sequences from *Deschampsia flexuosa* tissues in this study with 99–100% sequence identity (16S rRNA gene, nucleotides 390–900) to 16S rRNA gene sequences from other endophytic (Nissinen *et al*., 2012) or soil bacterial isolates (Männistö and Häggblom, 2006) from subarctic–low arctic Finnish Lapland in NCBI nucleotide collection.

Tissue	Arctic endophytic bacteria (%)	Arctic soil bacteria (%)	Number of *D. flexuosa* endophyte isolate sequences analysed
Seed	16	0	32
Experimental seedlings	0	0	16
Field seedlings	100	44	9
Root	58	58	26
Leaf	59	22	27
Average/total	41	23	110

The first two columns in the table indicate the percentage of isolates with 99–100% identity; the last column indicates the number of isolate sequences from different *D. flexuosa* tissues analysed.

bacterial counterpart, or that, unlike fungi, bacteria have developed lineages endemic to cold climates.

To the best of our knowledge, this is the first report on diversity of culturable endophytes of both bacteria and fungi in an arctic plant species. The isolated putative endophytes were tissue and habitat specific to various degrees. Overall, *Actinobacteria* and *Alphaproteobacteria* were affiliated with sand whereas *Bacteroidetes* and *Gammaproteobacteria* with forest habitat. Most of the bacterial isolates possessed capacity to solubilize organic phosphate. Also, many of our bacterial isolates had high level of homology to isolates from other cold environments. They are potential candidates for plant growth promoting bacteria in the cold habitat. Although the isolation method does not reveal the full microbial richness, it is necessary to work on arctic microbial isolates as they may be used in future experiments and ultimately in restoring vegetation in extreme environmental conditions.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Phylum level distribution based on partial 16S rRNA sequence of endophytic bacteria (total isolates) associated with *Deschampsia flexuosa* expressed as relative abundance in early (sand) and late (forest) successional stages. Bacteria were identified and classified using Ribosomal data base (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Fig. S2. Distribution of different endophytic fungal genera (total isolates) from different *Deschampsia flexuosa* plant parts expressed as relative abundance in early (sand) and late (forest) successional stages. Fungi were identified and classified using NCBI nucleotide database (http://

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Table S1. Percentage of phosphate (mineral or organic phosphate solubilizing assay) solubilizing bacterial isolates (out of 178 total isolates) from different parts (leaf, root, seed and seedlings) of *Deschampsia flexuosa* in early (sand) and late (forest) successional stage.

Appendix S1. Materials and methods.

APPENDIX S1

Experimental procedures

Study site

The study site is located in an Aeolian sand dune area in subarctic Northern Fennoscandia (68° 29' 16" N, 24° 42' 13" E). The region belongs to the belt of discontinuous permafrost. The sand dunes were formed after Ice Age and vegetated. Some parts of the dunes have lost the vegetation and wind has created blow-outs and drifting sands. Sand drifting has been occurring at least for the last 700 years (Seppälä 1995). Between the non-vegetated blow-outs different successional stages occur. The climax vegetation consists of mountain birch (*Betula pubescens ssp. czerepanovii*) forest, with the ground vegetation of dwarf shrubs such as *Vaccinium uliginosum, V. vitis-idaea, Empetrum nigrum, Betula nana, and Pleurozium schreberii.* In other successional stages *Arctostaphylos uva-ursi, A. alpina*, the grasses *D. flexuosa* and *Festuca ovina*, and the herbs *Antennaria dioica* and *Solidago virgaurea* occur. The 1981-2000 average annual temperature in the region is -1.3 °C (Pirinen et al., 2012). The area has a highly variable temperature with maximal temperatures between -51.5 °C (winter) to +30 °C (summer) (Pirinen et al., 2012), and large temperature fluctuations are common during the 100 days of growing period. The dune sands consist of Aeolian quartz particles with median grain size close to 0.2 mm (Seppälä 1995). Due to the porosity of the sand substrate and the low amount of annual precipitation (400 mm), the vegetation is subjected to periods of drought. Some vegetation types have developed organic matter layer (e.g. 10 cm in mountain birch forest), but some are without (e.g. *D. flexuosa* in deflated sand areas). The top soil pH varies between 4.7 (birch forest) and 5.8 (*D. flexuosa* in deflated sand areas) and total phosphorus between 1.91 mg/kg (*D. flexuosa* in deflated sand areas) and 14.12 mg/kg (birch forest), respectively.

Collection of *Deschampsia flexuosa* material

Sampling was conducted during the growth season in 2011 in two successional stages. Early successional stage (hereafter referred to as 'sand') was characterized by the grass *D. flexuosa* growing as monoculture in the blow-out areas. The sparse *D. flexuosa* tussock biomass was 9 grams per m^2 (Francini et al., unpublished). Late successional stage was mountain birch forest vegetation with continuous ground cover vegetation composed of abundant *D. flexuosa* together with *E. nigrum* and the moss *P. schreberi* under the cover of mountain birch trees (hereafter 'forest'). Samples of *D. flexuosa* were collected from four different blow-out areas between 150 and 2250 meters apart. In each area, we established three plots 20 to 30 meters apart. In each plot, we harvested one plant in both the two successional stages situated within 10 meters from each other. Altogether, the plant material consists of (4x2x3) 24 plants.

Deschampsia flexuosa shoots and roots were collected in 24th July 2011. In addition, small seedlings less than 3 cm in height were collected from sand (called 'field seedlings' hereafter) at the same time. *Deschampsia flexuosa* seeds were collected 29th August 2011 from both sand and forest in all the four areas.

Endophytic microbe isolation

Preweighed plant tissues of root, shoot, seedlings and seeds were surface sterilized by soaking in 70% ethanol for 1 min, 3% sodium hypochlorite for 3 minutes (except seeds, 6 minutes) , 1% sodium thiosulphate for 3 minutes, and washing three times with sterile deionized water for 3 minutes. Surface sterilized seeds were divided in to two portions. One portion was directly used for isolating microbes. Another portion was germinated on a sterile wet filter paper in Petri dishes for 10 days in greenhouse. The germinated seeds were used for microbe isolations ('experimental seedlings' hereafter).

In order to isolate bacteria, 1g of sterilized tissue (except seed) was homogenized in 3-4 ml of 50 mM potassium phosphate buffer at pH 6.5, and dilution plated as described below. Sterilized seeds were homogenized in 8 ml of BSE buffer (50 mM Tris–HCl [pH7.5], 1% Triton X-100 and 2 mM 2-mercaptoethanol) and centrifuged at 300×g for 5 min (at 15°C). Supernatant was transferred to new tubes and centrifuged at 12 000 ×g for 15 min (at 10°C). Pellets were suspended in 400µl of 50 mM potassium phosphate buffer set to pH 6.5. Serial dilutions were prepared and plated on the R2A media, pH 6.5. Plates were incubated at room temperature for a week whereafter they were moved to +4°C and monitored for new colonies. Single colonies of bacteria were transferred to new plates to obtain pure cultures. Pure strains were stored at -80 °C in R2A-glycerol solution.

In order to isolate fungi, sterilized tissues were cut into 1 cm pieces and plated directly into malt extract fungal media (Zijlstra et al., 2005). Plates were incubated in room temperature for a month or more. Hyphal tips of the developing fungal colonies were transferred to fresh malt extract agar plates.

16S rRNA and ITS rDNA amplification

Single bacterial colonies were transferred to tubes containing 100 µL of sterile deionized water and suspensions were heat lysed at 95 °C for 10 minutes and centrifuged at 13000x g for 5 minutes. Heat lysed suspensions were used as the DNA templates for PCR reactions. The 16S rRNA amplification was performed in a 50-µl reaction mixture including 1-µl DNA template, 0.25 µM of primers 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5'-GGYTACTTGTTACGACTT-3'), 0.25 µM dNTPs, 1X of *Taq* buffer, and 1 U *Taq* DNA polymerase (Fermentas). The PCR amplification was performed as follows: one cycle of 5 minutes at 94 °C, followed by 30 cycles of 30 seconds at 94 °C, 45 seconds at 51 °C, and 1:30 minutes at 72 °C, followed by one cycle of 7 minutes at 72 °C.

Fungal genomic DNA was isolated from fresh mycelia scraped from plates using the Qiagen DNA isolation kit according to manufacturer's protocol and used as template in PCR amplifications. The ITS region was amplified from genomic DNA using the forward primer ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and the reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR amplification was performed in a

50-µl reaction mixture including 1-µl DNA template, 0.25 µM of primers, 0.25 µM dNTPs, 1X of *Taq* buffer, and 1 U *Taq* DNA polymerase (Fermentas).The PCR program consisted of one initial denaturation step at 95 °C for 5 min followed by 30 cycles at 95 °C for 30 sec, 52 °C for 30 sec, 72 °C for 1 min, with a final extension at 72 °C for 7 min.

Sequencing and sequence analysis

The PCR products were purified by ethanol/EDTA precipitation and purified PCR products were sequenced with an automated multicapillary DNA sequencer, ABI Prism 3130xl genetic analyzer (Applied Biosystems, USA). 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. The RDB SEQMATCH (https://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch) were used to identify the closest phylogenetic relatives. The sequences were aligned, trimmed and analysed with MEGA 5 software (Tamura et al., 2011). The phylogenetic trees were visualized and annotated with iTOL tool at http://itol.embl.de/. These sequence data have been submitted to the GenBank databases under accession number KJ528986-KJ529110

Phosphate solubilization assays

All bacterial strains were tested for their ability to solubilize mineral as well as organic forms of phosphate using National Botanical Research Institute's phosphate growth medium (NBRIPM) supplemented with (g /L) 15 agar, 10 glucose, 5 Ca3(PO4)2, 5 MgCl2• 6H2O, 0.25 MgSO4• 7H2O, 0.2 KCl and 0.1 (NH4)2SO4 (Nautiyal 1999) and phytase screening medium (PSM) supplemented with (g /L) 15 agar, 10 D-glucose, 2 CaCl2, 5 NH4NO3, 0.5 KCl, 0.5 MgSO4 . 7H2O, 0.01 FeSO4 .7H2O, 0.01 MnSO4.H2O, and 3 phytate (phytic acid sodium salt hydrate) (Jorquera et al., 2011) . Seven strains per plate were stabbed in triplicate using inoculation loop. The ability to utilize tricalcium phosphate and phytate on NBRIPM and PSM agar was examined after incubation for 4 days at room temperature. The development of clearing zone around the colonies was used as an indicator of phosphate solubilization by the isolates.

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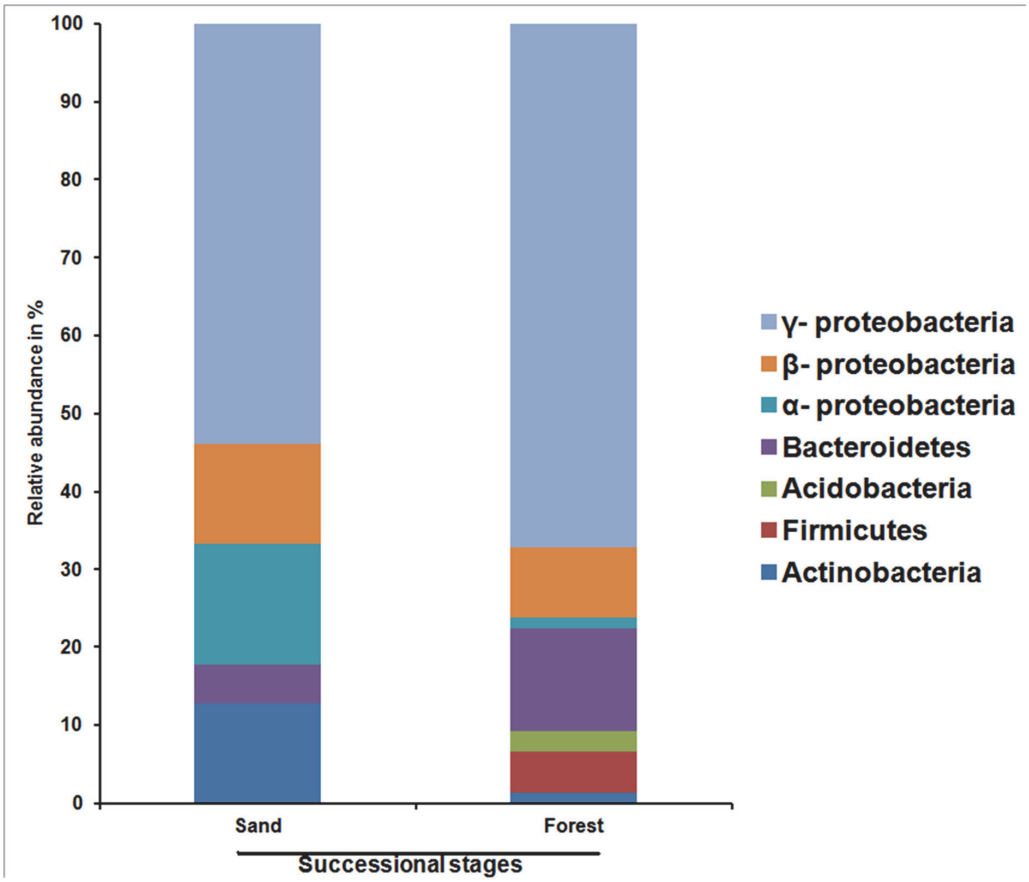

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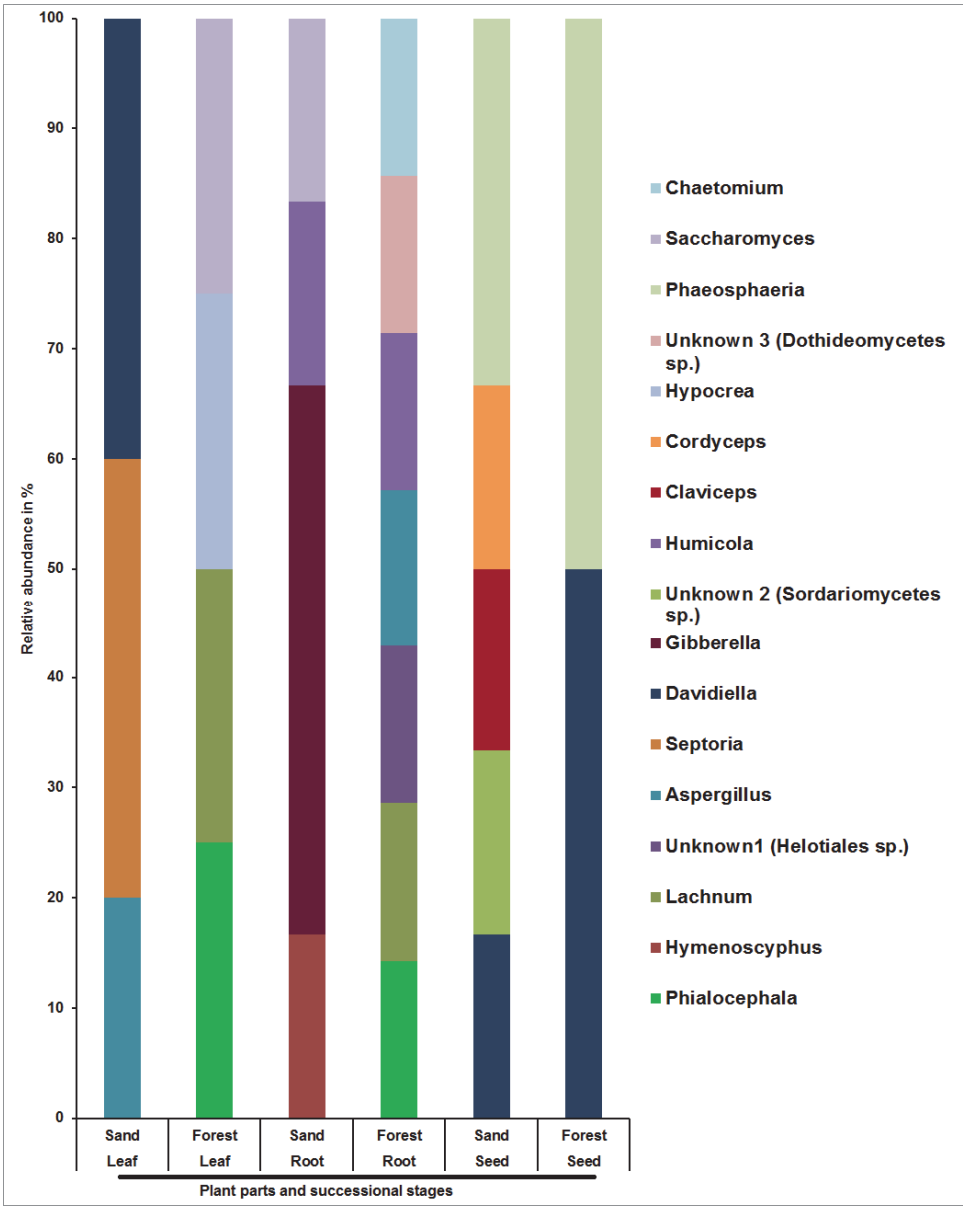

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Successional stage and plant parts	**Percentage of positive isolates**		
	Mineral phosphate (tricalcium phosphate)	**Organic phosphate (phytate)**	**Both (tricalcium phosphate + phytate)**
Sand leaf	50	63	50
Sand root	39	57	39
Sand seed	67	100	67
Sand experimental seedlings	73	100	73
Sand field seedlings	50	50	50
Forest leaf	59	76	59
Forest root	73	80	73
Forest seed	61	87	61
Forest experimental seedlings	55	70	55
Total	57	74	57

II

MICROBIAL COMMUNITY COMPOSITION BUT NOT DIVERSITY CHANGES ALONG SUCCESSION IN ARCTIC SAND DUNES

by

Anbu Poosakkannu, Riitta Nissinen, Minna Männistö & Minna-Maarit Kytöviita 2016

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III

ENDOSPHERE MICROBIAL LEGACY AFTER PLANT TRANSPLANTATION IN ARCTIC SAND DUNES

by

Anbu Poosakkannu & Minna-Maarit Kytöviita 2016

Manuscript

IV

NATIVE ARBUSCULAR MYCORRHIZAL SYMBIOSIS CHANGES LEAF ENDOSPHERE BACTERIAL COMMUNITY COMPOSITION IN *DESCHAMPSIA FLEXUOSA*

by

Anbu Poosakkannu, Riitta Nissinen & Minna-Maarit Kytöviita 2016

Manuscript