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

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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),

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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 $\mu L^{-1}$ DreamTaq DNA Polymerase and