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Changes in the root-associated fungal communities along a primary succession gradient analysed by 454 pyrosequencing

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Abstract

We investigated changes in the root-associated fungal communities associated with the ectomycorrhizal herb *Bistorta vivipara* along a primary succession gradient using 454 amplicon sequencing. Our main objective was to assess the degree of variation in fungal richness and community composition as vegetation cover increases along the chronosequence. Sixty root systems of *B. vivipara* were sampled in vegetation zones delimited by dated moraines in front of a retreating glacier in Norway. We extracted DNA from rinsed root systems, amplified the ITS1 region using fungal-specific primers and analysed the amplicons using 454 sequencing. Between 437 and 5063 sequences were obtained from each root system. Clustering analyses using a 98.5% sequence similarity cut-off yielded a total of 470 operational taxonomic units (OTUs), excluding singletons. Between eight and 41 fungal OTUs were detected within each root system. Already in the first stage of succession, a high fungal diversity was present in the *B. vivipara* root systems. Total number of OTUs increased significantly along the gradient towards climax vegetation, but the average number of OTUs per root system stayed unchanged. There was a high patchiness in distribution of fungal OTUs across root systems, indicating that stochastic processes to a large extent structure the fungal communities. However, time since deglaciation had impact on the fungal community structure, as a systematic shift in the community composition was observed along the chronosequence. Ectomycorrhizal basidiomycetes were the dominant fungi in the roots of *B. vivipara*, when it comes to both number of OTUs and number of sequences.

Keywords: 454 pyrosequencing, *Bistorta vivipara*, community structure, ectomycorrhiza, primary succession, root-associated fungi

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Introduction

As glaciers retreat because of climate change, new terrestrial habitats are exposed. In these previously uninhabited and barren areas with little to no organic soil, biotic communities develop gradually via the process known as primary succession. Typical characteristics of glacier forelands are short growing seasons, shallow soils that rapidly dry out, low air temperatures and high UV radiation, factors limiting establishment success in plants. Various micro-organisms in these systems, including fungi, have key roles in pedogenesis, biogeochemical cycling and facilitation of colonization by plants, yet little is known about these processes

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(Fierer *et al.* 2010). Some input of organic material might also come from cryoconite, which is a powdery windblown dust, which is deposited and builds up on glaciers. Most studies of biological succession in glacier forelands have focussed on plant communities (Matthews 1978; Vetaas 1994; Freynot *et al.* 1998; Raffl *et al.* 2006). Microbial communities have so far been addressed only in a few studies (Ohtonen *et al.* 1999; Fierer *et al.* 2010; Schütte *et al.* 2010), probably due to methodological limitations. However, with the recent developments in next-generation sequencing (NGS) techniques, comprehensive surveys of microbial diversity and patterns of succession can be carried out with unprecedented ease. For example, a study of bacterial diversity in a glacier foreland in the high arctic using 454 pyrosequencing (Schütte *et al.* 2010) revealed a high species turnover along the chronosequence. NGS technologies have shown great potential for the analyses of fungal diversity (Buée *et al.* 2009; Jumpponen & Jones 2009; Öpik *et al.* 2009). Most noticeably, 454 pyrosequencing enables the analysis of a huge amount of sequences simultaneously, making it more likely to detect infrequent taxa (Öpik *et al.* 2009).

The succession of macrofungi following glacier retreat has been addressed in several studies by fruiting body inventories (Baxter & Middleton 1961; Helm *et al.* 1996; Jumpponen *et al.* 1999, 2002; Alfredsen & Høiland 2001; Nara *et al.* 2003a). These studies demonstrated that ectomycorrhizal (ECM) fungi are present and potentially important players during plant establishment in glacial forefronts. Extraradical mycelia of ECM fungi are able to form common mycorrhizal networks that may function as a source of ECM fungal infections to neighbouring ECM plants, i.e. to seedlings that establish themselves close to mature plants (Onguene & Kuyper 2002; Nara 2006a,b). Moreover, spores from ECM fungi can play a crucial role during the establishment of remote seedlings (Ishida *et al.* 2008). Many ECM-forming fungi are able to colonize a variety of plant hosts and thereby facilitate the establishment of new plant species. These attributes point towards a potentially significant role of ECM fungi in the dynamics of primary succession.

In most of the fruiting body–based studies of primary succession, an increase in the number of macrofungi with increasing terrain age and time since deglaciation has been observed (Baxter & Middleton 1961; Jumpponen *et al.* 1999). Other more recent studies have relied on morphotyping of ECM root tips and/or molecular analyses (Nara *et al.* 2003b; Jumpponen 2004; Cázares *et al.* 2005; Nara 2006b). In a study focusing on primary plant establishment in a recently emerged volcanic landscape, Nara & Hogetsu (2004) observed that the survival of seedlings of *Salix reinii* was dependent on colonization by ECM fungi. Jumpponen *et al.* (2002) observed that the early fungal communities of ECM-forming fungi establishing in the forefront of a retreating glacier comprised only a few species. In later successional stages, additional taxa were recruited leading to an increase in the species richness (Jumpponen *et al.* 2002). In several studies, it has been indicated that some fungal taxa show tendencies of being pioneers, others are second-stage and late colonizers (Jumpponen *et al.* 2002; Nara *et al.* 2003a,b; Nara & Hogetsu 2004). Further, using microscopy of plant root samples, Cázares *et al.* (2005) observed systematic shifts in the frequency of different mycorrhizal types along a chronosequence towards a retreating glacier.

Most ECM-forming plants are trees and shrubs, and because of their size, it is difficult to explore their entire root-associated fungal assemblages. However, also some smaller herbs, including the circumpolar perennial herb *Bistorta vivipara* (L.) Delarbre (Polygonaceae) syn. *Polygonum viviparum*, form ectomycorrhiza. The small and condensed root system of *B. vivipara* allows the entire fungal community associated with each plant to be sampled and analysed (Kauserud *et al.* 2011). *B. vivipara* is a plant species with a wide ecological amplitude that often occurs as a pioneer species in arctic and alpine environments (Dormann *et al.* 2002). ECM in *B. vivipara* was first recognized by (Hesselmann 1900) and has been confirmed in later studies (Read & Haselwandter 1981; Lesica & Antibus 1986; Väre *et al.* 1992; Eriksen *et al.* 2002; Sønstebø 2002). Massicotte *et al.* (1998) concluded that *B. vivipara* has mycorrhizal associations comparable to those described for woody angiosperms, and at least 18 well-known ECM fungi have been reported in association with *B. vivipara* (Mühlmann *et al.* 2008).

In this study, we used 454 sequencing to analyse changes in the fungal community associated with *B. vivipara* roots along a primary succession gradient in front of a glacier in central Norway. We had the following main hypotheses that we wanted to test: (i) mainly ECM fungi are associated with *B. vivipara* along the successional gradient; (ii) the fungal diversity is lowest in the early successional stages close to the glacier and highest at intermediate stages; and (iii) a turnover in species composition occurs along the gradient where different fungi are associated with different parts of the gradient.

Materials and methods

Fieldwork

The study site is a glacier foreland in front of Blåisen, a northeastern outlet valley glacier to the plateau glacier Hardangerjøkulen that is situated in Ulvik municipality, Hordaland County, Southern Norway (60°33′N, 7°25′E). Precisely, dated moraine ridges exist at the study site

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(Nesje & Dahl 1991). Although critical remarks have been made on the use of chronosequence as a substitute for time (Johnson & Miyanishi 2008; Walker *et al.* 2010), the study by Nesje & Dahl (1991) indicates that the study area has followed the same trajectory. The ridges are the result of glacier retreat since 1750, which is known as the peak of 'the little ice age'. This enabled us to indirectly study fungal colonization over a 250-year time span. The glacier foreland was divided into four zones based on the dated moraines (Fig. 1). We established three parallel transects in the glacier foreland across the dated moraines from the glacier forefront (zone 1) to the fully established alpine vegetation outside the 1750 moraine (zone 4). The deglaciation of zone 4 might have happened long before the little ice age, because the moraine was deposited when the glacier reached its maximum extent. Hence, zone 4 can probably be considered as representing the climax vegetation in the area. Along each transect, we sampled five plants (including their root systems embedded in soil) at random within each zone, totalling to 60 plants. Soil was sampled approximately one metre away from each sampled plant for determination of pH and organic soil content (measured as loss on ignition; Table S1, Supporting information).

Morphometric data, such as shoot height and root size, were obtained from each sampled plant (Table S1, Supporting information). To prevent degradation of fungal DNA within the plant roots before DNA extraction, plants were stored at 4°C and treated within 36 h after sampling. We first rinsed the plant root systems in tap water to remove visible soil and plant debris and removed roots not attached to the plant rhizome. Subsequently, the plant roots were rinsed thoroughly in distilled autoclaved water one time for at least 5 min. All roots were then cut off and transferred to Eppendorf tubes containing cetyltrimethylammonium bromide (CTAB) lysis buffer for DNA extraction. Fresh root weights were obtained by weighing the Eppendorf tubes before and after adding the root systems. Entire root systems were stored in CTAB buffer at –20°C until DNA extraction.

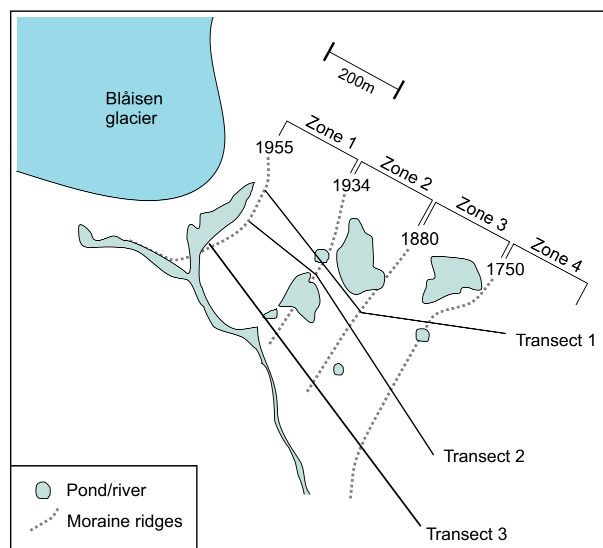

Fig. 1 Schematic illustration of the glacier foreland in front of Blåisen glacier displaying the three transects along the chronosequence. The moraine ridges indicate the boundaries between the four identified zones. Along each transect, five replicate root systems were collected from each zone, yielding a total of 60 samples.

Molecular analyses

We extracted the DNA from the entire plant root systems using CTAB extraction (Murray & Thompson 1980; Gardes & Bruns 1993). Samples were prepared for 454 pyrosequencing by performing nested PCR amplification using the fungal-specific primers ITS1F and ITS4 (White *et al.* 1990; Gardes & Bruns 1993) in the first step and fusion primers including ITS5 and ITS2 (White *et al.* 1990) in the nested step. Fusion primers were constructed by adding five different 6 bp unique tags and 454 pyrosequencing adaptors A and B to both ITS5 and ITS2, respectively. Tags were at least 3 bp different from each other in both directions. PCR was performed in 20 μL reaction volumes containing 2 μL template DNA and 18 μL reaction mix. Final concentrations in the PCR were 0.16 mM dNTP mix, 0.2 μM of each primer and 0.4 units Finnzymes Phusion polymerase. The amplification programme for both steps of the nested PCR was as follows: 30 s at 98 °C, followed by 30 cycles of 10 s at 98 °C, 20 s at 55 °C, 20 s at 72 °C and a final extension step at 72 °C for 7 min before storage at 4 °C. PCR products were cleaned with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), quantified using a bioanalyzer (Algilent 2100) and pooled into 12 equimolar amplicon libraries. Notably, samples from different zones were included in the different libraries. GS FLX sequencing of the tagged amplicons was performed at the Ultra-high Throughput Sequencing Platform at the University of Oslo using 12 lanes of one 454 plate. We included two negative controls during the entire analyses from the DNA extraction step. The raw data have been accessioned in the European Nucleotide Archive, accession no. SRP006836.1.

Bioinformatics and statistical analyses

We analysed the raw data, including 119 054 reads, using CLOTU (Kumar *et al.* 2011), a newly developed

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bioinformatics pipeline available at http://www.bioportal.uio.no. Reads with more than two sequencing errors in the forward primer and tag were removed from the data set. Sequences among the full-length reads that had non-congruent tags were also removed. After filtering short (<150 bp) and low-quality reads (all reads including one or more *N*'s), there were 101 870 remaining reads of which 20 700 were identified as unique.

The sequences were clustered into operational taxonomic units (OTUs) as a crude approximation of species, using BLASTCLUST, with the requirement of 75% overlap in sequence length between reads in the pairwise alignments. Two different cut-off values for sequence similarity (98.5% and 97%) were used during clustering. In studies of fungal diversity based on the ITS region, a 97% cut-off level has often been used (Smith *et al.* 2007; Bjorbækmo *et al.* 2010; Mohamed & Martiny 2010; Tedersoo *et al.* 2010). However, because of extensive inter- and intraspecific ITS divergence across species (Nilsson *et al.* 2008), no generally preferable cut-off level exists. The study by Ryberg *et al.* (2008) indicated that 97% might be a too conservative cut-off level leading to a massive pooling of species. In Fig. S1 (Supporting information), the effect of different similarity levels (80-100%) on the number of obtained clusters is illustrated, which shows that below 99% sequence similarity, the number of non-singleton clusters is not changing dramatically. With respect to the purpose of this study, in which the changes in the community structure along the chronosequence are emphasized, we would argue that it is less detrimental to split species having the same ecology (i.e. to use a strict cut-off level) than to group species with divergent ecological requirements. Hence, in the following, we put most emphasis on the results obtained using 98.5% sequence similarity, in line with Wallander *et al.* (2010).

We obtained a high number of singletons, OTUs including only one read. The debate over singletons in 454 data representing true diversity or sequencing errors is important and still not settled (Quince *et al.* 2009; Tedersoo *et al.* 2010). We have not addressed this topic specifically, but deleted all singleton OTUs from downstream analyses, as recommended by, e.g., Tedersoo *et al.* (2010). In CLOTU, one representative sequence from each OTU was submitted to BLASTn (Altschul *et al.* 1997) for comparison against the GenBank non-redundant (NCBI-nr) database. The interpreted taxonomy of the OTUs follows the NCBI taxonomy of 2009.

We used ANOVA, as implemented in R (R Development Core Team 2008), to test whether the number of OTUs per root system deviated from an even distribution across vegetation zones. A generalized linear model was applied to analyse the relationship between number of OTUs in root systems (response) and environmental and plant morphometric variables as explanatory variables. We included the number of sequences per root system as a covariate to correct for putative bias because of uneven sampling. Species accumulation curves and extrapolated species richness were calculated according to the approach given in Ugland *et al.* (2003). Number of shared OTUs between root systems within and across the four different vegetation zones was calculated in EstimateS (Colwell 2009), using the similarity index Chao-Sørensen (Chao *et al.* 2005).

The structure of fungal communities was examined by multivariate analyses of all non-singleton OTUs (470 OTUs) detected in the 60 plants. Two ordination methods, detrended correspondence analysis (DCA) (Hill 1979; Hill & Gauch 1980) and global non-metric multidimensional scaling (GNMDS) (Kruskal 1964a,b; Minchin 1987), were applied in parallel to describe patterns of variation in fungal OTU composition. The ordination analyses were conducted on raw data, 4th root-transformed data and presence/absence data. In addition, a separate GNMDS analysis was conducted on a presence/absence data set only including presumably ECM OTUs (i.e. OTUs with best Blast matches against ECM fungi). Axes identified by both methods are likely to represent true structure in the data, while axes not identified by both methods may or may not represent true gradients in fungal OTU composition (Økland 1996). Correspondence between ordination axes was determined by calculating pairwise Kendall's rank correlation coefficients (τ). DCA and GNMDS were performed using the packages vegan (Oksanen *et al.* 2009) and MASS (Venables & Ripley 2002) implemented in software R (R Development Core Team 2008) and using the Bray–Curtis measure of dissimilarity (Bray & Curtis 1957). Vectors for relevant predictors (zone, pH and root weight) were fitted to the best GNMDS solution, and their significance was assessed by a correlation test.

The multivariate analysis of variance (PERMANOVA) (Anderson 2001; McArdle & Anderson 2001) was conducted to test for differences in community structure using distance from glacier as a fixed factor with four levels that corresponded to the zones along the chronosequence. The analysis was conducted on presence/absence data, using pairwise tests between each zone with 4999 permutations.

Results

Data characteristics

After filtering out low-quality and short sequence reads, 101 870 reads were retained for further analyses. We obtained between 437 and 5063 reads from each of the

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60 root systems. Using a 98.5% similarity cut-off for clustering, the reads grouped into 1663 OTUs of which a high number (1193) were singletons. However, the 1193 singletons only made up 1.6% of the total number of reads. Clustering using 97% sequence identity returned also a high number of singletons (>50%). After all singletons had been discarded, 470 OTUs remained and were used in the further analyses. A few OTUs accounted for a large fraction of reads. The ten most common OTUs made up 58% of the reads, while many OTUs were represented by few reads (Fig. 2a). The frequency of the 470 OTUs across the 60 root systems varied between 1 and 47 (Fig. 2b). Notably, we found no significant relationship between the number of OTUs and the number of reads per root system ($P > 0.05$).

Taxonomic coverage

All sequences had best BLAST matches to fungi, indicating that the implemented primer pairs were highly fungal specific. Seventy-one percentage of the 470 OTUs belonged to Basidiomycota and 19% to Ascomycota (Fig. 3). The number of OTUs detected from Zygomycota, Chytridiomycota and Glomeromycota constituted 2.3% each. A vast majority (~90%) of the reads represented Basidiomycota, as did also most of the abundant OTUs, both in numbers of sequences and occurrences across root systems. Agaricales, Thelephorales and Sebacinales were the most frequent orders encompassing 31%, 17% and 9% of the OTUs, respectively. The abundance of the twelve most common fungal groups at a genus/family level in the four zones is shown in Fig. S2 (Supporting information). Most groups were detected in all zones, but especially, *Hebeloma* and *Laccaria* were far more abundant in zone 1 compared to the other zones. On the other hand, *Russula* and *Lactarius* were almost absent in zone 1. A total of 28 OTUs were widespread by definition of being present in more than 10 root systems (Table 1). The most widespread OTU, detected in 47 of 60 root systems and accounting for a total of 27 776 reads, had 95% pairwise similarity to a GenBank accession (AY669673) of *Cortinarius rubricosus*. The second most widespread OTU, detected in 32 of 60 root systems and represented by 4601 reads, showed 96% pairwise similarity to an accession of *Hebeloma mesophaeum*

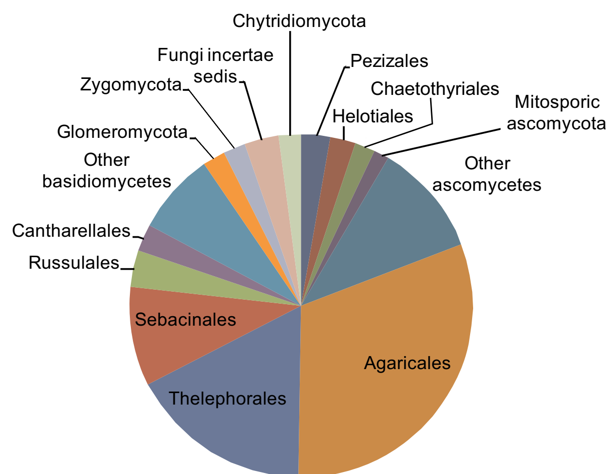

Fig. 3 Taxonomic distribution of the 470 operational taxonomic units mainly at the order level, according to the top BLAST hits against the NCBI database.

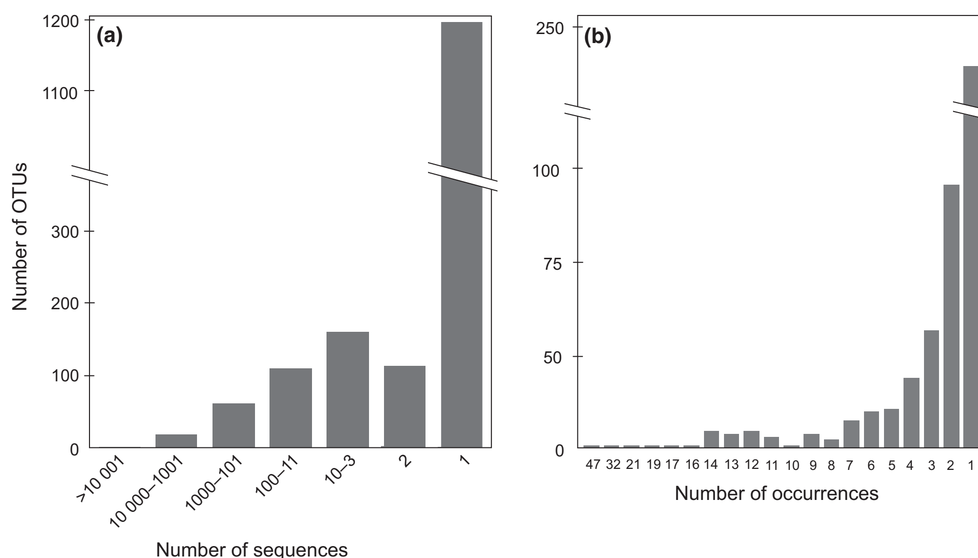

Fig. 2 (a) Distribution of number of reads within the 1663 identified operational taxonomic units (OTUs), singletons included. (b) Number of occurrences of the 470 OTUs across the 60 root systems.

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Table 1 The 28 most frequently detected operational taxonomic units in term of number of root systems, their taxonomic affiliation and distribution

Top hit	Taxonomic group	Cov.*	Sim.†	Freq.‡	Zone 1§	Zone 2	Zone 3	Zone 4
Cortinarius rubricosus	Agaricales (B)	98	95	78.3	46.7	86.7	100.0	80.0
Hebeloma mesophaeum	Agaricales (B)	100	96	53.3	40.0	53.3	53.3	66.7
Gyoerffyella sp.	Mitosp. Asco. (A)	95	98	35.0	26.7	53.3	40.0	20.0
Zygomycete	Zygomycota (Z)	93	98	31.7	33.3	33.3	20.0	40.0
Spadicoides bina	Sordariomycetes (A)	99	80	28.3	33.3	26.7	33.3	20.0
Hydnum rufescens	Chantarellales (B)	27	94	26.7	20.0	20.0	6.7	60.0
Lactifluus griseus	Russulales (B)	100	93	23.3	26.7	20.0	13.3	33.3
Tilletia barclayana	Tilletiales (B)	21	100	23.3	26.7	6.7	20.0	40.0
Inocybe egenula	Agaricales (B)	100	94	23.3	20.0	13.3	13.3	46.7
Sebacinaceae sp.	Sebacinales (B)	99	87	23.3	20.0	26.7	33.3	13.3
Thelephoraceae sp.	Thelephorales (B)	100	96	23.3	20.0	33.3	20.0	20.0
Tetracladium furcatum	Mitosp. Asco.	95	98	23.3	13.3	26.7	20.0	33.3
Geopora cervina	Pezizales (A)	83	91	21.7	13.3	26.7	40.0	6.7
Laccaria montana	Agaricales (B)	100	96	21.7	20.0	33.3	6.7	26.7
Russula pascua	Russulales (B)	99	99	21.7	20.0	6.7	33.3	26.7
Xenostigmina zilleri	Capnodiales (A)	94	93	21.7	20.0	46.7	13.3	6.7
Mortierella sp.	Mucoromycotina	90	98	21.7	20.0	20.0	20.0	26.7
Tomentella badia	Thelephorales (B)	99	94	20.0	26.7	6.7	13.3	33.3
Tomentella atromentaria	Thelephorales (B)	99	96	20.0	26.7	20.0	26.7	6.7
Inocybe curvipes	Agaricales (B)	96	90	20.0	26.7	13.3	20.0	20.0
Phialocephala fortinii	Helotiales (A)	100	98	20.0	13.3	26.7	26.7	13.3
Leohumicola minima	Leotiomycetes (A)	95	94	20.0	33.3	33.3	6.7	6.7
Typhula variabilis	Agaricales (B)	100	94	20.0	13.3	33.3	20.0	13.3
Inocybe egenula	Agaricales (B)	100	92	18.3	20.0	13.3	13.3	26.7
Mycena amabilissima	Agaricales (B)	35	92	18.3	13.3	20.0	13.3	26.7
Thelephora americana	Thelephorales (B)	100	96	18.3	13.3	6.7	33.3	20.0
Cladophialophora minutissima	Chaetothyriales (A)	95	96	18.3	6.7	13.3	13.3	40.0
Cortinarius sp.	Agaricales (B)	100	99	16.7	13.3	13.3	26.7	13.3

*Sequence coverage in BLASTn search.
†Sequence similarity in BLASTn search.
‡Per cent occurrence across the 60 root systems.
§Per cent occurrence across the 15 root systems in each vegetation zone.

(FJ845404). Although most ECM fungi were detected, some putative root pathogens appeared. For example, one frequently occurring OTU detected in all zones with taxonomic affinity to *Tilletia* (Table 1) could represent a systemic pathogen.

Fungal diversity along the primary succession gradient

The number of OTUs increased significantly along the primary succession gradient from 163 in zone 1 closest to the glacier to 179, 205 and 213 in zones 2, 3 and 4 (chi-square test, $P = 0.038$), respectively. Species accumulation curves were calculated for the four vegetation zones, separately (data not shown) and combined (Fig. 4). When the number of analysed root systems was extrapolated to 1000, we estimated 596 OTUs to appear in zone 4, followed by 557, 489 and 452 in zones 3, 2 and 1, respectively. Regarding average number of observed OTUs per root system, no significant difference was observed across the zones (ANOVA, $P = 0.36$). Variation in the number of OTUs per root systems was not explained either by zone, pH, organic soil content or root size (GLM, $P > 0.05$).

Changes in community structure along the primary succession gradient

On average, 2.8 OTUs were shared between pairs of root systems (Table S2, Supporting information). However, there was a significant difference in average number of shared OTUs between pairs of root systems within and across vegetation zones (ANOVA, $P < 0.0001$). Within the four zones, the average number of OTUs shared between pairs of root systems was 3.1–4.2, while the corresponding number between pairs in different zones was 2.0–3.8.

In spite of the general low overlap in OTUs between root systems, the GNMDS and DCA ordination analyses

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based on presence/absence data revealed a structure in the fungal community composition along the chronosequence (GNMDS plot shown in Fig. 5). The GNMDS ordination axis 1 was strongly correlated with the corresponding DCA axis 1 (Kendall's τ = 0.50). The first axis in the GNMDS plot largely reflects the primary succession gradient, where the plant roots largely distribute according to zones (Fig. 5). However, the plant roots from the climax vegetation (zone 4) deviate from this pattern and are widely dispersed along GNMDS axis 2. In the GNMDS analysis, the factors zone, pH and root weight all had significant effects on the ordination configuration (all with $P < 0.001$, Kendall's τ = 0.3 for root weight, 0.4 for pH and 0.4 for zone), where high pH values and large root systems are associated with the early part of the gradient (Fig. 5). GNMDS and DCA ordination analyses of raw data and 4th root-transformed data matrices largely revealed similar patterns, although less structure was observed in these analyses (data not shown). Furthermore, a separate GNMDS analysis including only OTUs with a presumably ectomycorrhizal nutritional mode gave also a similar pattern (Fig. S3, Supporting information). In a PERMANOVA analysis, the effect of zone was also found to be significant in comparisons among all possible pairs of zones, $P < 0.05$ (data not shown).

In Fig. S4 (Supporting information), the frequency distributions of the 36 most frequent OTUs across the zones are shown, as measured by presence/absence data in root systems. Ten OTUs were significantly deviating from a homogeneous frequency distribution (chi-square tests, $P < 0.05$). An OTU with 94% identity to an accession of *Laccaria montana* and another with 96% identity to an accession of *Thelephora americana* showed a clear affinity for the early part of the gradient (Fig. S4, Supporting information). Several OTUs had a distinct peak in zone 2 and 3 while a few, including

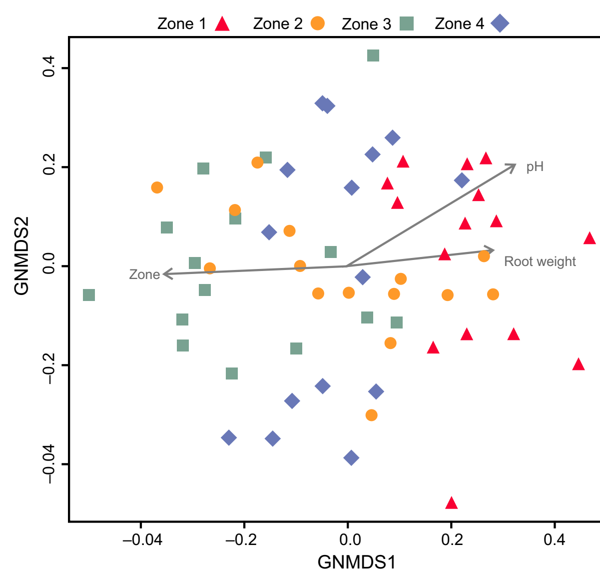

Fig. 5 GNMDS ordination diagram (axes 1 and 2) for the fungal operational taxonomic unit composition in the 60 root systems. The arrows point in direction of maximum increase in the three variables pH, zone and root weight. These factors had significant effects on the ordination configuration.

one with 96% identity to an accession of *Sebacina vermifera*, were significantly more abundant in the established vegetation (zone 4).

Discussion

Taxonomic coverage

Basidiomycetes were the dominant group of fungi in the *B. vivipara* root systems along the chronosequence, with respect to both number of OTUs and number of sequences. Interestingly, the basidiomycete OTUs had,

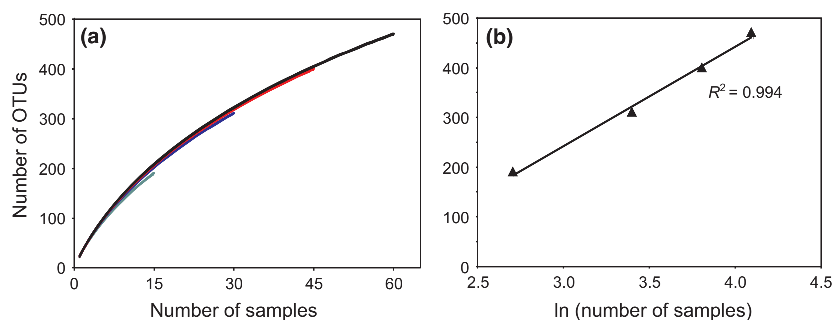

Fig. 4 (a) Accumulation curves of operational taxonomic units (OTUs) against sampling effort calculated according to the approach introduced by Ugland *et al.* (2003). The increasing sequence of accumulation curves, given by different colours, represents the accumulation curves based on an increasing random subset of samples: in our case 15, 30, 45 and 60. (b) The total species (TS) curve is defined as the function passing through the endpoints of the different accumulation curves depicted in (a), showing an almost linear relationship between ln (number of samples) and number of OTUs.

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in general, a far higher number of reads per OTU compared to the ascomycetes that might indicate that basidiomycetes are dominant in terms of mycelial biomass as well. This speculation rests on the assumption that there is a correlation between numbers of sequence reads and the initial mycelial biomass present in the samples. Although this relationship was not tested, we consider such a correlation plausible, although taxonomic biases certainly are introduced during extraction, PCR and 454 sequencing (Feinstein *et al.* 2009; Bellemain *et al.* 2010; Tedersoo *et al.* 2010). A low number of OTUs had best BLASTn match against Zygomycota, Glomeromycota and Chytridiomycota, suggesting that non-dikarya fungi are present in low frequencies in the root systems. Freeman *et al.* (2009) found dominance of chytrids in the fungal communities in high elevated and recently de-glaciated soils, in contrast to the taxonomic distribution observed in our study. However, Freeman *et al.* (2009) obtained their samples from pure soil, where chytrids may be more dominant. In theory, some of the chytrids detected in our study could be loosely associated with the roots. The employed ITS primers might also mismatch against chytrid fungi leading to a lower representation of these fungi.

As judged by the BLASTn matches, most of the frequent OTUs, in terms of both number of sequences and number of occurrences in the 60 root systems, had affinity with well-known ectomycorrhizal fungi in the orders Agaricales, Thelephorales, Sebacinales and Russulales. These ECM groups have also earlier been demonstrated to be associated with *B. vivipara* (e.g. Sønstebø 2002; Mühlmann *et al.* 2008). Among the ascomycetes, we detected some widespread OTUs with putative ectomycorrhizal ecology, such as *Geopora* and *Leohumicola*. Furthermore, OTUs with affinity to widely distributed dark septate endophytes like *Phialocephala fortinii* were detected frequently. These fungi might also have a mycorrhizal and beneficial function for the host plant in these habitats (Jumpponen 2001; Newsham *et al.* 2009; Newsham 2011).

Notably, OTUs with affinity to aquatic hyphomycetes (Ingoldian fungi) like *Articulospora tetracladia* and *Tetracladium furcatum* were widespread, appearing in 21 and 14 root systems, respectively. It has earlier been shown that aquatic hyphomycetes might have a plant endophytic stage during their life cycle (Sridhar & Bärlocher 1992). Hence, there might be a possibility that these Ingoldian fungi have an endophytic stage in the *B. vivipara* roots.

Changes in diversity

Most studies on diversity patterns along primary succession gradients have focused on aboveground plant species richness (Matthews 1978; Vetaas 1994; Freynot *et al.* 1998; Raffl *et al.* 2006) or animals (Kaufmann 2001; Hodkinson *et al.* 2004; Hågvar 2010). In several of these studies, the highest species richness has been observed at intermediate stages of succession (Matthews 1978; Vetaas 1994; Raffl *et al.* 2006). A general trend observed in these studies is the colonization of newly exposed areas by a few opportunistic species, followed by an increase in species richness associated with an increase in accumulated resources (biomass), number of trophic levels and intensity of biotic interactions. At later stages, when the ecosystem approaches a mature stage, strong competitors may dominate, resulting in a decline in species richness. The richness of root-associated fungi, as observed in this study, does not follow this pattern. Fungal richness is relatively high already in the recently exposed areas, followed by a weak, but significant increase in total richness towards more established ecosystems. This result is similar to the findings of Schütte *et al.* (2010) where they observed that diversity of soil-inhabiting bacteria in an arctic glacier forefront was generally high and increased significantly along the gradient. Competition might be a less important factor for limiting the richness of micro-organisms in a climax system compared to macro-organisms, as more niches could be available. Our results do not support earlier proposed models of fungal primary succession (Jumpponen *et al.* 2002; Nara *et al.* 2003a,b), which postulate that only few ruderal and r-selected species are able to colonize root systems in the newly exposed areas by spores and that with time, they are joined and sometimes replaced by additional, more competitive species. However, these studies were conducted using fruit body surveys, which give limited information about the extant fungal diversity. Moreover, our results suggest that the species richness patterns of fungal communities are more similar to those of bacteria, and probably other microbes, compared to those of the macrobiota, i.e. plants and animals.

Community structure

A high patchiness in the distribution of fungal OTUs was observed, which could indicate that the colonization process has a strong stochastic element. High patchiness seems to be a common feature in most investigated fungal communities (Horton & Bruns 2001; Jumpponen *et al.* 2002; Lilleskov *et al.* 2004; Stukenbrock & Rosendahl 2005; Taylor *et al.* 2010). However, in several of these studies, it is argued that the patchiness may be due to under-sampling (Lilleskov *et al.* 2004; Taylor *et al.* 2010). Our results, including a high number of sequences per root system, as well as a fairly high number of samples (60), indicate that this pattern

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rather reflects the extant spatial distribution of fungi in this habitat. In support of this, no significant relationship was observed between number of sequences per root system and number of OTUs, indicating that the root systems were not under-sampled. Our results largely corroborate those of Jumpponen (2003) who found very high heterogeneity of soil fungi in a glacier forefront. He concluded that stochastic dispersal events are important during the assembly of early fungal communities. In line with this, Dumbrell *et al*. (2010) showed that stochasticity is important, even in root-associated microbial communities of closed and mature vegetation.

Despite that only a few OTUs were shared across root systems and that most of the frequent OTUs did not have a clear affiliation to any zone (Table 1), the ordination analyses revealed a structure in the fungal community composition that correlated with time since deglaciation. However, the plant roots from zone 4 deviated from this pattern, which supports the hypothesis that the climax vegetation in zone 4 probably is significantly older than the other zones and might not have been glaciated for the last thousands of years. The three factors that were related to the ordination configuration, i.e. zone, pH and root weight, more or less paralleled ordination axis one that reflected the chronosequence. pH and root weight decreased along the gradient towards more continuous vegetation, while organic soil content increased.

Among the 36 most frequent OTUs, some had distinct affinities with the most recently exposed zones, a few more to the intermediate stages (zones 2 and 3), while relatively few OTUs peaked in the latest zone. However, almost all (92%) of the frequent OTUs were found in either three or all zones, demonstrating that the OTUs not have a distinct connection to just a part of the chronosequence. Among the early pioneer species were two OTUs with taxonomic affinity with *Laccaria montana* and *Thelephora americana*, respectively. *Laccaria* is from beforehand known to include pioneer species appearing early during primary successions (Nara *et al*. 2003a,b).

Conclusions

Our results demonstrate that a high fungal diversity, dominated by basidiomycetes, was present in the root systems of *B. vivipara* occurring in the most recently exposed areas. The total fungal diversity increased slightly but significantly towards the climax vegetation. There was a high patchiness in the fungal community structure, indicating that stochastic processes are important during fungal colonization and community development. One putatively important factor here is aerial random spore dispersal. In spite of the high patchiness, there was a systematic turnover in species composition along the chronosequence.

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This work forms a part of R.B.'s PhD thesis focussing on molecular ecology of ECM communities. S.K. is a PhD student working on bioinformatics applications for genomics data. G.F. and K.I.U. are interested in biodiversity analyses in general and of benthic fauna in particular. R.H. is mainly focusing on plant community structure at different spatial scales. T.C. and H.K. are working on different issues within fungal molecular ecology.

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Data accessibility

The raw 454 data have been accessioned in the European Nucleotide Archive, accession no. SRP006836.1. The representative sequences of the 470 non-singleton OTUs are available in the supporting information in fasta format.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 The relationship between numbers of clusters obtained and sequence similarity level (80-100%) used during clustering of sequences.

Fig. S2 The abundance of the 12 most abundant genera/families across zones, as measured by presence/absence of OTUs in root systems and best blast hit.

Fig. S3 GNMDS ordination diagram (axes 1 and 2) for the composition of fungal OTUs in the 60 root systems, including only OTUs with best blast match to ectomycorrhizal taxa.

Fig. S4 Frequency distributions of the 36 most abundant OTUs as measured by presence/absence in root systems.

Table S1 Characteristics for the 60 root samples.

Table S2 Number of shared OTUs across root systems.

Table S3 Representative sequences of the 470 non-singleton OTUs.

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