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Decaying *Picea abies* log bark hosts diverse fungal communities

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ABSTRACT

We examined taxonomic composition of fungal communities in *Picea abies* log bark using next generation sequencing. Three successional stages along gradients of log attributes were identified. In the initial stage, the communities were composed by yeasts, plant pathogens and cosmopolitan saprotrophic fungi with broad substrate utilization. In the intermediate stage, bark was colonized mainly by saprotrophs common in decaying wood, symbionts of epixylic plants and nematode-trapping fungi. The final stage was characterized by the dominance of mycorrhizal fungi. Wood-decaying fungi occurred in all stages. However, their sporadic appearance in bark samples suggests that they are not essential for bark decomposition. Our results provide an insight into the hidden diversity of wood-inhabiting communities – fungal communities, associated with decomposition of bark as a component of coarse woody debris.

1. Introduction

Information on the structure and successional dynamics of wood-inhabiting fungal communities is needed to develop proper strategies for their conservation (Heilmann-Clausen et al., 2015). Furthermore, understanding how the structure of wood-inhabiting fungal communities affects rate of decomposition of coarse woody debris (CWD) and its components links biodiversity and ecosystem functioning (Valentín et al., 2014; Hoppe et al., 2015).

Tree bark makes up to 25% of the stem volume and 16% of the stem dry mass of main boreal tree species (Ugolev, 2002; Lestander et al., 2012). After tree death, all bark tissues are subjected to decomposition when fallen off, or as a component of woody debris (Harmon et al., 1986). The process of bark decay is much less studied than decomposition of wood (Shorohova and Kapitsa, 2014; Shorohova et al., 2016) partly because of the highly complex, heterogeneous structure of bark (Corder, 1976; Harmon et al., 1986). Resistance of spruce (*Picea*) bark to pests and phytopathogens is provided by a combination of the mechanical properties of lignified cell walls and calcium oxalate crystals, the hydrophobic barrier of suberized cells, as well as by phenolic and terpenoid substances for chemical defence (Franceschi et al., 2005).

In naturally decomposing Norway spruce (*Picea abies*) bark, the cellulosic parenchymatous cells decay rapidly and extensively (Parameswaran and Wilhelm, 1979). Decay of lignified cells is noticeable after the second year of exposure, with characteristics similar to soft and white-rot in wood. Suberized cells start to decompose in the third year of exposure. Hydrolysis of the phenolic compounds begins later suggesting that the whole bark decomposition period may be extended by several more years (Parameswaran and Wilhelm, 1979). In *in vitro* experiments, milled Scots pine (*Pinus sylvestris*) bark and water bark extracts added to a standard nutrient medium had a significant inhibitory effect on the growth ability of wood-decayers (Rypacek, 1966). However, some fungi have adapted to the spruce chemical defence so that they are even able to use phenolic compounds as a sole carbon source (Hammerbacher et al., 2013).

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In forest ecosystems, because of differences in chemical and anatomical structure of bark and wood (Corder, 1976; Polubojarinov and Sorokin, 1992, 1997), different structures and compositions of microbial communities can be expected. In addition to wood-decaying fungi, and detritivores common in both wood and bark, the communities associated with bark may also include fungi unable to breakdown ligno-cellulose (Stokland et al., 2012), as well as epiphytic and endophytic microorganisms capable of using bark constituents as substrates (Stone et al., 2000; Schmull and Hauck, 2003). There are also propagules of incidental transient microorganisms. Some of the microorganisms associated with bark can protect a tree against phytopathogens and herbivorous invertebrates through induced resistance, nutrient acquisition or toxin production (Saikkonen et al., 1996; Vega et al., 2008). Entomopathogenic fungi (e.g. *Beauveria bassiana*, *Verticillium lecanii, Paecilomyces farinosus*) have been isolated from the bark of living trees, which may provide evidence of their mutualistic interactions and the ability for interim endophytic growth (Bing and Lewis, 1991; Stone et al., 2000). Some fungi can cause important bark diseases in living trees (Schmidt, 2006).

It can be assumed that the succession of bark mycobiota is to a large extent driven by the transmission of fungal propagules by insects. Bark beetles and other phloeophagus and xylophagous insects facilitate fungal colonisation of both wood and bark (Giordano et al., 2012). The composition of fungal communities associated with both bark and wood may be related to the succession of epixylic vegetation on logs which advances through time since tree death (Shorohova et al., 2016).

Our general knowledge of communities of microorganisms associated with bark is limited. There are scattered reports of species associated with the bark of a few tree species, located in a limited number of regions (Stone et al., 2000; Stokland et al., 2012) identified using either fungal fruit body surveys or traditional culture-based microbiological methods. However, most of the microorganisms can be revealed only with molecular methods (Buée et al., 2009; Stokland et al., 2012; Su et al., 2012).

Norway spruce is one of the dominant boreal forest tree species in Europe. The succession of fungal communities during decomposition of spruce wood have been relatively well studied using molecular and microbiological methods (Allmér et al., 2006; Kubartová et al., 2012; Ovaskainen et al., 2013). Most fungi used as indicators of old-growth forests are associated with decomposing spruce logs (Kotiranta and Niemelä, 1996). However, the pattern of fungal diversity and its change during decomposition of spruce CWD remains incomplete without data on the species associated with bark.

We aimed to identify the taxonomic composition of fungal communities associated with spruce bark using next-generation DNA sequencing, which facilitates better detection of non-cultivable or hardly identifiable microorganisms than using fruit body surveys or culture-based methods. We hypothesize that the composition of bark-associated fungal communities is subject to succession related to changes in the physical characteristics of bark.

2. Materials and methods

2.1. Study area

Bark samples were collected on the 23–27th of June 2015 in the middle boreal old-growth forest located in the State Strict Nature Reserve 'Kivach' in the Republic of Karelia, Russia (62°28′N, 33°95′E). The climate is characterized by a mean annual temperature of +2.4 °C, a growing season of 90 d, and mean annual precipitation of 625 mm (Skorohodova, 2008).

The forest stands are Norway spruce (*P. abies*) dominated with an admixture of silver and downy birches (*Betula pubescens* and *Betula pendula*), trembling aspen (*Populus tremula*) and Scots pine (*P. sylvestris*). The forest type is *Piceetum oxalidosum* with patches of *Piceetum fontinale* and *Piceetum oxalidoso-myrtillosum* (Fedorchuk et al., 2005). The soils are classified as humic-gley and superficially eluvial gleish sandy-loamy and loamy (Fedorets et al., 2006). In the year of inventory, the tree stand volume was 347 m^3 ha^{-1} and the volume of CWD was 52 m^3 ha^{-1}. See Shorohova et al. (2016) for details.

2.2. Sampling

The bark attached to 20 logs of Norway spruce from 20 to 50 cm in diameter at a length of 1.3 m from root collar (DBH) was sampled. The age of trees before death ranged from 80 to a few hundred years old. The dating of CWD pieces (identifying time since tree death for trees that had died in the previous 41 y) was conducted using the dendrochronological methods of cross-dating, growth release patterns and evaluating mechanical scars on neighboring trees (Dynesius and Jonsson, 1991). The control bark samples were taken from trees that died in the current year or one year before sampling. The cover of bark remaining on the log characterizing bark fragmentation (F, %) was visually estimated to account for fragmentation.

3-4 bark samples of approximately 1–3 cm^2 were taken by hatchet and forceps from 0 to 3 m from the stem base of each log (at 1.3 m, when possible) and measured in three dimensions. These samples were used to determine physicochemical parameters. Approximately 20–25 cm^2 of bark was also collected from adjacent areas of the log for subsequent molecular studies. Necessary precautions to avoid contamination and cross-contamination of the samples were applied: all instruments were sterilized between collections with blowtorch flame; the samples were packed in sterile polypropylene tubes.

The decay class for each log was recorded according to the CWD five stage decay class system (Shorohova and Shorohov, 2001; Shorohova et al., 2016). The stage of epixylic succession on decaying logs was identified for the stem base (Shorohova et al., 2016). Those stages were classified using the features of epixylic vegetation as a baseline (Kushnevskaya et al., 2007). The zero stage was characterized by no vegetation growing on a log. The earliest first stage was characterized by a sparse cover of bryophyte-lichen groups. The closed cover of ≥70% of mainly non-epigeous species that are not the dominant ground vegetation species characterized the second stage. Dominance of ground cryptogam species without significant contribution of vascular plants was considered indicative of the third stage. Where wood was overgrown by vascular plants designated the fourth stage.

2.3. Physicochemical analyses

The moisture of bark in % was calculated based on the fresh weight at the time of sampling and constant weight after drying at 103 °C. The fresh mass measurements were made at temperatures ranging from 13 to 18 °C with 18 mm of rainfall, which was accumulated during the measurement period followed by a week without precipitation. The specific mass of bark (mass per unit surface area, g cm^{-2}) was calculated by dividing the dry mass of a bark sample by the surface area of sample. The completely dried samples were coated with paraffin wax and sunk into water. The dry bulk density of bark (g cm^{-3}) was calculated by dividing the dry mass by the dry volume of the sample assessed using water displacement method (Polubojarinov, 1976).

Chemical analysis of bark samples was conducted as follows. The samples were lyophilised at −30 to −40 °C. Mechanical

trituration of the samples by hand-mill using liquid nitrogen (t = −190 °C) was used for tissues fixation. Total carbon (C) and nitrogen (N) concentrations were determined on freeze-dried bark material (1.5–2.5 mg) using a Perkin Elmer 2400 Series II CHNS/O Analyzer (USA), calibrated with the organic analytical standard, acetanilide (Perkin-Elmer № 0204–1121). The pH value was measured in water extraction (ratio bark: water = 1: 25) by potentiometric pH-sensor (Hanna, Germany). All analytical procedures were performed in 2–6 replicates.

2.4. Extraction of DNA

Before molecular studies, the bark samples were frozen with liquid nitrogen and stored at −45 °C. Prior to extraction of DNA, the samples were defrosted and subjected to superficial sterilization with blowtorch flame to reduce the amount of surface contamination by airborne microorganisms. After this samples were homogenized in sterile disposable chambers using a Tube Mill Control (IKA, Germany) grinder.

A part of homogenized sample (240 mg) was transferred to a 2 ml eppendorf tube, and DNA was extracted with the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, USA) according to the centrifugal protocol for plant tissues and fungal mycelium (provided by manufacturer). The DNA concentrations were measured with a Qubit 2.0 (Thermo Fisher Scientific, USA) using a dsDNA HS Assay Kit. Extracted DNA was used for library preparation of universal tailed amplicon sequencing as described for 454 Sequencing System.

2.5. Pyrosequencing and primary data analyses

The taxonomically significant internal transcribed spacer (ITS2) region was amplified with eukaryote-specific ITS3 and ITS4 primers because it was previously reported that they have a good ability to amplify fungal ITS regions (White et al., 1990; Bellemain et al., 2010). Multiplex identifiers (MIDs) were attached to the primer ends to carry out the simultaneous analysis of all samples.

Amplicon library pyrosequencing was performed using the GS Junior sequencer (Roche, USA) in the Core Centrum 'Genomic Technologies, Proteomics and Cell Biology' at the All-Russia Research Institute for Agricultural Microbiology (ARRIAM), St. Petersburg, Russia.

The ITS2 locus reads were processed by QIIME version 1.8.0 (Quantitative Insights into Microbial Ecology; Caporaso et al., 2010). To reduce the number of erroneous sequences and thus increase the accuracy of the whole pipeline, the denoising procedure was employed (Reeder and Knight, 2010). After this, the reads were demultiplexed based on their specific MIDs. Simultaneously, low quality or ambiguous reads were removed and primers were truncated. Fungal ITS2 reads were then established using ITSx (Bengtsson-Palme et al., 2013), which uses hidden Markov models and the HMMER package (Eddy, 2011). The chimeric sequences were detected using the UCHIME algorithm (Edgar et al., 2011) with UNITE database (Kõljalg et al., 2005; Abarenkov et al., 2010). All reads were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the UCLUST method (Edgar, 2010). The representative sequences were chosen according to their abundance between similar reads. The OTUs that had less than two copies over the complete study (singletons) were deleted (Lindahl et al., 2013). All 454-pyrosequencing data from this study is available through the Sequence Read Archive (SRA) (Leinonen et al., 2010) under BioProject **PRJNA378586**.

2.6. Phylogenetic and statistical analyses

Taxonomic assignment of representative sequences was carried out by the Ribosomal Database Project (RDP) Classifier method with minimum confidence 0.8 (Wang et al., 2007) with UNITE database (Kõljalg et al., 2005; Abarenkov et al., 2010). Additionally, Nucleotide-Nucleotide Basic Local Alignment Search Tool (BLASTN) was performed for identification with the use of the GenBank databases (Altschul et al., 1990), when the RDP Classifier method gave unsatisfactory results. When the query coverage was ≥99%, the result was recognized as significant. The query identity of ≥99% was used for positive identification to the species level, and the query identity of ≥98% was considered as probable identification to the species level, denoted with the abbreviation cf. The 97–95% identity was considered as a reliable identification to the genus level.

When possible, the identified fungal taxa were classified by taxonomic groups as well as by functional groups according to their ecological strategies. We were able to distinguish the following groups: pathogens – this group contained plant and invertebrate pathogens; wood-decayers – saprotrophic and biotrophic fungi capable of degrading coarse and fine woody debris; other saprotrophs – non substrate-specific ascomycetous and basidiomycetous fungi that are able to develop on various lignocellulosic residues; lichens – lichenized fungi; mycorrhizal fungi; other fungi – fungi characterized by an uncommon ecological role or by a combination of several life strategies; unknown fungi – the group contained fungi with unknown ecological specialization or with insufficient taxonomic information obtained.

Diversity indexes of communities were obtained with alpha_rarefaction.py workflow in Quantitative Insights Into Microbial Ecology (QIIME), which sequentially generate rarefied OTU tables, compute measures of alpha diversity for each rarefied OTU table, collate alpha diversity results and generate alpha rarefaction plots. The total number of OTUs (OTU richness), as well as the OTU richness and number of reads for each functional and systematic group were counted for each sample.

We modelled the effect of a suite of log attribute predictors on the Shannon index, total OTU richness and OTU richness per sample for fungal systematic and functional groups using generalized linear models (GLMs). We selected our final models based on the lowest Akaike Information Criterion (AICc) and the highest model weight (Burnham and Anderson, 2002). The predictors included time since tree death (TTD), log diameter at 1.3 m (DBH), area-specific mass of bark (SM), bulk density of bark (BD), bark cover (F), log decay class (DC), stage of epixylic succession (SES), bark C/N ratio, bark moisture (M) and bark pH. When classifying DC, classes 4 and 5 were grouped (for more details see Sampling). Correlation between predictors was tested before modelling. The analyses were performed in R software package (R Core Team, 2015).

The Bray-Curtis dissimilarity matrices were calculated using function vegdist. R functions hclust (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/hclust.html) and heatmap.2 (https://www.rdocumentation.org/packages/gplots/versions/3.0.1/topics/heatmap.2) were used for visualisation of heatmap with hierarchical clustering using average linkage based on the estimated matrix indices of relative abundance data (R Core Team, 2015).

To compare fungal community structure the non-metrical multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity matrices was conducted using metaMDS function (vegan, Oksanen et al., 2007). The resulted stress value was 0.1265473. External variables were used to interpret the structure using the vector-fitting procedure and the significance was tested with 999 permutations. The variables tested were the same as in GLMs.

Alignment of representative sequences was carried out with the

use of the MAFFT algorithm G-INS-1 (Katoh et al., 2002). The phylogenetic tree was generated using Maximum Likelihood with Mega 6 (Tamura et al., 2011, 2013).

The phylogenetic and compositional dissimilarity matrices were estimated by beta_diversity.py script in QIIME (Lozupone and Knight, 2005; Caporaso et al., 2010). This data was then used for measuring the strength and significance of sample groupings with Permutational Multivariate Analysis of Variance (PERMANOVA) and Analysis of Similarity (ANOSIM) with script compare_categories.py.

To test the hypothesis that all sample groups identified in this study have equal OTU frequencies, the G-test was performed with QIIME script group_significance.py. Based on the results of the G-test, the OTUs were assigned into three succession groups (SGs). Additionally, the OTUs were selected based on simultaneous maximal parameters of abundance and occurrence within each group.

3. Results

3.1. Library composition and fungal diversity

In this study, 23451 reads were generated for bark samples collected from twenty spruce logs of different decay classes and time since tree death. After quality filtering, denoising and removal of non-fungal and chimeric sequences 18936 reads remained. They were clustered in 435 OTUs; after singletons removal this number decreased to 285 OTUs. The range of sequencing depth was nonuniform across the samples and varied from 235 to 1996 reads.

The average number of observed OTUs in the bark samples was 37 and ranged from 22 to 58. The diversity indexes calculated for those samples ranged from 2.8 to 4.2. The estimated rarefaction curves indicated that the diversity of some samples with low depth of sequencing might be underestimated, although all calculated rarefaction curves were beyond the linear ranges.

3.2. Taxonomic observations

Among all observed OTUs ca. 67.4% belonged to Ascomycota, 24.2% to Basidiomycota and 8.4% were identified only to kingdom level. The proportions by abundance coincided with those by the number of OTUs. The fungal communities were dominated by Ascomycota (68%), whereas Basidiomycota and other fungi represented 30% and 2% of the total abundance, respectively (Suppl. 1). The phylum Ascomycota was represented by 23 orders, and the most abundant were Helotiales (19.1%), Sordariales (13.9%) and Saccharomycetales (10.3%). Helotiales contained 8 identified genera, and the predominant was *Infundichalara* (4.6%). The high number of reads (11.7%) annotated as Helotiales were not identified to the genus level. The abundance of Sordariales was entirely achieved by the presence of genera *Chaetomium* (9.8%) and *Humicola* (2.9%). Saccharomycetales contained 3 estimated genera from which the most abundant was *Nakazawaea* (6.1%). The phylum Basidiomycota was represented by 12 orders, from which the most abundant was Agaricales (13.4%). Among 6 genera identified in Agaricales, the predominant were *Mycena* (7.3%) and *Hypholoma* (1.5%).

Twenty-six percent of OTUs were identified to the genus level and 17% to the species level. The highest incidence (number of samples in which a certain OTU occurred) was determined for OTU98 (*Chaetomium* sp.), which was observed in 18 samples. Other OTUs with high incidence were OTU57 (*Albifimbria* sp. 14 samples), OTU253 (Helotiales sp. 12 samples), OTU395 (*Infundichalara* sp. 11 samples), OTU93 (*Humicola* sp. 11 samples), OTU390 (*Nakazawaea holstii*, 10 samples).

3.3. Relationships between fungi and log attributes

Almost all bark and log parameters related to degree of decomposition such as TTD, SM, C/N, BD, DC, SES, M and F were significantly at $p < 0.05$ either positively or negatively correlated with Pearson's or Spearman's correlation coefficients >0.5 (Suppl. 2). Consequently, due to the correlation among predictors and the small sample size, only one-predictor GLMs were considered. The diversity index decreased with the decrease of C/N ratio in bark. The total OTU richness was negatively associated with bark moisture content and had a parabolic relationship with SM (Table 1). Basidiomycetes showed a relationship with all log

Table 1
Fungal diversity parameters and the number of OTUs by systematic and functional groups related to log characteristics as defined by GLM. 0 – no relationship, ↑ – positive relationship, ↓ – negative relationship, ↑↓ - a parabolic-shape relationship. The link function is indicated for each predictor. All non-zero relationships were significant at the $p < .05$ level. Abbreviations: G – Gaussian variance function; P – Poisson variance function. The best predictors for each dependent variable are denoted with bold font.

Dependent variables	Variance function	Time since tree death (TTD)	Specific mass of bark (SM)	Bulk density of bark (BD)	Bark cover (F)	Log decay class (DC)	Stage of epixylic succession (SES)	Bark C/N	Bark moisture (M)	Bark pH	Log diameter at 1.3 m (DBH)
Diversity											
Shannon index	G	0	0	0	0	0	0	**↑ log**	0	0	0
Total OTU richness	G	0	↑↓ log	0	0	0	0	0	**↓ power (-3)**	0	0
***Systematic groups* (OTU richness)**											
Basidiomycetes	P	↑ identity	**↓ log**	↓ identity	↓ identity	↑ identity	↑ identity	↓ log	↑ log	0	↑log
Ascomycetes	P	0	↑↓ square root	0	0	0	↓ identity	↑ identity	**↓ identity**	↑ log	**↓ power (-3)**
***Functional groups* (OTU richness)**											
Mycorrhizal fungi	P	↑ log	↓ log	↓ log	↓ log	↑ log	↑ log	↓ log	**↑ log**	0	↑log
Saprotrophs	P	0	0	**↓ log**	0	0	0	0	0	0	0
Pathogens	P	0	0	↑ log	↑ square root	0	0	↑ log	**↓ square root**	0	0
Wood-decayers	P	0	0	0	0	0	0	0	0	0	0
Lichens	P	0	0	0	0	0	0	0	**↓ square root**	0	0

attributes, except bark pH. Their OTU richness increased as the bark became significantly decomposed (TTD, DC, SES and M increased, whereas BD, SM, C/N ratio and bark cover decreased, Table 1). The OTU richness of ascomycetes decreased with the increase of SES and M and the decrease of bark C/N and pH. Ascomycetes showed maximum richness at the intermediate values of bark specific mass (Table 1, Fig. 1). Among functional groups, only mycorrhizal fungi were associated with all tested predictors, except pH. Their OTU richness followed the same pattern as that of basidiomycetes (Table 1). The OTU richness of saprotrophs depended only on BD. Pathogens had a positive relationship with BD, bark cover and C/N and a negative relationship with bark moisture content, i.e. were inversely related to bark decomposition. The OTU richness of lichenized fungi was negatively related to bark moisture content. Wood-decayers did not show any dependence on tested predictors. Bark specific mass significantly predicted the total OTU richness, as well as the richness of basidiomycetes, ascomycetes and one functional group – mycorrhizal fungi (Fig. 1).

The heatmap and hierarchical clustering based on the relative abundances of the fungal OTUs allowed grouping of the samples and OTUs, providing distinct patterns of their joint occurrence (Fig. 2). We distinguished three main groups of bark samples, which contained divergent fungal taxonomic characteristics. Noticeably, these groups were related to physical parameters of bark. For example, the first succession group (1st SG) was represented by 8 bark samples with estimated nonoverlapping specific mass values in the range of 0.2–0.3 g cm^{-2}. The second group (2nd SG) was represented by 7 samples with the specific mass of 0.11–0.18 g cm^{-2}. The third group (3rd SG) was represented by 5 samples with the specific mass of 0.03–0.06 g cm^{-2}.

Bark samples were separated along the gradient of physical parameters of the bark (Table 2, Fig. 3). The effect of all tested variables, except DBH and pH, significantly explained bark fungal community structure (Table 2). The differences in fungal communities in bark samples in the three succession groups (1st SG, 2nd SG, 3rd SG) were significant at $p < 0.05$ (Fig. 3, Table 3).

The G-test revealed the OTUs that were significantly related among previously distinguished sample groups (Table 4). In the 1st SG, the fungi from Ascomycota *N. holstii*, *Albifimbria* sp., *Candida* sp., *Chaetomium* sp., *Cladosporium* sp. and *Humicola* sp. prevailed. The ascomycetous OTUs designated as *Hyphodiscus* cf. *brachyconius*, *Cladonia* sp., *Pseudoplectania* sp., *Infundichalara* sp., Pseudeurotiaceae sp., Tympanidaceae spp., Sebacinales sp., Orbiliales sp., Hypocreales sp., Helotiales spp.; basidiomyceteous – *Mycena* spp. and Agaricales sp. belonged to the 2nd SG. In this group, it was also possible to allocate OTU63, OTU111 and OTU384, which were identified only to the Ascomycota phylum. In the 3rd SG, we could designate the OTUs from Ascomycota identified as Dermateaceae sp., Helotiaceae sp. and Ascomycota sp. The OTUs from Basidiomycota were identified as *Tylospora fibrillosa*, *Hypholoma capnoides*, *Mycena* sp. and Agaricales spp.

Not all OTUs passed the classification criteria and were not confidently assigned to any successional group. For example, *Cladophialophora* cf. *chaetospira* (OTU194), by abundance of reads,

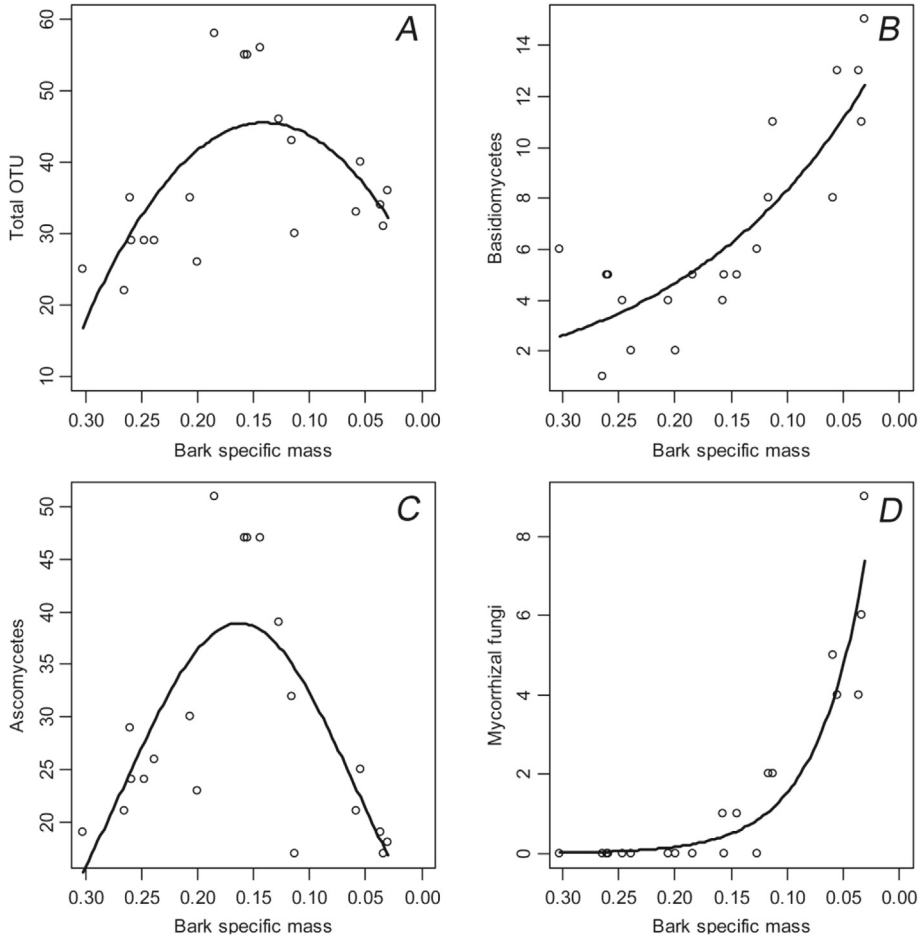

Fig. 1. Response of the total number of OTUs (A), and the number of OTUs by fungal phyla (Basidiomycota (B), Ascomycota (C)) and in one functional group - mycorrhizal fungi (D) - to decreasing gradient of bark specific mass. Estimated GLM (generalized linear model) response curves. Original observations are shown by open symbols.

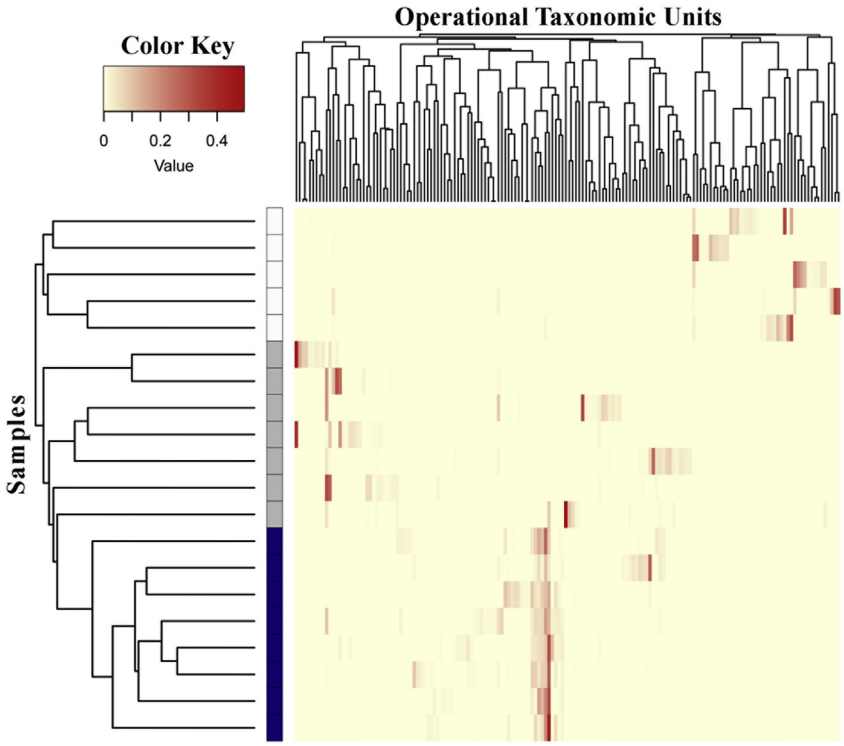

Fig. 2. Heatmap of the abundance of each OTU within each sample hierarchically clustered with UPGMA on the Bray-Curtis dissimilarity distance matrixes for the samples and OTUs. Different colours of vertical scale denote successional groups with individual taxonomic structure (the 1st successional group – blue, the 2nd successional group – grey, the 3rd successional group – white).

Table 2
Determination coefficients (r^2) and significance (p) of fitted vectors with ordination configuration on Fig. 3. Correlation value significant at the $p < .05$ level is denoted by bold font. Abbreviations: TTD – time since tree death; DBH – log diameter at a length of 1.3 m from root collar; DC – log decay class; C:N – carbon - nitrogen ratio; F – bark cover; SM – specific mass of bark; BD – bulk density of bark; M – moisture of bark; SES – stage of epixylic succession; pH – potential of hydrogen.

Variables	NMDS1	NMDS2	r2	Pr (>r)
TTD	−0.87341	−0.48699	0.733	**0.001**
DBH	−0.47128	−0.88198	0.2111	0.132
F	0.97268	0.23215	0.7222	**0.001**
SM	0.99981	0.01949	0.8179	**0.001**
BD	0.91783	0.39697	0.3876	**0.014**
DC	−0.89567	−0.44472	0.6027	**0.001**
SES	−0.84553	−0.53393	0.8392	**0.001**
C:N	0.92917	0.36966	0.5642	**0.001**
M	−0.80617	−0.59169	0.7232	**0.001**
pH	−0.10402	0.99457	0.0249	0.809

prevailed in the 2nd SG (67.7%) versus the 3rd SG (32.3%). However, its occurrence in the 3rd SG (5 samples) exceeded that in the 2nd SG (3 samples). Consequently, it cannot be reliably related to any of the groups. Several taxa such as Helotiales spp. (OTU353, OTU97), Eurotiomycetes sp. (OTU377), *Coniophora olivacea* (OTU90), *Resinicium bicolor* (OTU314), unknown Basidiomycota sp. (OTU104) had ambiguous parameters in several groups. Therefore, it was problematic to classify them into any group despite significant p-values.

The OTUs, which occurred only in one sample, even if they met statistical criteria, cannot provide significant information about their belonging to a certain group. Therefore, we did not include them in Table 4. Some of these were identified to the species level. This identification made it possible to assign them to a certain group based on ecological habitat preferences known from the literature. Such OTUs were in the second group (2nd SG – *Dichostereum granulosum*, *Mollisia minutella*, *Psilocybe medullosa*) and in the third group (3rd SG – *Hyphodontia pallidula*, *Sistotremastrum* cf. *suecicum*, *Tomentella terrestris*, *Russula* cf. *griseascens*) (Suppl. 2).

Other OTUs were identified to the species level and strongly demonstrated occurrence within one succession group, but insignificantly due to a low abundance of reads. In the 1st SG, this part was represented by *Fomes fomentarius*, *Cryptococcus victoriae*, *Fusarium oxysporum, Kuraishia capsulata*, *Ceratocystis polonica*, *Blumeria graminis*, *Ophiostoma penicillatum*, *Trichaptum abietinum*, *Candida nitratophila, Aspergillus* cf. *tamari*. In 2nd SG, such OTUs were *Sistotrema brinkmanni*, *Basidiodendron caesiocinereum*, *Dactylellina mammillata*, *Cladonia coniocraea*, *Eucasphaeria* cf. *capensis*, *Zalerion* cf. *arboricola*. In the 3rd SG, they were *Pseudotomentella mucidula*, *Resinicium furfuraceum*, *Mycena amicta*, *Lactarius vietus*, *Lactarius* cf. *trivialis*, *Pseudotomentella* cf. *mucidula*, *Xeromphalina campanella*, *Amphinema* cf. *byssoides*, *Elaphomyces muricatus*, *Tomentella stuposa*.

The listed OTUs (Table 4, Suppl. 2) give a complete summary of fungal community structure across the defined successional stages. For more information, see Suppl.2.

4. Discussion

4.1. Log attributes influence the diversity and successional changes of fungal communities, associated with bark

Wood decay stage, density, moisture and C:N ratio influenced fungal diversity and community structure in *P. abies* wood (Rajala et al., 2012, 2015; Ovaskainen et al., 2013; Hoppe et al., 2015). Our results demonstrated that in addition to the above-mentioned wood and bark attributes, bark fungal community changed along

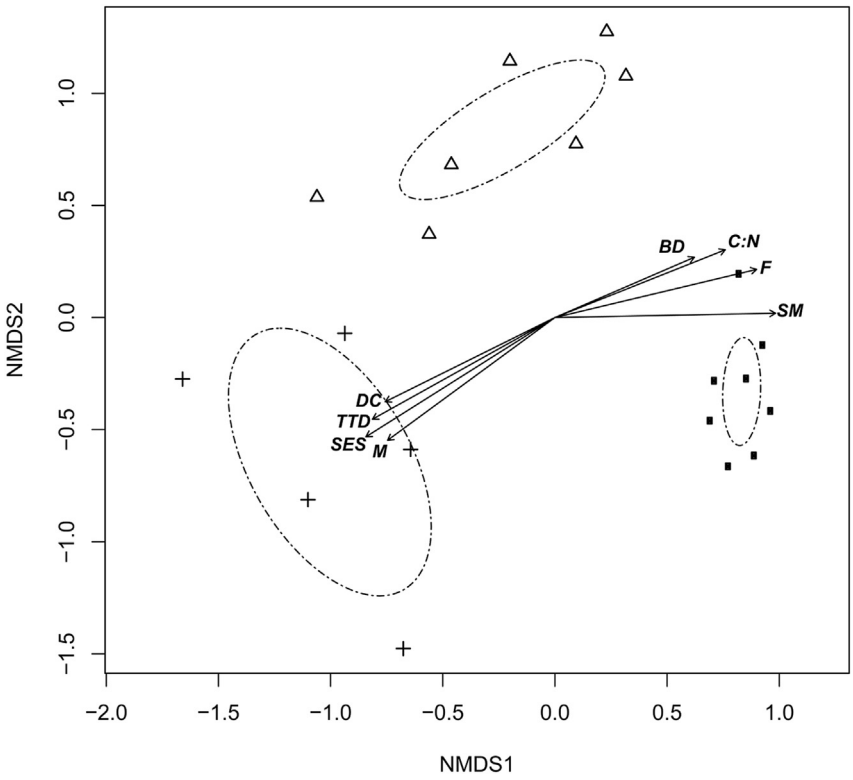

Fig. 3. NMDS ordination of bark samples. Environmental variables are shown by lines with arrows. The samples are grouped according to the successional groups (SG) corresponding to the heatmap (Fig. 2) as classified in this study. Squares indicate the 1st group, triangles indicate the 2nd group and crosses indicate the 3rd group. The ellipses around centroids show the standard error with a confidence level of 0.95. Abbreviations: TTD – time since tree death; DC – decay class; C:N – carbon - nitrogen ratio; F – bark cover; SM – specific mass of bark samples; BD – bulk density of bark samples; M – moisture; SES – stage of epixylic succession.

the gradients of bark specific mass, bark cover, stage of epixylic succession and time since tree death. Thus, successional trends were revealed for both wood and bark fungal communities. However, the comparison of community composition reveals both differences and similarities between these trends. Fungal richness in wood increased towards the end of succession (Ovaskainen et al., 2013; Rajala et al., 2015). Bark fungal diversity decreased as the C/N decreased, and the total OTU richness in bark reached its maximum at the intermediate stage (our study). In wood, at the onset of decay, fungal species in logs were mainly ascomycetes, but as decomposition continued, the community was increasingly dominated by basidiomycetes (Rajala et al., 2012). In bark, the richness of ascomycetes reached its maximum at the intermediate stages (our study). In contrast to *P. abies* wood-inhabiting communities with ca. 60%–80% of Basidiomycota reads (Hoppe et al., 2015; Ottosson et al., 2015), in our study Ascomycota were relatively more abundant than Basidiomycota. In wood, brown and white-rot decaying fungi dominate in the community (Rajala et al., 2015), whereas in bark the saprotrophs with more general life strategy dominate (our study). The OTU richness of mycorrhizal fungi both in wood and bark increased as the logs became increasingly decomposed (Ovaskainen et al., 2013; Rajala et al., 2015; our study).

Bark moisture was a good predictor of bark degradation affecting fungal diversity and richness of systematic and functional groups and community structure. Moisture content was strongly

Table 3
Variation in the composition of fungal communities related to the stages of succession of epixylic vegetation, log decay class and successional stages of bark fungal communities (SG) identified based on heatmap (Fig. 2) as evaluated by two nonparametric tests. Abbreviations: ANOSIM - analysis of similarities; PERMANOVA - permutational multivariate analysis of variance. Significant p-values are marked with bold font.

Distance	Type of analysis		Stage of epixylic succession	Decay class	SG
Weighted UniFrac	ANOSIM	R statistic	0.15	0.35	0.76
		P-value	0.087	**0.005**	**0.001**
	PERMANOVA	pseudo-F statistic	1.35	2.17	8.27
		P-value	0.189	**0.012**	**0.001**
Unweighted UniFrac	ANOSIM	R statistic	0.25	0.29	0.93
		P-value	**0.019**	**0.01**	**0.001**
	PERMANOVA	pseudo-F statistic	1.59	1.47	5.99
		P-value	**0.018**	**0.026**	**0.001**
Bray curtis	ANOSIM	R statistic	0.48	0.54	0.86
		P-value	**0.002**	**0.001**	**0.001**
	PERMANOVA	pseudo-F statistic	1.35	1.59	4.53
		P-value	0.056	**0.006**	**0.001**

Table 4
Distribution of OTUs among fungal successional groups with significant statistics in accordance to the g-test and Bonferroni corrected p-value <.05. SG are the successional groups. The percent abundance of OTU reads/the number of OTU incidences are presented for each group.

OTU	Taxonomic status	Asco/Basidiomycetes	**1**st**SG**	**2**nd**SG**	**3**rd**SG**	G-test	Bonferroni p
OTU138	Dermateaceae sp.	**a**	0	0	100/3	135.7	0
OTU189	*Pseudoplectania* sp.	**a**	0	100/2	0	337.5	0
OTU353	Helotiales sp.	**a**	0	0.2/1	99.8/1	258.1	0
OTU390	*Nakazawaea holstii*	**a**	98.0/8	0.2/1	1.8/1	102.9	0
OTU395	*Infundichalara* sp.	**a**	1.9/3	97.9/6	0.2/2	373.4	0
OTU41	Sebacinales sp.	**a**	0	100/2	0	100.9	0
OTU5	Helotiaceae sp.	**a**	0	0.3/1	99.7/5	123.9	0
OTU97	Helotiales sp.	**a**	1.1/3	0	98.9/1	119.3	0
OTU98	*Chaetomium* sp.	**a**	74.9/8	23.3/5	1.8/5	89.4	0
OTU44	Tympanidaceae sp.	**a**	0	100/4	0	36.6	<0.001
OTU93	*Humicola* sp.	**a**	98.1/8	0.5/1	1.4/2	49.7	<0.001
OTU57	*Albifimbria* sp.	**a**	93.0/8	3.7/3	3.3/3	43.8	<0.001
OTU380	Ascomycota sp.	**a**	0	0	100/2	40.8	<0.001
OTU194	*Cladophialophora* cf. *chaetospira*	**a**	0	67.7/3	32.3/5	39.8	<0.001
OTU343	*Candida* sp.	**a**	100/8	0	0	31.6	<0.001
OTU290	Orbiliales sp.	**a**	0	100/2	0	31.2	<0.001
OTU182	Helotiales sp.	**a**	0	100/3	0	30.3	<0.001
OTU377	Pseudeurotiaceae sp.	**a**	0	100/2	0	28.7	<0.001
OTU74	Hypocreales sp.	**a**	1.0/1	99.0/2	0	27.3	<0.001
OTU214	Eurotiomycetes sp.	**a**	62.3/2	37.7/2	0	22.5	0.004
OTU1	*Cladosporium* sp.	**a**	92.7/7	7.3/2	0	21.6	0.006
OTU21	*Hyphodiscus* cf. *brachyconius*	**a**	2.9/1	92.2/6	4.9/1	21.1	0.007
OTU148	Tympanidaceae sp.	**a**	12.1/2	87.9/5	0	20.1	0.012
OTU253	Helotiales sp.	**a**	10.5/4	68.9/6	20.6/2	19.4	0.018
OTU63	Ascomycota sp.	**a**	7.2/1	92.8/3	0	19.2	0.019
OTU111	Ascomycota sp.	**a**	0	100/3	0	19.0	0.021
OTU384	Ascomycota sp.	**a**	0	84.9/6	15.1/1	18.5	0.028
OTU162	*Cladonia* sp.	**a**	0	100/3	0	17.5	0.046
OTU376	Helotiales sp.	**a**	22.7/3	77.3/4	0	17.3	0.049
OTU121	*Mycena* sp.	**b**	0	0.2/1	99.8/3	215.1	0
OTU139	Agaricales sp.	**b**	0	100/3	0	193.2	0
OTU159	*Tylospora fibrillosa*	**b**	0	0.3/1	99.7/5	159.4	0
OTU167	Agaricales sp.	**b**	0	0	100/2	98.7	0
OTU181	*Mycena subcana*	**b**	0	100/5	0	208.9	0
OTU430	*Hypholoma capnoides*	**b**	0	0	100/2	235.8	0
OTU8	*Mycena* sp.	**b**	1.0/2	98.9/4	0.1/1	211.5	0
OTU90	*Coniophora olivacea*	**b**	0	99.8/1	0.2/1	145.7	0
OTU314	*Resinicium bicolor*	**b**	0	92.6/1	7.4/1	48.0	<0.001
OTU104	Basidiomycota sp.	**b**	0	97.1/1	2.9/1	26.8	<0.001
OTU389	Agaricales sp.	**b**	0	0	100/2	25.0	0.001

($r = 0.699$) positively correlated with time since tree death, i.e. water saturation of bark increased with time. Bark changed its physical characteristics and hygroscopicity increased along decay (Shorohova et al., 2016). Bark absorbs water through different paths such as precipitation, metabolic water produced by microorganisms (Schmidt, 2006) as well as accumulated by epixylic vegetation (Shorohova et al., 2016). The epixylic vegetation may influence the richness and structure of fungal communities by changing bark moisture regimes and influencing its N content.

Decreasing C/N ratio during decay has been previously reported for wood (Rajala et al., 2012) and bark (Palviainen et al., 2010). The absolute N concentration in dead wood may increase due to fungal translocation from soil (Laiho and Prescott, 2004), atmospheric deposition (Harmon et al., 1986) and biological fixation (Rinne et al., 2017). The amount of nitrogen in plant tissues is very low. For growth and enzyme production fungi need to get it from external sources. The variation in ascomycetes-basidiomycetes proportions in bark fungal communities showed a relationship with C/N ratio. Stress tolerant or ruderal species (predominantly ascomycetes) which initially occupy the bark, as well as subsequent colonizers may facilitate progressive accumulation of nitrogen through their translocation from other environmental pools into bark.

The fungal taxonomic diversity and community structure data related to the physical parameters of bark revealed three main successional groups. The first group included mainly widespread cosmopolitan ascomycetous fungi, which were mostly saprotrophs with broad substrate use (Fig. 4). This group, more than others ones, contained fungi which can be pathogens of plants and invertebrates. The group also contained various ascomycetous and basidiomycetous yeasts with unknown specialization.

In the second successional group, a large number of saprotrophs also occurred. However, they were more specialized on decaying wood than the saprotrophs from the first group. The abundance of lichens was higher than in the first group, in contrast to the abundance of pathogens, which decreased (Fig. 4). This successional group also contained more nematode-trapping fungi from the order Orbiliales. They may appear due to the persistence of parasitic plant nematodes, which may remain after the tree dies and change their feeding strategy from phytophagous to mycophagous (Hunt, 1993). Nonpathogenic saprophagous, mycophagous and predacious nematode species can inhabit wood also after tree fall (Braccia and Batzer, 1999; Zhao et al., 2008).

In the third successional group, we detected the highest abundance of mycorrhizal fungi and almost complete disappearance of lichenized and pathogenic fungi. Nematode-trapping fungi were almost undetectable. Wood-decayers became more common in the last two groups. In all succession stages, despite changes in taxonomic structure, unknown fungi shared about a half of the reads' abundance.

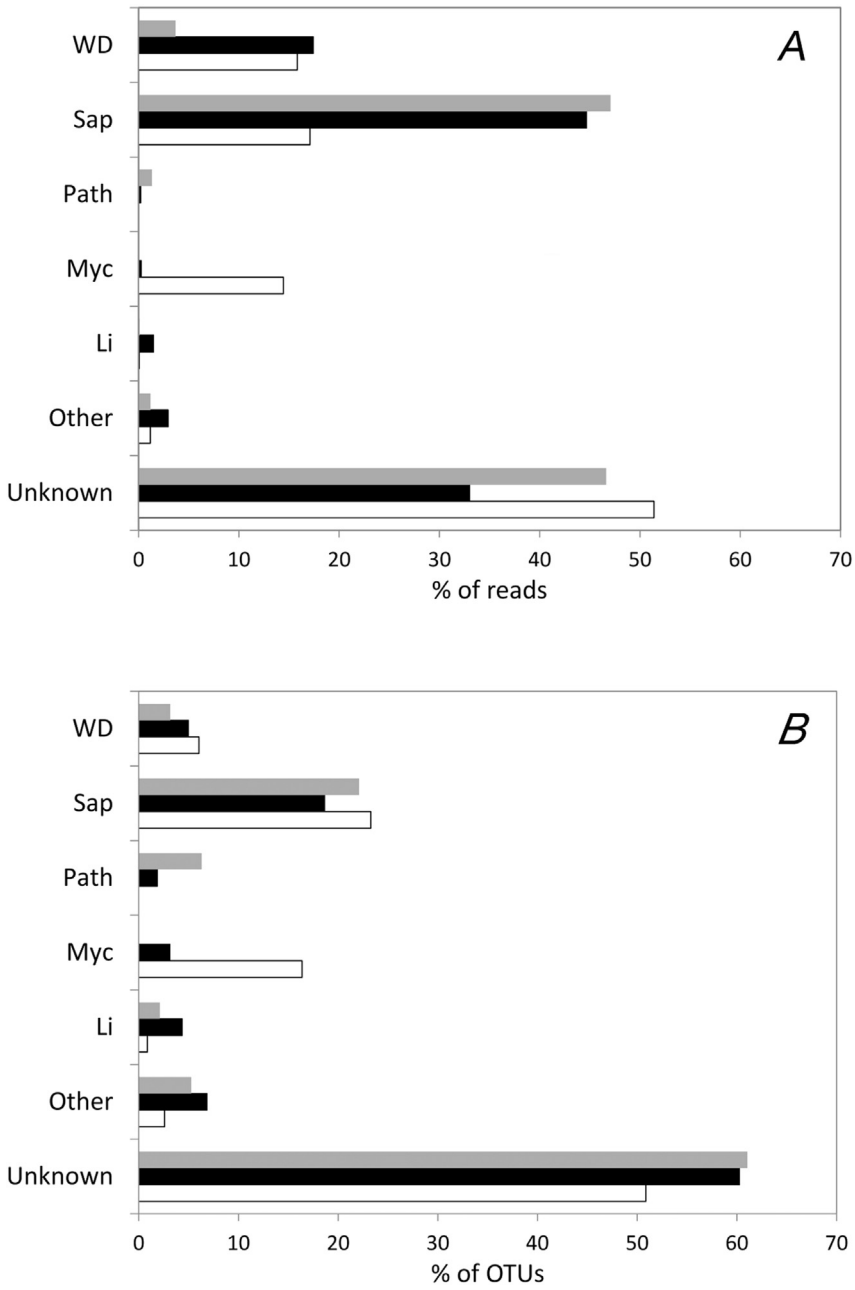

Fig. 4. The abundance of fungal functional groups based on the percent of abundance of reads (A) and OTUs (B) represented in the 1st successional group – grey, the 2nd successional group – black, the 3rd successional group – white. Abbreviations: Unknown – unknown strategy; WD – wood-decaying saprotrophs; Sap – saprotrophs with broad substrate utilization; Path – pathogens; Other– other groups; Myc – mycorrhizal fungi; Li – lichens.

4.2. Taxonomic characteristics of communities

The first successional group contained several ascomycetous and basidiomycetous yeasts. *C. victoriae* is considered to be an obligate component of most epiphytic yeast complexes (Glushakova et al., 2015). *N. holstii* is commonly associated with conifers, bark beetle frass and larvae tunnels (Kurtzman et al., 2011). It has also been isolated from other substrates such as cider, apples and embalmed cadavers. The assignment of *K. capsulata* to the first successional group is questionable due to low statistical parameters. In this study, it had the same wood-inhabiting specialization (tree association) as *N. holstii*. Wickerham (1951) assumed that such yeasts have become adapted to grow in the presence of toxic pitch. This ability and its association with plants protect these physiologically weaker yeasts from microorganisms that are more competitive. The association of these fungal species with bark beetles and their larvae has also been shown. *N. holstii* and *K. capsulata* produce large quantities of extracellular of phosphorylated mannans, allowing an adherence of cells to bark beetles, which facilitates their transport from one tree to another. In turn, the yeasts may serve as a food for insects and together with other microorganisms form walls around the galleries, which prevent drowning of the beetles by blocking flow of sap (Wickerham and Burton, 1961). Above-mentioned wood-inhabiting yeasts are able to convert cis-verbenol aggregation pheromone of bark beetles to other compounds, thereby reducing the insect's attraction to the

trees (Barbosa, 1991). This information is rather contradictory and prevents assigning wood yeasts to any ecological niche.

The filamentous fungi *C. polonica* and *O. penicillatum* are also associated with bark beetles. These fungi expand through the insect underbark galleries, breeding chambers and adjacent sections of bark and sapwood, causing blue-staining, while the insects use them as a nutrient resource. These species are clearly related to *Ips typographus,* and their propagules are transferred on exoskeleton elements or in the digestive tracts of insects (Solheim, 1993; Brignolas et al., 1995; Jacobs et al., 1997). The European spruce bark beetle (*I. typographus*) can carry ophiostomatoid fungi from the genera *Ophiostoma*, *Ceratocystis*, *Grosmannia*, *Ceratocystiopsis*, *Leptographium*, *Pesotum* and also from several Zygomycota, Ascomycota, Basidiomycota, anamorphic fungi and yeasts (Jankowiak, 2005; Giordano et al., 2012).

The genera *Albifimbria*, *Chaetomium*, *Cladosporium* and *Humicola* revealed in the present study are most likely soil-borne filamentous ascomycetes displaying a saprobic lifestyle. *Chaetomium* sp. has previously been shown to decompose beech bark, with an average weight loss of 15% after 12-week-incubation (Parameswaran et al., 1976). Weight loss was lower than that associated with brown and white-rot fungi in the same experiment. However, this fungus showed distinct decomposition of parenchyma cells and sclereids and demonstrated a tolerance to bark phenolic components. The *Chaetomium* sp. predominantly decomposed carbohydrates and, to some extent, lignin.

In the second stage of succession, together with saprotrophs and lichens, fungi with an alternative nutritional mode were detected. These were predatory fungi including *D. mammillata* and other unspecified Orbiliales (Suppl. 2), which are able to form nematode-trapping devices. *H.* cf. *brachyconius* was detected in most samples in the second successional stage. This fungus is uniformly regarded as a saprotroph, but it is reported that other species within this genus, for example *Hyphodiscus hymeniophilus,* may act as mycoparasites (Kubartová et al., 2012).

The ecological role of OTUs, associated with the family Tympanidaceae, regularly observed in the second succession stage, is uncertain. This family includes fungi with worldwide distribution, often occurring on conifers and associated with necroses, but they may show a saprotrophic lifestyle on wood and resin. This group of fungi demonstrates a tolerance to desiccation (Baral, 2015). This tolerance may increase their competitiveness in a substrate with unstable moisture content such as bark.

Fungi from Sebacinales were abundant in the second succession stage. Several OTUs were identified to the family level Serendipitaceae, which was newly proposed as a second taxon in an earlier monophyletic order (Weiβ et al., 2004, 2016). In contrast to the family Serendipitaceae, which includes endophytes and lineages with different mycorrhizal abilities (e.g. ecto-, ericoid, jungermannioid and orchid mycorrhiza), Sebacinaceae contains mainly ectomycorrhizal and saprotrophic species (Kottke et al., 2003; Kühdorf et al., 2014; Weiβ et al., 2016).

The most abundant in the second succession stage were the ascomycetes from the genus *Infundichalara*. These fungi are frequently associated with decaying coniferous wood, bark residues and basidiomata of the Polyporales (Réblová et al., 2011). Other saprotrophic fungi from unrelated genera *Pseudoplectania* are also common on rotten wood and litter (Carbone et al., 2014).

One of the most abundant OTUs was *T. fibrillosa*, which was found in almost all samples from the third successional group. This ectomycorrhizal (ECM) species is regularly reported in molecular investigations of wood-inhabiting communities and is common inside decaying wood logs (Tedersoo et al., 2003; Kubartová et al., 2012; Rajala et al., 2012; Ovaskainen et al., 2013). *T. fibrillosa* is able to produce manganese and lignin peroxidase enzymes (Cairney and Burke, 1994; Chen et al., 2001; Tedersoo et al., 2003). Presumably, this ability makes microorganisms evolutionarily successful because, in addition to mycorrhizal nutrition, they can effectively obtain extra carbohydrates and other nutrients from woody debris as wood-decayers. Other fungi from the family Atheliaceae (e.g. *Piloderma* sp. and *A.* cf. *byssoides*) were less abundant in the present study.

In the first stage of succession, in six of eight samples (75%), the small quantity of reads belonged to *F. fomentarius*, *T. abietinum*, *Fomitopsis pinicola*. In the second and third stages, wood-decaying fungi were found in six of twelve samples (50%). In some instances, up to five different species found in one sample, but only a few communities were dominated by wood-decayers. Thus wood-decaying fungi were present in all successional stages, but their presence was sporadic or imperceptible. These results suggest that wood-decaying fungi are not essential for decomposition of bark. Earlier research showed that bark is an unsuitable substratum for wood-decaying fungi (Rypacek, 1966). On the one hand, extractive substances of bark inhibit the development of fungi. On the other hand, the cell walls impregnated with suberin interfere with fungal hyphae and the ability of their exoenzymes to penetrate deeply inside bark. During bark decomposition, wood-decaying fungi can lose their competitive advantage compared to the ascomycetes that cause soft-rot. It was noted that soft-rot fungi can develop on the lignocellulosic substrate under extreme ecological conditions, which are unsuitable for basidiomycetes (Schmidt, 2006). Tolerance of a wide range of water content, pH, and temperature plus low oxygen demand can predetermine the importance of soft-rot fungi in bark decomposition. Recent studies showed that ascomycetes may have a prominent role in wood decomposition (Van der Wal et al., 2015); this may hold true for bark decomposition as well.

5. Methodological considerations

We were only able to identify to species and genus levels ca. 40% of the processed OTUs. These results are typical in molecular studies of fungal communities due to the incompleteness of current fungal databases and the limited taxonomic information provided by the ITS2 region, as well as the insufficient length of sequencing fragments and incomplete liquidation of erroneous or chimeric sequences (Bellemain et al., 2010; Bazzicalupo et al., 2013). However, the next-generation sequencing technique allows a comprehensive look into microorganism communities compared to traditional microbiological methods, especially for the organisms which are difficult to identify or culture. As a result of incomplete OTU identification, the classification of fungi by their substrate preferences, functional groups, life strategies, etc. becomes a nontrivial challenge, which is complicated by a scarcity of available ecological data. Additionally, determining the ecological role of species can be complicated due to species representation in more than one ecological group. For example, wood-decaying fungi can act as saprotrophs, pathogens and predators (Stokland et al., 2012). Another example is *F. oxysporum*, revealed in several samples in the first successional stage. This species can act as a soil saprotroph, a mutualistic or commensal plant endophyte, with certain strains or forms exhibiting pathogenic abilities (Stergiopoulos and Gordon, 2014).

6. Conclusions

Bark decomposition is apparently a long complex multi-staged process with changes in various groups of fungi. The changes in fungal community, associated with decaying Norway spruce log bark, occur along the gradients of time since tree death, bark moisture, C/N ratio, specific mass, bulk density, bark cover, log

decay class and stage of epixylic succession. As ascomycetes are predominantly endophytes of living trees such as *P. abies* and are often pioneer species in dead wood, it is understandable that they also prevail in the earlier stages of bark decay. In intermediate stages, bark is colonized by saprotrophs common on decaying logs and symbionts of epixylic plants, nematode-trapping fungi and lichenized fungi. In the final stage of succession, bark is colonized mainly by mycorrhizal fungi. Wood-decaying fungi occurred in all stages. However, their sporadic appearance in bark samples suggests that they are not essential for bark decomposition.

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Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.funeco.2017.12.005.

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