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A cost-effective and efficient strategy for Illumina sequencing of fungal communities: A case study of beech endophytes identified elevation as main explanatory factor for diversity and community composition

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ABSTRACT

We describe an accurate and efficient workflow for highly multiplexed paired-end Illumina sequencing of fungal full-length ITS amplicons. The impact of habitat and substratum conditions on leaf-inhabiting fungal communities was analysed. Fully vital and clearly senescent leaves of European beech (*Fagus sylvatica*) were sampled along an elevation gradient of about 1000 m in the Bavarian Alps, Germany, in autumn 2013. Surface-sterilised leaves were used for genomic DNA extraction, tagging-by-amplification and high-throughput sequencing. Significant correlation of community composition with elevation was observed. The mycobiome was little affected by the physiological state of the leaves, because only a partial shift of taxonomic composition was observed from vital towards clearly senescent leaves.

1. Introduction

Fungal endophytes colonise living tissues of their plant hosts without causing visible disease symptoms (Sieber, 2007; Rodriguez et al., 2009; Unterseher, 2011; Bullington and Larkin, 2015).

Community composition of leaf mycobiomes is influenced by numerous and often interacting environmental factors including host plant identity (Unterseher et al., 2007, 2012; Peršoh, 2013; Weig et al., 2013), genotype (Cordier et al., 2012b; Bálint et al., 2013), the structure and diversity of the surrounding vegetation (Helander et al., 2007), elevation (i.e. temperature; Hashizume et al., 2008; Cordier et al., 2012a; Zimmerman and Vitousek, 2012; Davey et al., 2013) or geographical location (U'Ren et al., 2012; Blaalid et al., 2014). Most recently, Matulich et al. (2015) discovered an overshadowing effect of temporal variation (seasonality and interannuality) on long-term responses of microbial communities to increased drought and nitrogen availability.

In general, changes in the composition of micro- and mycobiomes along elevational gradients are interpreted as a consequence of changing environmental parameters such as temperature, atmospheric pressure and light intensity (e.g. Streit et al., 2014). Altered abiotic conditions with elevation also induce biochemical and physiological responses in the host plants (e.g. Reich and Oleksyn, 2004; Witzell and Martin, 2008). It has been shown previously that leaf photosynthetic capacity (measured as chlorophyll concentration) increased with increasing altitude (Oleksyn et al., 1998; Vitasse et al., 2009; Bresson et al., 2011). Leaves exposed to broader day–night temperature ranges at higher altitudes had higher flavonoid concentrations than control groups (Jaakola and Hohtola, 2010), suggesting a need for such molecules to protect against stressful conditions, such as herbivory, drought,

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nitrogen depletion or solar irradiation in mountains (Harborne and Williams, 2000; Winkel-Shirley, 2002; Meyer et al., 2006; Brossa et al., 2009).

Leaf ageing and leaf litter transformation influence diversity and composition of phyllosphere mycobiomes (Jumpponen and Jones , 2010; Baldrian et al., 2013; Scholtysik et al., 2013; Voříšková and Baldrian, 2013). Leaf endophytes are known to become saprobes during leaf senescence and to influence early litter decomposition (e.g., Promputtha et al., 2010; Peršoh, 2013; Unterseher et al., 2013). Meta-analyses revealed a major fraction (67%) of endophytic fungi to be present in litter (Osono, 2006), with even more endophytes expected in fresh litter for a short time. Endophytic fungi involved in litter decomposition are obviously most abundant and active during early decomposition stages, when readily available sugars or easily degradable cellulose are still present (Peršoh et al., 2013). These substances are soon depleted leading to a community shift towards typical litter and soil fungi (Peršoh et al., 2013; Voříšková and Baldrian, 2013).

The last few years have seen rapid progress in fungal biodiversity research of the phyllosphere and other species-rich habitats with cultivation-free high-throughput sequencing (HTS) technologies (Jumpponen and Jones, 2009; Cordier et al., 2012a; Kemler et al., 2013; Schmidt et al., 2013). The critical evaluation of HTS technologies and data (Amend et al., 2010; Gilles et al., 2011; Krueger et al., 2011; Baldrian et al., 2013; Peršoh, 2015) and constantly developed, refined and improved bioinformatic pipelines (Schloss et al., 2009; Caporaso et al., 2010; Køljalg et al., 2013; Vetrovský and Baldrian, 2013;Bálint et al., 2014) allow for a broader understanding of fungal communities. Among the available high-throughput technologies, Illumina-based sequencing provides unprecedented sequencing capacities and the ability to multiplex hundreds of samples (Caporaso et al., 2011; Smith and Peay, 2014). Currently paired-end Illumina MiSeq data can cover either full ITS1 or ITS2 regions.

The present paper focuses on a method with the detailed description and evaluation of a cost-effective and accurate amplicon library preparation followed by an accurate and easy-to-use bioinformatic workflow. A comprehensive biodiversity assessment allowed us to approach our main hypothesis of changing fungal richness, community composition and phylogenetic signals along the present elevation gradient. More specifically we analysed whether the favourable environmental conditions for beech trees at low elevations support a higher richness of phyllosphere endophytes compared with the more extreme site conditions towards the natural timber line of the tree species.

Considering recent observations of a rapid community turnover of leaf-inhabiting microfungi in late attached, vital leaves and fresh leaf litter (Voříšková and Baldrian, 2013), we aimed to assess hypothesised shifts in fungal community composition on an even finer time scale (Peršoh, 2013) by analysing fully vital and clearly senescent autumn leaves.

2. Materials and methods

2.1. Sampling design and field work

Three sites were selected at different altitudes in a beech (*Fagus sylvatica*) dominated forest (*Helleboro-Fagetum*) of the mountain massif 'Untersberg' in the Berchtesgaden Alps near the city of Marktschellenberg, Bavaria, Germany (Fig. 1; valley site; Lat. 47.712946, Long. 13.040101, 517 m a.s.l.; mountain site: Lat. 47.683158, Long. 13.002102, 975 m a.s.l.; treeline site; Lat. 47.714043, Long. 13.010827, 1381 m a.s.l.). To ensure that altitude accounted for most of the environmental variation, locations with the same soil type (Leptosol over limestone) and similar surrounding plant species (e.g. *Acer pseudoplatanus*, *Picea abies*, *Daphne mezerium*, *Dentaria enneaphyllos*, *Helleborus niger* and *Hepatica nobilis*) were selected. In this area the natural mountain treeline at ca. 1500 m a.s.l. is identical to the natural mountain line of the target tree species *F. sylvatica*. (Fig. 1). At each site (elevation) five trees with a similar diameter at breast height of ca. 20 cm were chosen within an area of 50 × 50 m. From each tree ten vital and ten senescent shaded autumn leaves were collected randomly from approximately 5 m above the ground in October 13th 2013. The living green leaves showed no visible signs of ageing and disease, whereas the strongly aged leaves were mostly yellow to brown but not yet desiccated, undamaged and still attached to the twigs. Leaves were submerged in 70% ethanol for 3 min immediately after sampling to inhibit activity of surface microbiota during transport. Vital and senescent leaves of each tree were stored separately in paper bags at 4–7 °C. Within 48 h after collecting, leaves were thoroughly surface sterilised according to standard methods (Unterseher et al., 2013) and frozen at −80 °C until further processing.

2.2. DNA extraction

Vital and senescent leaves of each sample (individual tree) were homogenised with sterile distilled water in a commercial blender for 1 min at full speed and filtered through analytical sieves as described in Unterseher et al. (2013). Approximately 100 mg (fresh weight) of the retained leaf particles were used for extraction of genomic DNA with Charge Switch gDNA Plant Kit (Invitrogen) according to the manufacturer's instructions. A total of 30 samples (3 sites, 5 trees per site, 2 leaf types per tree) were extracted for this study. Together with another 300 samples from different studies, they were used for amplicon library preparation and sequenced in a single run. Preliminary amplifications of the internal transcribed spacer region (ITS) with the fungus-specific primer pair ITS1F/ITS4 (White et al., 1990) resulted in infrequent successful amplification. Therefore, we prepared a nested PCR, starting with the amplification of ITS with the universal primer pair V9G/ITS4 (de Hoog and Gerrits van den Ende, 1998) under common conditions with 30 cycles (Unterseher et al., 2013). Amplicons were purified with 1:5 diluted ExoSap-IT (Affymetrix UK Ltd., United Kingdom) according to the manufacturer's instructions and then served as template DNA for the Illumina library preparation. Whereas most studies use either ITS1 or ITS2, full-length ITS amplicons were sequenced in the present study. An overlap of 50–70 bp in the conserved 5.8S region was calculated for the 300 bp paired-end sequencing and sufficiently high read quality for successful contig assembly was expected.

2.3. Multiplexing and library preparation

Library preparation consisted of two consecutive amplification steps. The first PCR introduced internal barcoding tags of varying length, selected from previously approved tag sequences (Poland et al., 2012, Table 1). The corresponding primers (PCR_{TA} in Table 1) consisted of the commonly used primers ITS1F and ITS4 (White et al., 1990) extended by four different forward and four different reverse tags and 21 bp of the Illumina sequencing primer, which served as binding site for the primer used in the second amplification (PCR_{IN} in Table 1).

PCR_{TA} reactions (MangoTaq kit from Bioline GmbH, Germany) contained 15.64 μl H_2O, 5 μl buffer (5×, containing gel-loading dye), 1.7 μl $MgCl_2$ (50 mM), 0.5 μl dNTP (10 mM), 0.5 μl primer 1 and 2 (10 mM), 0.16 μl polymerase (5 U/μl) and 1 μl of DNA in a total volume of 25 μl. Amplification started at 94 °C for 3 min followed by 33 cycles of 94 °C for 27 s, 56 °C for 60 s, 72 °C for 90 s and final