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Do foliar fungal communities of Norway spruce shift along a tree species diversity gradient in mature European forests?

Diem Nguyen*, Johanna Boberg, Katarina Ihrmark, Elna Stenström, Jan Stenlid

Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Box 7026, 75007 Uppsala, Sweden

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ABSTRACT

Foliar fungal species are diverse and colonize all plants, though whether forest tree species composition influences the distribution of these fungal communities remains unclear. Fungal communities include quiescent taxa and the functionally important and metabolically active taxa that respond to changes in the environment. To determine fungal community shifts along a tree species diversity gradient, needles of Norway spruce were sampled from trees from four mature European forests. We hypothesized that the fungal communities and specific fungal taxa would correlate with tree species diversity. Furthermore, the active fungal community, and not the total community, would shift along the tree diversity gradient. High-throughput sequencing showed significant differences in the fungal communities in the different forests, and in one forest, tree diversity effects were observed, though this was not a general phenomenon. Our study also suggests that studying the metabolically active community may not provide additional information about community composition or diversity.

1. Introduction

Diversity of species is considered beneficial for most ecosystems (Cardinale et al., 2012; Gamfeldt et al., 2015). Mixed forests have been associated with higher levels of ecosystem services (Gamfeldt et al., 2013; Carnol et al., 2014) and are thought to reduce the risk of fungal pathogen disease susceptibility as compared to monoculture stands (Pautasso et al., 2005; Felton et al., 2016). High tree species diversity, as proposed by the insurance hypothesis, may maintain the overall integrity of a forest ecosystem by reducing this risk (Yachi and Loreau, 1999). Both foliar pathogens and endophytes may be affected by the tree diversity in the stand (Müller and Hallaksela, 1998; Hantsch et al., 2013; Nguyen et al., 2016), although the mechanism for such an effect is not yet clear.

Tree leaves and needles host a range of organisms, including fungi. These foliar fungal species may have beneficial, antagonistic or no apparent impact on the tree (Carroll, 1988; Rodriguez et al., 2009). Fungi can be found on the leaf surface as epiphytes (Legault et al., 1989; Santamaría and Bayman, 2005) or inside leaves as endophytes, not causing any obvious symptoms (Carroll and Carroll, 1978; Petrini, 1992; Müller and Hallaksela, 2000). The distribution and abundance of foliar fungi vary not only among host species (Deckert and Peterson, 2000) but also among genotypes of one species (Bálint et al., 2013). At the individual plant level, the fungal communities are diverse and can differ within a plant (Müller and Hallaksela, 2000; Arnold et al., 2003; Cordier et al., 2012a) and also within a leaf (Lodge et al., 1996; Arnold et al., 2000). Across large geographical scales, variation in the composition of foliar fungi has been observed in conifers (Terhonen et al., 2011; Millberg et al., 2015). Such differences have often been attributed to variations in temperature, precipitation patterns, vegetation zones, geographic distance and other environmental factors (Helander, 1995; Vacher et al., 2008; Zimmerman and Vitousek, 2012). Fungal communities of coniferous hosts have higher diversity at higher latitudes (Arnold and Lutzoni, 2007; Millberg et al., 2015). At smaller spatial scales, differences in the foliar fungal community may be caused by forest structure, host density, microclimate and the surrounding environment (Saikkonen, 2007). Given that foliar fungal communities respond to all of these factors, they might be expected to vary with the surrounding vegetation, although this has not been studied in detail.

The fungal community can be studied in a number of ways. Molecular-based approaches, in contrast to culture-based methods, allow the detection of many more species (Amann et al., 1995),

* Corresponding author.
E-mail address: Diem.Nguyen@slu.se (D. Nguyen).

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including species that cannot be obtained in culture. The recent advances in high-throughput sequencing technology have increased both the resolution and scope of fungal community analyses and have revealed a highly diverse and complex mycobiota of plant foliage (O'Brien et al., 2005; Jumpponen and Jones, 2009; Jumpponen et al., 2010; Cordier et al., 2012b; Menkis et al., 2015; Millberg et al., 2015). Most studies have so far described the fungal community by sequencing the ribosomal RNA genes (rDNA), which provide a description of all members of the community, regardless of activity level. For example, dead organisms with intact genetic material, resting spores and vegetative mycelia may be detected by sequencing methods (England et al., 1997; Demanèche et al., 2001). However, sequencing the ribosomal RNA transcripts (rRNA) instead will reveal the metabolically active and functionally important taxa of the community and provide insights into the activity of these fungi in environmental samples (Pennanen et al., 2004; Baldrian et al., 2012; Delhomme et al., 2015). The metabolically active fungi would presumably reflect the portion of the fungal community that is responding to variation in the environment, such as vegetation gradients. By focusing on these fungi, the influence of external factors shaping foliar fungal communities can be elucidated.

The overall aim of the study was to determine whether fungal communities associated with Norway spruce (*Picea abies*) needles correlate with variation in tree species richness along a tree species diversity gradient. Furthermore, we investigated whether the metabolically active community responds differently than the total community to this tree diversity gradient. To that end, current-year needles were collected from Norway spruce trees across a tree species diversity gradient in four mature European forests that represent different mature forest types (Baeten et al., 2013), and the fungal communities were analyzed using 454 pyrosequencing. Additionally, at one forest site, both RNA and DNA were sequenced from the same foliar samples to compare the metabolically active fungal community (RNA) relative to the total fungal community (DNA), respectively. We hypothesized that the fungal communities and specific fungal taxa would correlate with varying mixtures of tree species along a tree species diversity gradient. Further, we expected that the metabolically active fungal community (RNA), and not the total fungal community (DNA), would shift along the tree species diversity gradient, as they are likely to actively respond to changes in the microenvironment created by changes in tree species composition in the mixtures.

2. Material and methods

2.1. Sampling sites and collection

The study was conducted in mature forests in four countries (i.e. Finland, Romania, Germany and Poland), spanning four major European forest types, established within the FunDivEUROPE Exploratory Platform (Baeten et al., 2013) (Fig. 1, Table 1). Sampling was conducted over approximately 2 weeks in 2012 or 2013 for each of the four forests during the vegetation period.

Standardized plots of 30 × 30 m were delimited within each forest, where different compositions of tree species were targeted to create a tree species diversity gradient with richness levels ranging from monoculture to three- (North Karelia, Finland), four- (Râşca, Romania and Hainich, Germany) or five-species (Białowieża, Poland) mixtures and different tree species assemblages at each level of species richness (Table 1). Focal Norway spruce trees were randomly selected from a pool of those trees with the largest diameter at breast height (19–79 cm). Within each plot, six trees in monoculture plots and three trees in mixtures were sampled. In total, 220 trees in 64 plots were sampled from the four forests (Table 1).

From each tree, two branches were cut from the southern exposure: one from the sun-exposed upper part of the canopy, and one in the lower third of the canopy. Shoots were collected from these branches from each tree. Per branch, five current-year shoots were sampled resulting in a total of 10 shoots per tree that were placed into one paper bag per tree. Each sample represented a tree. To prevent changes in the fungal community that could result from growth of opportunist organisms, shoots with needles still attached were immediately dried at 60 °C for 3 d (samples from forests in North Karelia and Hainich). Dried needles, detached from their shoots, were mixed in their respective paper bags. When it was not possible to dry the shoots within 24 h, which was the case for samples from forests in Râşca and Białowieża, samples were stored at 4 °C for a maximum of 2 weeks, and at −20 °C until further processing. These samples were then freeze dried for 3 d. Needles were removed from the shoots and mixed in their respective papers bags. For all samples, a subsample of 20 needles was removed randomly from each bag for further analysis of the total fungal community.

In addition to the total fungal community from these four forests, an assessment of the metabolically active and the total fungal community was studied in current-year needles from the forest in North Karelia, Finland. The same branches and shoots collected as described earlier were used. Needles were collected immediately after cutting down branches from each of the 60 trees. Two needles from each of the five shoots from the top branch and two needles from each of the five shoots from the bottom branch, 20 needles in total, were collected directly into 2 mL screw-cap centrifuge tubes with 1 mL of RNAlater (Thermo Fisher Scientific, Waltham, USA), without any sterilization procedure, and stored at 4 °C for a maximum of 2 weeks, until longer term storage at −20 °C was possible.

2.2. Molecular detection of the needle-associated fungal community

Samples originating from Râşca, Hainich, Białowieża and North Karelia that were used to study the total fungal community (*all sites* study) were prepared separately from those samples from North Karelia that were used for the study of the metabolically active and total fungal community (*Finland RNA* study). The fungal communities from both studies were determined with high-throughput sequencing of the internal transcribed spacer (ITS) region of the ribosomal RNA genes.

Prior to DNA extraction for the *all sites* study, desiccated needles were washed with 0.1% Tween-20 solution to remove ephemerally attached organisms and particles. Subsequently, the needles were dried on clean filter paper and transferred to a 2 mL screw-cap centrifuge tube together with two metal nuts that fit into the tube, and homogenized using a bead beater (Precellys 24, Bertin Technologies, Rockville, USA) at 5500 RPM for 20 s twice, with a 10 s pause in between, until a powder was produced. Genomic DNA was extracted with CTAB buffer (3% cetyltrimethylammonium bromide (CTAB), 2 mM EDTA, 150 mM Tris–HCl, 2.6 M NaCl, pH 8). Chloroform was used to remove protein contaminants. DNA was precipitated with 2-propanol, washed with 70% ethanol, and resuspended in water. Extraction negative controls (i.e. centrifuge tubes that did not include samples) were also included.

The fungal ITS2 region was amplified with primers gITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990), with ITS4 extended with a unique 8-base pair (bp) sample identification barcode for each sample. The resulting amplicons were 250–400 bp in length. Amplification of each sample occurred in 50 μL reactions [0.025 U μL^{-1} DreamTaq DNA Polymerase and

Fig. 1. Map of European sampling sites in different forests utilized to study the foliar fungal community of current-year Norway spruce needles.

Table 1
Sampling site locations, forest type and composition of forest mixture with respect to Norway spruce.

Forest site	Râşca	Hainich	Białowieża	North Karelia
	Romania	Germany	Poland	Finland
Forest type	Mountainous beech	Temperate Beech	Hemi-boreal	Boreal
Species pool	*Picea abies*	*Picea abies*	*Picea abies*	*Picea abies*
	Abies alba	*Acer pseudoplatanus*	*Pinus sylvestris*	*Pinus sylvestris*
	Fagus sylvatica	*Fagus sylvatica*	*Betula pendula*	*Betula pendula*
	Acer pseudoplatanus	*Fraxinus excelsior*	*Carpinus betulus*	
		Quercus robur	*Quercus robur*	
Location	47.3° N, 26.0° E	51.1° N, 10.5° E	52.7° N, 23.9° E	62.6° N, 29.9° E
Study area size (km × km)	5 × 5	15 × 10	30 × 40	150 × 150
Mean annual temperature, °C	6.8	6.8	6.9	2.1
Mean annual precipitation, mm	800	775	627	700
Sampling period	July 2013	July 2012	July–August 2013	August 2012
	Number of plots (trees) with *Picea abies*			
Monoculture	2 (12)	2 (12)	2 (12)	4 (24)
Two species	5 (15)	3 (8)	5 (15)	8 (24)
Three species	5 (15)	3 (8)	6 (18)	4 (12)
Four species	3 (9)	2 (6)	8 (24)	
Five species			2 (6)	
Total	15 (51)	10 (34)	23 (75)	16 (60)
	Number of trees sampled and sequenced			
	51	34	75	60/55 (*all sites/Finland RNA*)

buffer (Thermo Fisher Scientific, Waltham, USA) 200 μM dNTP, 500 nM gITS7, 300 nM ITS4, 2.75 mM $MgCl_2$, and 0.125 ng μL^{-1} genomic DNA template (or 1:10 dilution of extraction controls) was performed using an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Carlsbad, USA). PCR negative controls (no samples added) were used to evaluate that there was no contamination during the preparation for PCR. The PCR cycle parameters consisted of an initial denaturation at 95 °C for 5 min, 26–35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s, followed by a final elongation step at 72 °C for 7 min. The number of amplification cycles was determined individually for each sample to preserve the fungal genotype composition (Lindahl et al., 2013). Thus, PCR was interrupted while in the exponential phase to yield weak to medium-strong amplicons as visualized by gel electrophoresis on 1% agarose gels. The amplicons were purified with the Agencourt AMPure XP kit (Beckman Coulter, Brea, USA) and the concentration was determined using the Qubit Fluorometer and dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, USA). Each sample was amplified in triplicate and each replicate was individually purified and concentration determined. PCR amplicons were mixed in equal mass proportion into a general sample that was further purified using E.Z.N.A. Cycle-Pure Kit (Omega Bio-tek, Norcross, GA, USA) for samples from Hainich and North Karelia, or JETQUICK DNA Clean-Up Spin Kit (Genomed, Löhne, Germany) for samples from Râşca and Białowieża. There were two libraries, namely one consisting of samples from Hainich and North Karelia, and another with samples from Râşca and Białowieża. Each library was subjected to ligation of sequencing adaptors and separately sequenced with the 454 sequencing technology using GS FLX Junior (Roche, Switzerland) that is equivalent to an eighth of a plate, with Titanium series chemistry, and carried out by Eurofins Genomics (Ebersberg, Germany). DNA extraction controls were sequenced, while PCR negative controls were not sequenced, as nothing was amplified from them.

Samples for the *Finland RNA* study, i.e. those from North Karelia, Finland that were used for the analysis of the metabolically active and total fungal community, were subjected to RNA and DNA extraction as follows. Needle samples were removed from RNAlater and ground with nucleic acid-free mortar and pestle, both of which were pre-treated to above 450 °C, with sand and liquid nitrogen to maintain below freezing conditions that ensured RNA stability. Needles were pulverized and transferred to 15 mL plastic tubes and stored at −70 °C until further processing. RNA extraction was performed according to Chang et al. (1993). To each tube, 4 mL of extraction buffer (CTAB extraction buffer supplemented with PVPP [2% CTAB, 25 mM EDTA pH 8.0, 0.1 M Tris-HCl pH 8.0, 2 M NaCl, 2% mercaptoethanol and 2% PVPP]) was added and incubated at 65 °C for 10 min. Two chloroform purification steps to remove proteins were performed with centrifugation at 6800*g* for 5 min. Overnight precipitation at 4 °C with 2 M lithium chloride and centrifugation at 6800*g* for 40 min to pellet the RNA were carried out. The RNA pellet was resuspended in 100 μL RNase-free water, transferred to 1.5 mL tubes and precipitated at −20 °C with 2.5 vol ethanol and 1/10 volume of 3 M NaOAc, pH 5.2. The precipitated RNA was centrifuged for 15 min at 16200*g*, followed by pellet resuspension with 16 μL RNase-free water. DNase I (Sigma, St. Louis, USA) digestion of the RNA removed any remaining DNA contamination. To confirm that there was no DNA remaining in the extracted RNA, amplification with primers gITS7 and ITS4 of the treated material was conducted as above. RNA concentrations were determined fluorometrically with Qubit RNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, USA). The cDNA synthesis was carried out by using the iScript Select cDNA synthesis kit (Bio-Rad, Hercules, USA) with 5 μL of DNase treated RNA (approximately 1–500 ng of RNA) and 1 μL ITS4 primer, followed by 90 min incubation at 42 °C. PCR with iScript alone resulted in no visible bands on 1% agarose gels.

The lithium chloride supernatant was retained following the RNA pelleting step (where the DNA fraction was expected to be) and DNA was precipitated with one volume 2-propanol, followed by 70% ethanol wash and resuspended in 200 μL molecular-grade water. Half of the samples had residual lithium chloride, and these were further purified with JETQUICK DNA Clean-Up Spin Kit. Amplification of DNA and cDNA with gITS7 and barcoded ITS4 primers followed the above protocol. DNA concentration of 0.25 ng/μL and for the cDNA, 2.5 μL of the iScript reaction was used for the amplification of ITS2. Amplicon purification, concentration determination, pooling, purification with E.Z.N.A. Cycle-Pure Kit and 454 pyrosequencing occurred as above. There were no extraction controls for the RNA/DNA extraction, though all PCR controls were sequenced. Five samples from one of the three-species mixture plots could not be successfully amplified and thus were not sequenced. In total, there were 55 RNA samples that derived from RNA extraction and a matching 55 DNA samples that were from the same RNA samples.

2.3. Parsing the sequence data

The sequence reads ('*reads*') generated from three separate 454 pyrosequencing runs were subjected to quality control and single-linkage clustering in the SCATA bioinformatics pipeline (http://scata.mykopat.slu.se). All three datasets from each of the sequencing runs (two from the *all sites* study and one from the *Finland RNA* study) were combined in one SCATA analysis. Quality filtering of the sequences using the high-quality region (HQR) extraction option (HQR is the longest part of a read that fulfills all the quality thresholds) included: the removal of short sequences (<200 bp) and sequences with low mean read quality score (<20) or with a score below 10 at any position. Homopolymers were collapsed to 3 bp before clustering, and restored to their original length before final analyses and downstream sequence identification. Sequences that were missing primer sequences and/or identification barcode sequence were also excluded, allowing for two mismatches in each of the primer sequences. Primer sequences and barcodes were removed, but information on the read associated with the sample was retained as metadata. The sequences were then clustered into different operational taxonomic units (OTUs) using single-linkage clustering based on 1.5% dissimilarity (i.e. 98.5% similarity) using the USEARCH clustering engine. This clustering threshold approximately corresponds to species level, and was validated by including reference sequences. Clemmensen et al. (2015) further elaborates on the SCATA pipeline workflow and provides justification of our procedure here. The most abundant genotype for each cluster was used to represent each OTU. For OTUs containing two sequences, a consensus sequence was produced.

The construction of a neighbor-joining tree (Lindahl et al., 2013), based on MUSCLE alignment algorithms of sample and reference sequences, aided in the initial removal of non-fungal reads (e.g. plants, algae, one bacterium and a retroelement). OTUs were taxonomically identified by BLAST searches (Altschul et al., 1997) against the GenBank nucleotide database, optimized for somewhat similar sequences (blastn, http://blast.ncbi.nlm.nih.gov). Uncultured fungal clones or environmental samples were not excluded from the initial BLAST searches. To describe the taxonomic affiliations of the OTUs, two procedures were then used. In the first method, for the most abundant OTUs (as defined for each forest, and with a minimum of 10 reads per sample), species-level identification was attempted. For each of these OTUs, up to 100 matches from different authors were considered, preferably from published studies by authorities in taxonomy. For conflicting matches, the

lowest common rank level was used for taxonomic assignment (Peršoh et al., 2010). The sequence must align 100% over the entire length of the queried sequence. The ITS similarities for defining taxa in GenBank were delimited following Ottosson et al. (2015) as follows: species level – 98–100% similarity and e-values below e^{-100}; genus level – 90–97% and e-values below e^{-90}; and order level – 80–89% and e-values e^{-80}. These OTUs were used for further analyses specified below.

In the second method to describe the taxonomic affiliations of the OTUs, where the interest was to identify the taxonomic rank phylum, class and order for each OTU, several top GenBank matches providing taxonomic rank information were evaluated and phylum, class and order were recorded. Uncultured fungal clones or environmental samples do not provide taxonomic information and were thus excluded. If the top match was Phylum (or Class or Order) sp., for example Ascomycota sp. or Helotiales sp., the match was not selected for taxonomic rank identification. Rather the next best match to species, even if Genus sp. (e.g. *Hymenoscyphus* sp.) was included for taxonomic rank identification. Where there were multiple matches that had the same high bitscore and similarity, but provided conflicting taxonomic resolution, the lowest common rank level was used for taxonomic assignment. For conflicts at the phylum rank, i.e. one match was to a Basidiomycota species and another to an Ascomycota species, no taxonomic rank was assigned, and 'Fungus' was recorded. Phylogenetic classification was assigned after Schoch et al. (2006); Hibbett et al. (2007); Boehm et al. (2009); Hodkinson and Lendemer (2011); Rosling et al. (2011); Zhang et al. (2011); Boonmee et al. (2014); Ertz et al. (2014); Chen et al. (2015).

2.4. Statistical analyses of the fungal community diversity and composition

The study had a hierarchical design. Each forest consisted of many plots that were associated with different tree species richness levels based on tree species composition, as noted earlier. There were multiple plots within a forest with the same tree species richness level (i.e. in North Karelia, there were four plots of Norway spruce in monoculture plots, and in Hainich, there were two plots containing Norway spruce in the three-species mixture plots), and multiple Norway spruce trees (either three or six sampled) within each plot. To avoid pseudoreplication errors and allow for downstream analyses, the reads for each tree sample were pooled at the plot level for each OTU that resulted in plot-level read values.

Fungal diversity was analyzed using Hill numbers, which has been found to be a reliable metric with microbial sequence data from complex communities (Jost, 2006; Haegeman et al., 2013) and has previously been used for fungal community sequence data (Bálint et al., 2015). Hill numbers can be used to measure and incorporate richness (Hill 0), exponent of Shannon Index (Hill 1) and inverse Simpson (Hill 2) (Legendre and Legendre, 1998). A community is more diverse if all of its Renyi diversities, as represented by the Hill numbers, are higher than in another community. Fungal diversity was assessed at different levels: (i) between forests (that were sequenced and processed in the same way), (ii) across the tree species diversity gradient in each forest, with tree species richness specified as a discrete variable, and (iii) between community types, i.e. active (RNA) and total (DNA) fungal communities. As carried out by Bálint et al. (2015), linear models were used to explain Hill 0, Hill 1 and Hill 2 with the square root of total read numbers for a plot-level sample. The square root of the read numbers accounts for differential sequencing depth in different samples. The partial residuals of the Hill numbers, after accounting for differential sequencing depth, between forests, and among tree species richness levels within a forest or community type were compared with Tukey's HSD.

Rarefaction curves were constructed separately for each forest, as well as for community type (i.e. active (RNA) and total (DNA) separately), using sample-based data in the software EstimateS version 9.1.0 (Colwell, 2013). In the construction of these curves, each tree sample was individually specified, rather than using the pooled plot-level read values.

The relative abundance of reads was calculated for each OTU in each plot-level sample and was used for the following analyses. Non-metric multidimensional scaling (NMDS) ordination was used to visualize the multivariate community composition, based on the relative abundance of each OTU. The *metaMDS* function in the vegan package (Oksanen et al., 2013) was used to make NMDS plots. Bray-Curtis dissimilarity matrices, three dimensions and 100 random starts were specified. The first solution served as the starting point for a second NMDS analysis.

Additionally, permutational multivariate analysis of variance (PERMANOVA, *adonis* function (Anderson, 2001)) allowed the partitioning of the variance contributed by the explanatory variables, and thus tested significance of the difference between forests, between community types in North Karelia, and among levels of tree species richness for each forest in the *all sites* study and for each community type in the *Finland RNA* study, respectively. For all PERMANOVA analyses, sample distances were calculated with Bray-Curtis dissimilarity matrices and statistical significance was estimated with 999 permutations.

For the most abundant OTUs, as defined earlier, the relationship between their relative abundance as a function of tree species richness was investigated. Comparison of the relative read abundance for within-species comparisons is admissible, though between-species comparisons are problematic due to innate sequence structure (Amend et al., 2010). The most abundant OTUs were determined by setting a threshold of 10 reads per sample and calculating the minimum number of reads required. The threshold level for North Karelia was 580 reads, Białowieża 740, Hainich 340, and Råşca was 490.

Generalized linear mixed-effect models (GLMMs) were used to determine significance of the distribution of individual OTUs that were most abundant along a tree species diversity gradient for the *all sites* and *Finland RNA* studies. Specifically, we tested which, if any, of the most abundant OTUs correlated with tree species richness. GLMMs take into account the hierarchical sampling design and account for non-independence among observations and allow for nested and crossed random-effect terms (Zuur et al., 2009; Schielzeth and Nakagawa, 2013). Consequently, tree-level sequence reads were used for these analyses, and not plot-level reads. The response variable, which was the proportion of a specific OTU, had a binomial distribution. The explanatory variable tested was tree species richness (treated here as a continuous variable to aid in interpretation of the results). The random factors included plot, and to properly account for the variance (that does not vary freely), random residual was estimated by specifying the sampling unit, i.e. a unique identifier for each tree sample. All analyses, unless otherwise specified, were carried out in R version 3.1.3 (R Core Team, 2013). GLMMs were run with the *glmer* function, with the binomial distribution and logit link (logit = ln(response probability of OTU/probability of the non-response of OTU)) specified, in the *lme4* package (Bates et al., 2015). Odds ratios for the effects of explanatory variable can be calculated from the estimated regression coefficients as exponent of the estimated coefficient. In logistic regression, the odds ratios are interpreted as a change in the probability of an OTU to be present when tree species richness increases by one unit (Breslow and Day, 1987; Hosmer and Lemeshow, 1989).

3. Results

3.1. Fungal community diversity and composition

Following 454 sequencing of spruce needle samples across all four forests (*all sites* study) for the total fungal community, 209,267 sequence reads were generated. Of these, 142,578 (68%) passed quality filtering. In the part of the study aimed to compare the metabolically active fungal community to the total fungal community (i.e. community type) from trees in North Karelia, Finland (*Finland RNA* study), 454 sequencing of current year needles resulted in 108,144 reads of which 86,035 (80%) passed quality filtering. Across both studies, there were 1737 global OTUs (non-singletons) and 1127 global singletons. Non-fungal OTUs (30 OTUs, 29966 reads, 13% of all reads) were removed. The two datasets, namely the *all sites* study and the *Finland RNA* study, were analyzed separately. Following the removal of OTUs with fewer than 10 reads globally for each dataset, there were 513 fungal OTUs in the *all sites* study and 310 fungal OTUs in the *Finland RNA* study.

Some OTUs could be putatively identified in both datasets to species level. The abundant species included: *Ascochyta skagwayensis*, *Aureobasidium pullulans* (ubiquitous yeast-like fungus), *Celosporium larixicola*, *Ceramothyrium carniolicum*, *Chrysomyxa ledi* (rust pathogen of Norway spruce), *Epicoccum nigrum*, *Exobasidium arescens*, *Heterobasidion parviporum* (root and butt rot pathogen of Norway spruce), *Phaeocryptopus gaeumannii* (pathogen of Douglas-fir (*Pseudotsuga menziesii*)), *Ramularia vizellae* (plant pathogen), *Sydowia polyspora*, *Taphrina carpini* (pathogen of oak hornbeam (*Carpinus betulus*)), and *Taphrina vestergrenii* (pathogen of fern).

For the *all sites* study, a majority of the 513 OTUs were putatively assigned to Ascomycota (394 OTUs, 105,665 sequence reads) while Basidiomycota accounted for 115 OTUs (18,083 reads) (Supplementary Figure 1A). Four OTUs were putatively assigned to Chytridiomycota, Glomeromycota and unidentified fungi, accounting for 298 reads. At the class level, most OTUs were putatively assigned to Dothideomycetes (169 OTUs, 49,736 reads), Eurotiomycetes (60 OTUs, 12,799 reads) and Leotiomycetes (56 OTUs, 21,027 reads). There were 18 OTUs (5011 reads) that could not be assigned to class level. Overall, there were 35 Ascomycota orders, 21 Basidiomycota orders, 9 unknown orders in both phyla, all spanning 18 classes (and one unassigned Ascomycota class).

For the *Finland RNA* study, putatively assigned Ascomycota made up the majority of the 310 OTUs and reads (232 OTUs, 47,326 reads), followed by Basidiomycota (74 OTUs, 19,244 reads). In total, there were putatively 29 Ascomycota orders, 15 Basidiomycota orders, and 9 unknown orders in both phyla, in 21 classes (Supplementary Figure 1B). Two unidentified fungal OTUs (399 reads) and two putative Glomeromycota OTUs (28 reads) were also detected.

Furthermore, in the *Finland RNA* study, the number of OTUs in the total fungal community (DNA) (303 OTUs, 49,671 reads) and the metabolically active fungal community (RNA) (293 OTUs, 17,326 reads) were similar, although the number of reads was three times higher in the DNA fraction (Table 2). A majority of the OTUs were likewise putatively assigned to Ascomycota (active community: 219 OTUs (12,062 reads) and total community: 228 OTUs (35,264 reads)). There were 70 putative Basidiomycota OTUs (5103 reads) for the active community and 72 OTUs (14,141 reads) for the total community (Table 2). There were 17 OTUs (346 reads) that were uniquely in the DNA fraction, and seven OTUs (110 reads) that were uniquely in the RNA fraction. In general, the unique OTUs were very uncommon and the most abundant of these unique OTUs accounted for less than 0.16% of the total reads. Those OTUs uniquely in the DNA were predominantly Ascomycota (13 OTUs, 297 reads) and the remaining four OTUs (49 reads) were Basidiomycota. Similarly, OTUs uniquely in the RNA were predominantly Ascomycota (four OTUs, 71 reads), two OTUs (26 reads) were Basidiomycota and one OTU (13 reads) putatively assigned to Glomeromycota.

The distribution of reads for both the *all sites* and *Finland RNA* studies showed log-normal distribution, such that many OTUs had few sequence reads and a few OTUs had many reads, that is typical of this type of sequence data (data not shown). Rarefaction curves for the data separated by forest in the *all sites* study did not reach saturation, suggesting that deeper sequencing would uncover more taxa (Supplementary Figure 2A). On the other hand, rarefaction curves constructed for the data separated by community type in the *Finland RNA* study showed a nearly saturated curve for the total community (DNA), but not the active community (RNA), suggesting deeper sequencing of the total fungal community than the active

Table 2
Comparison between community type, i.e. the active (RNA) and total (DNA) fungal community, in the *Finland RNA* study. Number of OTUs and reads for each putatively assigned orders for the respective community type are presented.

	Number of OTUs in both RNA and DNA		Number of reads in both RNA and DNA	
			RNA	DNA
Ascomycota	215		11991	34967
Basidiomycota	68		5077	14092
Fungus	2		141	258
Glomeromycota	1		7	8
Total	286		17216	49325
	Number of OTUs		Number of reads	
	Only in RNA	Only in DNA	Only in RNA	Only in DNA
Ascomycota	4	13	71	297
Basidiomycota	2	4	26	49
Fungus	0	0		
Glomeromycota	1	0	13	
Total	7	17	110	346
	Number of OTUs		Number of reads	
	RNA total	DNA total	RNA total	DNA total
Ascomycota	219	228	12062	35264
Basidiomycota	70	72	5103	14141
Fungus	2	2	141	258
Glomeromycota	2	1	20	8
Total	293	303	17326	49671

fungal community (Supplementary Figure 2B).

3.2. Fungal community from the all sites study (total community analysis)

Fungal community composition differed strongly across the tree species richness levels in Hainich (R^2 = 0.50, P = 0.023; Fig. 2, Supplementary Table 1). However, fungal community diversity, as determined by Hill numbers, was not different along the tree species richness gradient in any of the four forests (data not shown).

The most abundant OTUs from each of the four forests varied in their relative read abundance, which was between 1% and 28% at the extremes, and found in varying number of samples (Supplementary Table 2). Negative tree species richness effects were observed for OTU_24 (Dothideomycetes sp.) in Râșca (Table 3). Tree species richness effects were also observed in Hainich; OTU_2 (Helotiales sp.) and OTU_39 (*Sporobolomyces* sp.) exhibited negative relationships. However positive relationships were seen for OTU_7 (*A. pullulans*), OTU_6 (*Ceramothyrium* sp.), OTU_35 (*Trichomerium* sp.) and OTU_61 (Chaetothyriales sp.) (Table 3).

Fungal community composition differed strongly between pairs of forests that were differentially processed and sequenced at different times, regardless of tree species diversity, namely between North Karelia and Hainich, and between Râșca and Białowieża (R^2 = 0.28, P < 0.001; Supplementary Table 1). Tree species richness did not influence the fungal community composition across forests (Supplementary Table 1). Fungal community diversity, as determined by Hill numbers, was not significantly different between the forests either (data not shown).

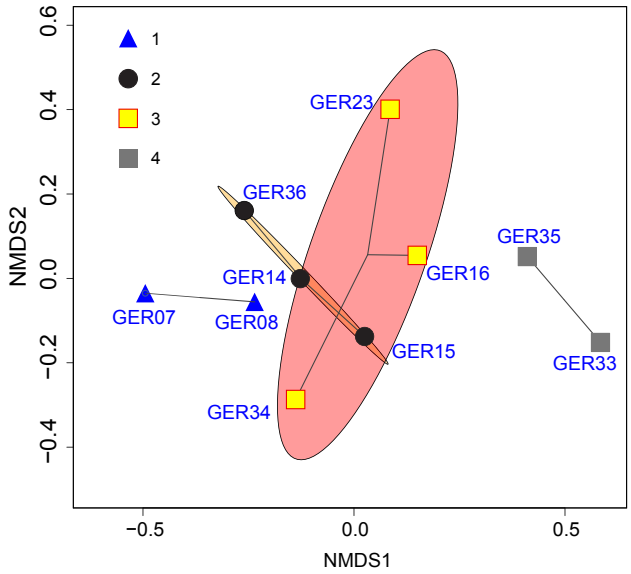

Fig. 2. NMDS results of the fungal community composition of plot-level OTUs for each plot (GERxx, where xx represents the plot number) in the Hainich forest in Germany. Axes are arbitrary, represent two NMDS dimensions and scaled in units of Bray-Curtis dissimilarity. A stable solution was reached (stress value = 0.076). The colored ellipses mark the 95% confidence interval of the group centroids for each tree species richness level. Lines link the samples pooled at the plot level to the group centroids. The blue triangles are plots from the monoculture, black circles enclosed within the orange ellipse represent the two-species mixture, yellow squares enclosed within the red ellipse represent the three-species mixture and the grey squares represent the four-species mixture.

3.3. Fungal community from the Finland RNA study (metabolically active and total community analysis)

Fungal community composition of each community type, i.e. active fungal community (RNA) and total fungal community (DNA), in North Karelia, Finland was distinct (R^2 = 0.08, P = 0.009; Fig. 3, Supplementary Table 1). However, the active (RNA) and total (DNA) fungal communities did not differ significantly among the tree species richness levels (P = 0.102 and P = 0.169, respectively, Supplementary Table 1) and this was further confirmed by the overlap in the community profile in the NMDS of the fungal communities from both the metabolically active and total community, respectively, along the tree richness gradient (data not shown). Fungal community diversity, as determined by Hill numbers, was tested between the community types, and separately for the metabolically active and total fungal communities. The active community and total community were equally diverse, and were not significantly different along the tree diversity gradient for either community (data not shown).

The most abundant OTUs from each of the community types varied in their relative read abundance, which was between 1% and 15% at the most extreme, and found in varying number of samples (Supplementary Table 3). Negative tree species richness effects were observed for OTU_16 (*H. parviporum*) in the active (RNA) community (Table 4). Tree species richness effects were also observed in the total (DNA) community. Here, OTU_11 (*T. carpini*) was negatively affected by the tree species richness, while positive effects were seen for OTU_20 (Eurotiomycetes sp.) and OTU_32 (Pleosporales sp.) (Table 4).

4. Discussion

The central question of our study was: Do foliar fungal communities of Norway spruce shift along a tree species diversity gradient in mature European forests? Indeed, we found that the fungal community varied along the tree species diversity gradient in the Hainich forest in Germany. However, we observed no overall trend in other mature forests in this study. Similar variable results to tree species diversity have been previously observed, where mixtures of Norway spruce with other tree species either resulted in increased, decreased or no changes in the susceptibility of Norway spruce to pathogen infection (Kató, 1967; Siepmann, 1984; Piri et al., 1990). In particular, Nguyen et al. (2016) found that depending on the forest site, different tree species diversity effects could be detected for foliar pathogens. In their study, there were either no correlations with tree species diversity and pathogen incidence, or an increase in disease incidence with diversity. It is thus still unclear to what degree and under what circumstances tree species diversity affects fungal communities of tree foliage.

Tree species richness effects were not generally observed for fungal communities in Râșca, Romania; Białowieża, Poland; and North Karelia, Finland. This is in contrast to what has been found previously in forests in Finland, where fungal diversity in needles was higher in pure spruce stands and mixed virgins stands (Müller and Hallaksela, 1998). Hoffman and Arnold (2008) highlighted that host identity shaped a given plant's endophyte community. One likely explanation for our lack of observing tree species diversity effects is the importance of tree species identity effects in the neighborhood of Norway spruce trees. The fungal community may be affected by the specific composition of tree species, which may contribute to alterations in the microenvironment. Tree species identity effects have been found to be often more pronounced than effects of species diversity (De Deyn et al., 2004; Nadrowski et al., 2010) and important for the distribution of foliar fungal pathogens (Hantsch et al., 2013, 2014). Foliar endophyte communities of conifer species exhibit patchy distribution among individual trees (Cordier et al., 2012a), and can vary even between needles of one tree (Deckert and Peterson, 2000). The variation may be large

Table 3
Generalized linear mixed-effects model results at the fungal taxa level for the most abundant OTUs. Effect of tree species richness on OTU abundance across all individual trees for each forest site is presented. Significant results (Bonferroni corrected a = 0.05/number of samples within each country) are indicated in bold print (Râşca α = 0.0045; Hainich α = 0.0056; Białowieża α = 0.00625; and North Karelia α = 0.008).

	Group[a]	Putative taxonomic assignment[b]	Parameter estimate for tree species richness			
			Estimate	StdError	Z-value	P
Râşca						
OTU_4	A	*Sydowia polyspora*	0.02	0.20	0.09	0.927
OTU_5	A	Arthoniomycetes sp.	0.08	0.14	0.60	0.547
OTU_12	A	*Tumularia* sp.	−0.10	0.12	−0.83	0.405
OTU_8	A	*Celosporium larixicola*	0.00	0.23	−0.02	0.988
OTU_30	A	Arthoniomycetes sp.	0.53	0.35	1.53	0.125
OTU_23	A	Leotiomycetes sp.	0.36	0.14	2.52	0.01
OTU_3	A	*Cladosporium* sp.	0.03	0.14	0.21	0.84
OTU_24	A	Dothideomycetes sp.	−0.72	0.16	−4.40	1.07E-05
OTU_26	A	*Cyphellophora* sp.	0.08	0.17	0.47	0.64
OTU_2	A	Helotiales sp.	0.15	0.21	0.71	0.48
OTU_7	A	*Aureobasidium pullulans*	0.45	0.19	2.44	0.01
Hainich						
OTU_2	A	Helotiales sp.	−0.34	0.08	−4.38	1.19E-05
OTU_3	A	*Cladosporium* sp.	−0.09	0.07	−1.25	0.213
OTU_7	A	*Aureobasidium pullulans*	0.95	0.20	4.83	1.35E-06
OTU_4	A	*Sydowia polyspora*	−0.31	0.20	−1.59	0.111
OTU_6	A	*Ceramothyrium* sp.	0.94	0.00	413.70	<2e-16
OTU_8	A	*Celosporium larixicola*	−0.16	0.21	−0.75	0.451
OTU_11	A	*Taphrina carpini*	0.07	0.17	0.41	0.680
OTU_18	A	*Epicoccum nigrum*	0.20	0.11	1.92	0.055
OTU_12	A	*Tumularia* sp.	0.06	0.21	0.29	0.770
OTU_49	A	*Ascochyta skagwayensis*	−0.07	0.35	−0.19	0.852
OTU_33	A	*Ramularia vizellae*	−0.25	0.21	−1.17	0.241
OTU_35	A	*Trichomerium* sp.	0.77	0.23	3.44	0.001
OTU_38	A	*Taphrina vestergrenii*	−0.03	0.19	−0.18	0.858
OTU_17	A	Chaetothyriales sp.	0.02	0.17	0.11	0.913
OTU_39	B	*Sporobolomyces* sp.	−0.43	0.00	−136.60	<2e-16
OTU_13	B	*Exobasidium* sp.	−0.09	0.30	−0.30	0.767
OTU_60	A	*Hortaea* sp.	−0.94	0.47	−1.99	0.046
OTU_61	A	Chaetothyriales sp.	0.12	0.00	49.70	<2e-16
Białowieża						
OTU_3	A	*Cladosporium* sp.	0.07	0.09	0.80	0.422
OTU_7	A	*Aureobasidium pullulans*	0.45	0.29	1.56	0.119
OTU_5	A	Arthoniomycetes sp.	0.20	0.18	1.08	0.281
OTU_4	A	*Sydowia polyspora*	0.04	0.13	0.27	0.785
OTU_6	A	*Ceramothyrium* sp.	0.15	0.15	1.02	0.308
OTU_40	A	*Metschnikowia* sp.	−0.49	0.37	−1.33	0.183
OTU_12	A	*Tumularia* sp.	−0.06	0.13	−0.47	0.642
OTU_15	B	*Exobasidium arescens*	−0.33	0.14	−2.34	0.019
North Karelia						
OTU_1	B	*Chrysomyxa ledi*	0.34	1.07	0.32	0.751
OTU_4	A	*Sydowia polyspora*	−0.78	0.44	−1.78	0.075
OTU_3	A	*Cladosporium* sp.	0.06	0.38	0.15	0.882
OTU_8	A	*Celosporium larixicola*	−1.04	0.50	−2.08	0.038
OTU_7	A	*Aureobasidium pullulans*	−0.80	0.49	−1.63	0.103
OTU_22	A	*Phaeocryptopus gaeumannii*	−0.34	0.87	−0.39	0.698
OTU_9	A	Dothideomycetes sp.	−0.60	0.56	−1.06	0.287
OTU_5	A	Arthoniomycetes sp.	−0.03	0.81	−0.04	0.966

[a] Group refers to taxonomic level of phylum, where A is Ascomycota and B is Basidiomycota.
[b] Putative taxonomic assignment of each OTU.

enough such that observation of patterns that may be affected by tree species diversity would be masked by such large differences.

We further observed that there was a relationship between the abundance of six OTUs in Hainich and whether Norway spruce was in monoculture plots or in mixed plots. Our observation confirms our hypothesis that there would be a relationship between specific fungal taxa and tree species diversity. An additional OTU in Râşca and four OTUs in North Karelia from the *Finland RNA* study were affected as well by tree species diversity. Possible reasons for tree species richness effects could be the microclimate created by presence of other tree species (Stone et al., 1996; Unterseher et al., 2007), dispersal limitation resulting from the complexity of the forest structure (Lodge and Cantrell, 1995) and inoculum potential (Moricca and Ragazzi, 2011). Decreased host density or the presence of other tree species may result in decreased fungal infections (Barbosa et al., 2009; Castagneyrol et al., 2013).

The direction of tree species diversity impact on individual fungal taxa may reflect the host range of these taxa. This could result in reduced contacts between the fungus and host, as suggested by the dilution effect (Keesing et al., 2006). Species that specialize on a single or few hosts would likely be negatively affected by tree species richness as the availability of suitable hosts decreases. Still, a specialist fungal species found in this study, OTU_1 (*C. ledi*), did not exhibit any tree species richness effects. This may, however, reflect long distance dispersal abilities of rust fungi and their lifecycle. *C. ledi* requires two hosts to complete its lifecycle, on Norway spruce and the alternate host, a shrub, *Ledum palustre*. However, in the present study, it is likely that the needles were infected by spores dispersed from *L. palustre* that were locally present in wet sites in the coniferous forest but that grew outside

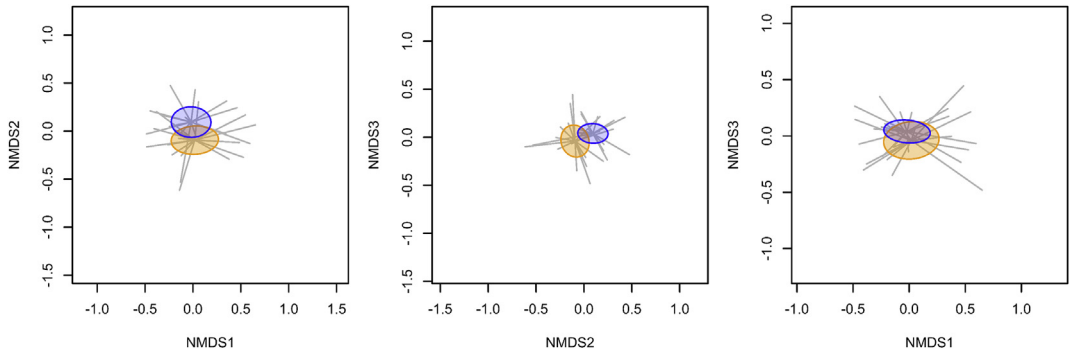

Fig. 3. NMDS results of the fungal community composition of plot-level OTUs for each community type, i.e. active (RNA) and total (DNA) fungal community, in the North Karelia forest in Finland. Axes are arbitrary, represent three NMDS dimensions and scaled in units of Bray-Curtis dissimilarity. A stable solution was reached (stress value = 0.13). The colored ellipses mark the 95% confidence interval of the group centroids for either the active RNA community (orange) or total DNA community (blue). Lines link the samples pooled at the plot level to the group centroids.

Table 4
Generalized linear mixed-effects model results at the fungal species level for the dominant OTUs. Tree species richness effect on OTU abundance across all individual trees for the fungal community type, i.e. active (RNA) and total (DNA), in North Karelia, Finland. Significant results (Bonferroni corrected a = 0.05/number of samples in each community type) are indicated in bold print (RNA $\alpha = 0.0009$; and DNA $\alpha = 0.0009$).

	Group[a]	Putative taxonomic assignment[b]	Parameter estimate for tree species richness			
			Estimate	StdError	Z-value	P
Active (RNA)						
OTU_1	B	*Chrysomyxa ledi*	0.12	1.22	0.10	0.922
OTU_19	A	Venturiales sp.	−0.01	0.31	−0.02	0.986
OTU_16	B	*Heterobasidion parviporum*	−3.67	0.95	−3.87	1.09E-04
OTU_9	A	Dothideomycetes sp.	0.25	0.22	1.14	0.254
OTU_20	A	Eurotiomycetes sp.	0.10	0.30	0.32	0.752
OTU_14	A	Arthoniomycetes sp.	0.34	0.34	1.00	0.318
Total (DNA)						
OTU_1	B	*Chrysomyxa ledi*	−0.34	0.64	−0.52	0.601
OTU_14	A	Arthoniomycetes sp.	0.46	0.44	1.06	0.291
OTU_19	A	Venturiales sp.	0.10	0.37	0.27	0.786
OTU_10	A	Hypocreales sp.	−0.81	0.56	−1.46	0.145
OTU_9	A	Dothideomycetes sp.	0.42	0.20	2.14	0.033
OTU_4	A	*Sydowia polyspora*	−0.16	0.17	−0.95	0.343
OTU_8	A	*Celosporium larixicola*	0.12	0.23	0.52	0.602
OTU_5	A	Arthoniomycetes sp.	0.78	0.66	1.17	0.241
OTU_6	A	*Ceramothyrium* sp.	0.58	0.22	2.60	0.009
OTU_20	A	Eurotiomycetes sp.	0.18	0.00	81.50	<2e-16
OTU_3	A	*Cladosporium* sp.	−0.19	0.30	−0.65	0.518
OTU_16	B	*Heterobasidion parviporum*	−2.80	1.03	−2.71	0.007
OTU_28	A	Botryosphaeriales sp.	1.54	0.60	2.57	0.010
OTU_21	B	*Exobasidium canadense*	−0.30	0.31	−0.97	0.334
OTU_32	A	*Phoma* sp.	0.17	0.00	87.50	<2e-16
OTU_22	A	*Phaeocryptopus gaeumannii*	−0.05	0.44	−0.11	0.915
OTU_43	A	Arthoniomycetes sp.	0.54	0.24	2.22	0.026
OTU_34	A	*Kirstenboschia* sp.	0.25	0.42	0.61	0.545
OTU_13	B	*Exobasidium* sp.	−0.02	0.25	−0.07	0.949
OTU_11	A	*Taphrina carpini*	−0.12	0.00	−58.50	<2e-16
OTU_7	A	*Aureobasidium pullulans*	−0.50	0.35	−1.41	0.158
OTU_62	A	*Dwayaangam* sp.	0.59	0.27	2.17	0.030

[a] Group refers to taxonomic level of phylum, where A is Ascomycota and B is Basidiomycota.
[b] Putative taxonomic assignment of each OTU.

the sampling plots.

Positive tree species richness effects on fungal species abundance may reflect properties of generalist fungal taxa. Increased tree richness would provide more possible host species for a generalist fungal species that has a broad host range. OTU_7 (*A. pullulans*) is a common and cosmopolitan species (Zalar et al., 2008) that had a positive relationship with tree species richness. However, we have limited information about the ecology of most of these fungal taxa thus hindering efforts to understand the observed patterns.

Analysis of the metabolically active fungal community presumably gives a picture of the functionally important members of the fungal community. In our study, fungal community compositional differences between the metabolically active fungal community and the total community were observed, though there was no shift along the tree species diversity gradient in either community type, and fungal diversity was likewise not different. We thus reject the hypothesis that community type (i.e. active (RNA) and total (DNA) fungal communities) composition and fungal diversity change with tree species richness. In general, we found that the active community did not provide better resolution of the fungal community than the total community. These findings contrast with that observed in soil fungal communities. While Baldrian et al. (2012) found no differences in the fungal richness

between the metabolically active and total soil fungal communities, they did observe greater differences in fungal diversity and community composition in metabolically active communities. In contrast and concurrent with our results, Pennanen et al. (2004) found relatively consistent patterns between the metabolically active and total soil fungal communities in response to treatment.

One interesting example was provided by OTU_16 (*H. parviporum*). OTU_16 was detected in the active fungal community and was negatively correlated with tree species diversity. *H. parviporum* is a common root and butt rot fungus causing severe damage in managed Norway spruce forests in Europe (Asiegbu et al., 2005). The fungus has been commonly detected in several environmental studies also from other sources (Korhonen and Stenlid, 1998; Persson et al., 2009, 2011; Strid et al., 2014). Basidiospore dispersal is much more efficient within short distances in a forest stand than farther away (Möykkynen et al., 1997). Since *H. parviporum* is a relatively narrow host range specialist on Norway spruce it was expected to be negatively influenced by a host dilution effect, which has been previously observed (Piri et al., 1990; Thor et al., 2005).

Our study revealed strong fungal community compositional differences between forest sites. The difference between the composition in the North Karelia forest in Finland and Hainich forest in Germany may reflect site-specific factors. Fungi respond to climatic factors such as precipitation and temperature (Gadgil, 1974; Stone et al., 2008), radiation and relative humidity (Juniper, 1991), and vegetation period length (Lodge and Cantrell, 1995). North Karelia has a mean annual temperature (MAT) of 2.1 °C, which in general is colder compared to Hainich (6.8 °C), though the mean annual precipitation (MAP) is relatively similar between the two forests, i.e. 700 mm and 775 mm, respectively. While MAT and MAP do not say anything about the conditions during the time when the needles are actively photosynthesizing, they serve as proxies for dissecting potential differences. Light regimes in the summer months in North Karelia are also different than in Hainich. An additional factor that may impact the fungal community is the land use history and the management practices (Siira-Pietikäinen et al., 2001; Jangid et al., 2011). The forest in North Karelia originated from clear-cuts with low management regimes, while the forest in Hainich is old-growth, uneven aged forest and has been unmanaged in the last 50 y. Additionally, the origin of Norway spruce populations may also explain the differences observed in this study, suggesting that host genotypes may play a role in determining the fungal community (Bailey et al., 2005; Bálint et al., 2013). While we have no data to clarify the population origins of the trees sampled in this study they may presumably have derived from different glacial refugia since the last ice age (Huntley and Birks, 1983). Further studies are required to disentangle the contribution of each of these biotic and abiotic factors shaping the fungal communities in forests.

An enormous diversity of fungal species was detected in the current-year needles, i.e. those that flushed during the sampling season, in our study. Older needles would presumably accumulate fungal species with age (Legault et al., 1989; Espinosa-Garcia and Langenheim, 1990). The richness and diversity of fungi associated with Norway spruce needles has been previously shown by different methods (Müller et al., 2001; Rajala et al., 2013; Menkis et al., 2015), and close to 100 species have been cultured (Sieber, 1988). Fungal species that are expected to be common on Norway spruce needles include *Lophodermium piceae*, *Rhizosphaera kalkhoffii*, and *Tiarosporella parca* (Heiniger and Schmid, 1986; Sieber, 1988; Solheim, 1989; Livsey and Barklund, 1992). *L. piceae* and *R. kalkhoffii* were detected in our dataset but in low abundance (<0.1% of reads, 14% of samples and 0.6% of reads, 39% of samples in the *all sites* study, respectively). *L. piceae* is generally a very common endophyte (Livsey and Barklund, 1992; Müller and Hallaksela, 1998, 2000; Müller et al., 2001), but was found in low abundance in young needles (Rajala et al., 2014) and abundantly in current-year needles. Barklund (1987) found that about 80% of the needles were infected with *L. piceae*. However, *T. parca* was not detected in our study. A search in GenBank revealed that primers used in this study do not match this fungus. It is oftentimes the case that primers designed to amplify many taxa do not detect all fungal species (Ihrmark et al., 2012), or that reference sequences are lacking all together.

Consistent with work by Arnold and Lutzoni (2007) and Higgins et al. (2007), our study found that Ascomycota were highly represented in the needle community of Norway spruce, with Dothideomycetes and Sordariomycetes being the predominate classes in the temperate and boreal forests. Recently, a metatranscriptomic study of the fungal community of Norway spruce (needles, buds and vegetative shoots) found that the active members are dominated by species from the class Dothideomycetes and specifically the orders Pleosporales and Capnodiales, Eurotiomycetes and Sordariomycetes (Delhomme et al., 2015). Not only were they abundant in our total community datasets, they were also metabolically active fungal taxa in the needles. The next question to address is the function of these species. Müller et al. (2001) suggested that a few species act as pioneer decomposers. Delhomme et al. (2015) identified highly expressed genes of oxidases, oxygenases, dehydrogenases and sugar transporters in spruce tissues. The high expression of these genes suggests that the fungi are actively impacting Norway spruce to gain access to nutrients. Whether these activities reflect the working contribution of endophytes that are long thought to be quiescent, of epiphytes, or both require further experiments to disentangle.

In conclusion, we observed tree species diversity effects on the fungal community composition in one mature forest. However, these effects were not a general phenomenon; no patterns could be determined from other studied mature forests. Differences in fungal community composition were observed in different forests that may reflect site-specific factors. Against our expectations, the total fungal community (from DNA) was as informative as the active fungal community (from RNA) and the latter did not contribute to any greater insights. More robust and general conclusions about potential tree diversity effects on fungal communities may require increased replication within one forest site and across similar forest types. Other associated factors such as tree species identity effects and the tree species compositions within the plots should also be disentangled.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2016.07.003.

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