

Fungal community composition shifts along a leaf degradation gradient in a European beech forest

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Abstract

Aims The fungal communities in living and decomposed leaves of European Beech (*Fagus sylvatica*) were compared to identify the phyllosphere fungi involved in litter decomposition at a site in Bavaria, Germany.

Methods New primers were designed to cover a broad range of fungal ribosomal DNA sequence diversity. Following ‘environmental PCR’, clone libraries from each of five samples of living leaves (surface-sterilized and untreated), freshly fallen, initially and highly decomposed leaves, were screened using RFLP fingerprinting.

Results Statistical analysis (ANOSIM) revealed that the fungal communities colonizing living (a) and initially decomposed leaves (c) significantly differed between each other and from freshly fallen (b) and highly decomposed leaves (d). Fungal assemblages of a and d were statistically indistinguishable from each other and from the endophyllous fungal community in living leaves.

Conclusions The results showed that endophyllous fungi play a role throughout the whole decomposition process of beech leaf litter. Therefore, clarification of the life cycle of certain endophytic and/or soil fungi

may only be achieved by considering both phyllosphere and soil habitats.

Keywords *Fagus sylvatica* (European Beech) · Phyllosphere · Endophytic fungi · Soil fungi · ITS rRNA gene · RFLP fingerprints

Introduction

Decomposition of plant litter is a key process with regard to carbon and nutrient cycling at ecosystem and global scales (Berg and McClaugherty 2008). Accordingly, strong efforts have consistently been undertaken to understand the underlying processes and were even intensified in view of climate change (Baldrian and Lindahl 2011). Basically, the plant tissue degradation process depends on availability of the substances that are most easily degradable by the decomposer community (Berg and McClaugherty 2008), and can be subdivided into three phases (Moorhead and Sinsabaugh 2006; Šnajdr et al. 2011): (a) the consumption of readily available sugars and other soluble substrates; (b) the degradation of structural polysaccharides such as hemicelluloses and celluloses; and finally (c) the most recalcitrant compounds such as lignin are attacked. Due to accumulation of the partly degraded litter, age of litter increases with depth within the organic layers covering the mineral soil. This leads to a vertical stratification with each of the layers harbouring different microbial communities, representing successional stages in the

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decomposition process (Lindahl et al. 2007; Baldrian et al. 2011a).

In forests, the degrader community is dominated by fungi, even though bacterial biomass may equal fungal biomass at later decompositional stages (Baldrian et al. 2011a). The primary decomposer community (a) consists mainly of ascomycetes, of which those fungi already colonizing the living leaves may constitute a significant proportion (Koide et al. 2005). Although epi- and endophyllous fungi are reported to be equally abundant in freshly fallen leaves (Osono 2006), epiphyllous fungi are considered to decrease more rapidly in numbers due to their limited capability for utilizing structural polysaccharides, as compared to endophytic fungi (Osono and Takeda 2001). In addition, ascomycetes cumulatively colonizing freshly fallen leaves from the underlying litter layer contribute to shifts in fungal community composition between phases a and b (Osono 2002). With the onset of lignin degradation limiting the decomposition process, basidiomycetes increasingly dominate the decomposer community (Lindahl et al. 2007; Baldrian et al. 2011a), because ascomycetes are, with a few exceptions such as the Xylariales, enzymatically less well-equipped for lignin degradation in comparison with basidiomycetes (Baldrian et al. 2011b).

Fungal diversity is known to be extremely high in soil (cf. Gewin 2006), but precise estimates have to be regarded with caution, because the extraordinary effort required prohibits exhaustive assessments using conventional methods. Species richness estimates from molecular surveys are prone to errors (Dickie 2010; Unterseher et al. 2011) and so far there has been no separate and comprehensive analysis of the different organic layers. An exception is the study of Lindahl et al. (2007), who recognized, for pine forest soil, a positive correlation between fungal diversity and increasing litter decomposition.

The significance of European Beech (*Fagus sylvatica* L.) for the vegetation in Central Europe has recently been underlined by the granting of UNESCO World Heritage status (whc.unesco.org/en/list/1133) to five German beech forests. Accordingly, beech is one of the most profoundly studied tree species (e.g., Magri et al. 2006), including the degradation of beech wood (Heilmann-Clausen and Christensen 2003; Liers et al. 2011) and nitrogen fluxes during leaf litter decomposition (d'Annunzio et al. 2008). However, although detailed studies on the fungal community involved in litter decomposition have been conducted for spruce and pine

forests (Lindahl et al. 2007; Baldrian et al. 2011a), there are still none for beech forest sites. In line with these studies, 'environmental PCR' was applied to assess fungal community composition along a vertical degradation gradient on a beech forest soil. Furthermore, we analysed the fungal assemblage in the phyllosphere of living leaves, to address the following hypotheses:

- 1) The fungal community structure differs along a vertical gradient representing successional stages of beech litter degradation.
- 2) Fungal community composition of the phyllosphere resembles that of recently fallen leaves due to previously endophyllous fungi participating in primary decomposition.
- 3) The phyllosphere and decomposer communities increasingly differ with increasing litter decomposition, due to the inability of phyllosphere fungi to utilize the recalcitrant substances accumulated during the decomposition process.

Materials and methods

Sampling site and sample preparation

The sampling site is located in the beech forest area 'Steigerwald' (Bavaria, Germany), at 480 m altitude. Mean annual precipitation is 800 mm, half of which falls during the growing season (from April until October) and the mean annual temperature is 7.5°C. At the sampling site, the tree population exclusively consists of *Fagus sylvatica* L., is 190 years of age, and has not been farmed for at least 30 years. The ground is even and consists of keuper arenite, covered by cambisol. Beneath the litter layer (Oi) of 1–2 cm depth there is a layer of fragmented litter (Oe), which varies in depth among the sampling plots (1–4 cm). The layer of completely decomposed litter (Oa) is either lacking or up to 0.5 cm thick. Understorey plants are scarcely present due to a rather dense leaf canopy cover.

Samples were collected in early autumn (2009/09/14), during the initial fall of leaves. Five plots with adult beech tree individuals represented five sampling plots (A–E). The plots were distributed along a circular line (Ø ca. 80 m) (A: 49° 55' 33.852" N, 10° 34' 12.428" E; B: 49° 55' 33.031" N, 10° 34' 8.99" E; C: 49° 55' 32.315" N, 10° 34' 8.591" E; D: 49° 55' 31.966" N, 10° 34' 8.137" E; E: 49° 55' 31.638" N, 10° 34' 12.749" E).

E). Four different samples, representing different stages of leaf decomposition, were collected at each plot. As the samples from different plots represented independent replicates, the layout was sufficient to meet the requirements of the most recently formulated demands for environmental studies (Prosser 2010). The following four degradation categories (DC) were distinguished: living shade-leaves attached to the tree (DC-0), freshly fallen, apparently still intact leaves from the ground surface (DC-1, corresponding to the upper Oi layer), initially decayed leaves, still recognizable as beech leaves but appearing 'mummified' due to loss of inner tissue structure (DC-2, on the border between the Oi and Oe layer), and fairly decomposed litter directly above the uppermost mineral horizon (DC-3, Oa or lower Oe layer adjacent to the A horizon). Altogether, twenty sample units were drawn: five plots \times four degradation categories.

Litter and organic layers were sampled directly beneath the respective trees from each of which 10 leaves were sampled 4–5 m above ground level (DC-0). Plant roots were discarded. Five sampled leaves of each tree were directly subjected to DNA-extraction (DC-0), while five were previously surface-sterilized according to the methodology of Unterseher and Schnittler (2009), to assess the endophytic fungal diversity (DC-0e) by incubation under a regime of careful stirring in distilled water (dH₂O, 1 min), 70 % EtOH (2 min), 1 % NaHClO (5 min), 70 % EtOH (1 min) and finally three times in dH₂O (1 min). While leaves for surface sterilization (accomplished within 24 h after sample collection) were transported at ambient temperature, all other samples were transferred to the laboratory in liquid nitrogen. There, the samples were subdivided into portions of 0.2 g (wet weight) for DNA extraction and stored at -80°C until further processing. Portions of intact leaves were obtained by pooling disks of about 0.5 mm in diameter cut from at least five different leaves. Decayed leaves were carefully homogenized by hand.

Molecular analyses

As detailed by Peršoh et al. (2008), Al₂(SO₄)₃ was added to the samples of fallen leaves, to flocculate humic substances prior to cell disruption by bead beating (FastPrep™ Instrument, Bio 101) in a nucleic acid extraction buffer (0.4 M LiCl, 100 mM Tris–HCl, 120 mM EDTA 2.5 % SDS, pH 8). Following a

phenol-chloroform purification, total genomic DNA was precipitated by the addition of NaCl and 2-propanol.

Coverage of fungal sequence diversity by the commonly applied primers for amplification of the internal transcribed spacer region (ITS) of the rRNA gene was verified against a subset representing the sequence diversity of all fungal small (SSU) and large subunit (LSU) rRNA gene sequences included in the ARB-SILVA databases (Pruesse et al. 2007), release nos. 94 (SSU) and 102 (LSU), respectively. Because this analysis revealed insufficient coverage and specificity of ITS4 (White et al. 1990) and especially of the ITS1F (Gardes and Bruns 1993) primer, a new set of six multiplex primers was designed (Table 1).

Each polymerase chain reaction (PCR) batch of 25 μl in total included 15.65 μl H₂O, 0.75 μl MgCl₂ (50 mM), 2.5 μl 10 \times PCR buffer (Invitrogen), 2.5 μl dNTPs (2 mM), 0.42 μl of each of the six primers (10 μM), 0.1 μl *Taq* polymerase (5 U/ μl , Invitrogen), and 1 μl of 100 or 1000-fold diluted DNA extract.

Cycling reactions started with an initial denaturation of 2 min at 95 $^{\circ}\text{C}$ followed by 35 cycles of 20 s at 94 $^{\circ}\text{C}$ (denaturation), 1 min at 59 $^{\circ}\text{C}$ (annealing), 2 min at 72 $^{\circ}\text{C}$ (elongation) and finished with an extension step of 15 min at 72 $^{\circ}\text{C}$. The PCR products were subcloned using the TOPO TA Cloning Kit for sequencing (Invitrogen), as recommended by the manufacturer. Colonies were picked into 16.65 μl water and the inserts were directly amplified using the primers M13f (5'-GTAAAACGACGGCCAG-3') and M13r (5'-CAGGAAACAGCTATGAC-3'). The PCR reactions were prepared and operated as described above, but with the initial denaturation step prolonged to 6 min and the annealing temperature decreased to 53 $^{\circ}\text{C}$.

In order to analyse restriction fragment length polymorphism (RFLP), amplicons were digested by incubation with the restriction enzymes *MspI* (5'C/CGG) and *AluI* (5'AG/CT) as recommended by the manufacturer (Fermentas, St. Leon-Rot, Germany). Undigested PCR products (0.5 μl) were merged with the corresponding digested PCR products (5 μl) for gel electrophoresis (3 % agarose; 20 min at 90 V+90 min at 120 V), using a 'Low Molecular Weight' DNA ladder (range: 25–766 bp; BioLabs, Frankfurt) as a length reference.

From each of the 39 most abundant RFLP types, at least two clones from 2 to 4 different

Table 1 Primer sequences. Sequences are given in 5'–3' orientation

Forward primers		Reverse primers	
Name	Sequence	Name	Sequence
fSSUh49F1	CCGATTGAATGGTTATAGTGAG	rLSUh11F1	CCTCCGCTTATTGATATGC
fSSUh49F2	GATTGAATGGCTCAGTGAG	rLSUh11F2	TTTCCTCCGCTTATTGATATG
fSSUh49F3	CGATTGAATGGCTTAGTGAG	rLSUh11F3	AGTTTCTTTTCCTCCGCT

samples were selected for sequencing. Amplified inserts were mixed with the primer ITS2 (White et al. 1990) and shipped to Macrogen Europe (Amsterdam) for purification and sequencing of the ITS1 region.

Data evaluation

Lengths of undigested amplicons served to verify the sum of the fragment lengths for coherence. *GeneProfiler* (v 4.05, Meyer Instruments, Houston) was used to quantify the fragment lengths. Bands less intense than the following smaller DNA fragment were discarded, as were fragments smaller than 50 bp. From two bands of similar size, only the smaller fragment was selected, if the sum of bp associated with PCR fragments was higher than the bp sum for undigested DNA.

A list of the fragment lengths was analysed using *R* (v 2.11.1, R Development Core Team 2008). Similarities among RFLP patterns possessing equal numbers of fragments were assessed using the function 'RFLPdist' (Flessa et al. 2010). Similar RFLP patterns were initially grouped to RFLP types by hierarchical cluster analysis. Thresholds for grouping were individually determined and differed according to the number of fragments involved. The initial groupings were checked by eye and refined when necessary. A data matrix comprising the abundance of each RFLP type detected in the 25 samples was further processed in *Primer 6* (Plymouth Routines, v 6.1.1) and used to identify frequent RFLP types, i.e. RFLP types present in at least three samples. Similarities among ITS1 sequences of the frequent RFLP types were calculated using Local BLAST (<ftp://ftp.ncbi.nih.gov/blast/executables/release/2.0.10/blast-2.0.10-ia32-win32.exe>) with the parameters '-m8 -r2 -G5 -E2'. The *R* function 'simMatrix' was applied to transform the calculated pairwise similarities into a similarity matrix, and a hierarchical cluster analysis

('hclust') was conducted to group RFLP types according to their ITS1 sequence similarity to ITS1 genotypes. The method 'average linkage' and a threshold of 90 % were applied. The sequences grouped to each genotype were checked by eye to identify that inconsistencies within genotypes were not being ascribable to sequencing artifacts. Taxonomic affiliation of the sequences was assessed as detailed by Peršoh et al. (2010). Briefly, a BLAST search was conducted against the GenBank database (www.ncbi.nlm.nih.gov) and all hits which attained at least 90 % of the best BLAST score were included. A taxonomic level was chosen for name assignment, unifying all names under which the respective sequences were deposited. However, environmental sequences and clearly outlying names were disregarded, but documented as ambiguities and outliers, respectively (supporting information, Table S1).

The abundance matrix of RFLP types per sample was transformed to a presence/absence matrix in *Primer 6*, because abundances are hardly comparable among different sample types (i.e. leaves, litter, soil). This data set served as a basis for calculating similarities among the samples according to the presence of each RFLP type, using Jaccard's Coefficient (Jaccard 1901). Similarities among the samples were visualized by using non-metric multidimensional scaling (NMDS). Furthermore, analyses of similarity (ANOSIM) among the different degradation categories (DC) were calculated. ANOSIM calculates a *p*-value that is generally accepted as indicating significant differences between groups when below 0.05. The *R*-value expresses the degree of dissimilarity. *R*-values above 0.75 indicate clear separation of the respective groups according to the respective grouping factor, while values above 0.5 indicate an overlap between otherwise well-separated groups, and *R*-values below 0.25 indicate barely separable groups (Chapman and Underwood 1999).

Results

Primer design

A comparison against publicly available sequences revealed the commonly used primer sequences (ITS1F, ITS4) only matched a fraction of the fungal sequences (supporting information, Table S2). Most strikingly, the ITS1F primer perfectly matched only 22 % of the sequence diversity in Zygomycota (represented by 58 different sequences), and showed at least two mismatches to 69 % of the sequences. The newly designed primer sets (fSSUh49F, rLSUh11F) covered higher sequence diversity among all fungal taxa, except for the Chytridiomycota. While the newly designed reverse primer set is less selective for fungi than the ITS4 primer, selectivity for fungi is mainly achieved by the new forward primer.

Amount and reliability of assessed data

In total, 834 different ITS-RFLP genotypes (i.e. ‘RFLP types’) were identified among the 1,774 analysed clonal PCR products (i.e. ‘clones’) from 25 environmental samples. The majority (78.5 %, $n=655$) of the RFLP types were singletons, recorded only from one sample, and 64 RFLP types (representing 503 clones) occurred in at least three samples. The number of RFLP types found at least twice, but only in a single category, was 76, and 103 RFLP types occurred in different degradation categories.

Sequence analysis of the 39 most abundant RFLP types (representing 369 clones) revealed three RFLP types each corresponded to a single ITS1 genotype. The majority (28) of the sequenced RFLP types represented altogether four genotypes. While the sequences

of each of these 28 RFLP types corresponded to the same genotype, eight RFLP types represented multiple ITS1 genotypes. However, repetition of the statistical analyses to exclude potential corruption of the results revealed only negligible differences between the results if either all RFLP types were included in the analysis, or only those confirmed by sequence analyses not to represent multiple ITS1 genotypes (Table 2).

Fungal community structure

The diversity in the four degradation categories (DC) clearly differed: most RFLP types were detected in living leaves (DC-0: 302 RFLP types), followed by the Oe-Ah interface (DC-3: 227), freshly fallen leaves (DC-1: 142) and partly decomposed leaf litter (DC-2: 115). Inside the living leaves (DC-0e), 187 RFLP types were detected. Singletons, i.e. RFLP types of any abundance found in only one sample, accounted for 96 % of the diversity in DC-3 (Fig. 1), the category showing the second highest richness of RFLP types. In the most diverse category (DC-0), only 82 % of the analysed clones were singletons, and in the least diverse (DC-2), 54 % of all clones originated from at least two independent samples.

These differences in fungal community structure also become obvious from the NMDS plot (Fig. 2), where the samples from DC-2 are located in close proximity according to similar RFLP type composition. The samples from DC-3 and DC-0e are widely scattered, indicating major differences among samples of each category; i.e. a patchy distribution pattern of the fungi within these categories.

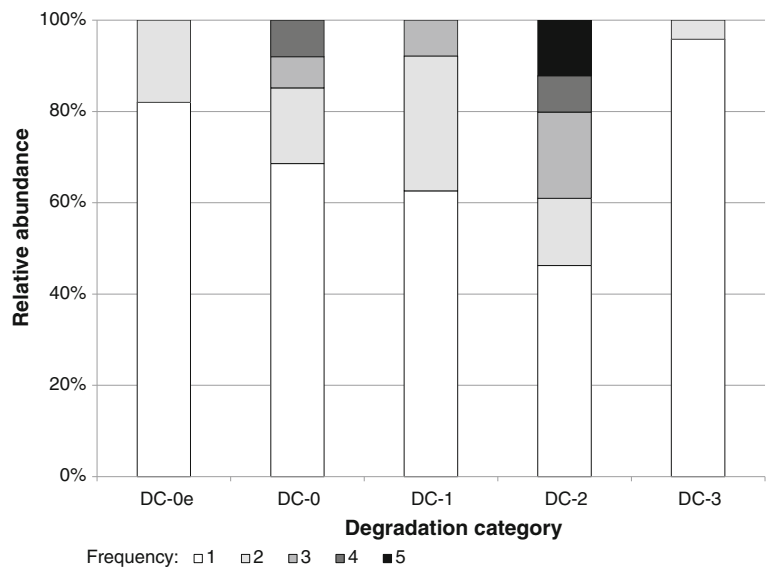
The communities in DC-0, DC-1, and DC-2 also differed significantly ($p<0.01$) according to ANOSIM

Table 2 Differences among degradation categories (DC) according to ANOSIM. R-values calculated from the presence or absence of all RFLP types (lower left) and RFLP types representing single ITS1 genotypes (upper right) in each sample, respectively. Numbers in bold highlight significance ($p<0.05$) and asterisks

indicate high significance ($p<0.01$) of differences between groups. DC-0: non surface-sterilized living leaves; DC-0e: surface-sterilized living leaves; DC-1: freshly fallen leaves; DC-2: initially decomposed leaves; DC-3: largely decomposed litter

	DC-0e	DC-0	DC-1	DC-2	DC-3
DC-0e		0.30*	0.09	0.53*	-0.03
DC-0	0.30*		0.66*	0.89*	0.31
DC-1	0.23	0.61*		0.80*	0.09
DC-2	0.66*	0.86*	0.84*		0.43*
DC-3	0.12	0.33	0.13	0.47*	

Fig. 1 Contribution of RFLP types occurring at different frequencies to the total number of clones in each degradation category. DC-0: non surface-sterilized living leaves; DC-0e: surface-sterilized living leaves; DC-1: freshly fallen leaves; DC-2: initially decomposed leaves; DC-3: largely decomposed litter



(Table 2). In addition, DC-0 significantly differs from DC-3 and DC-0e. The communities in DC-0e and DC-1, DC-0e and DC-3, and DC-1 and DC-3, were not distinguishable, as reflected by R-values <0.25 . Furthermore, DC-3 considerably overlaps with all other categories (R-values <0.5). Most distinctive is DC-2, with R-values mostly >0.6 .

Except for DC-2, which shared the most RFLP types with DC-3, all categories showed their highest overlap with DC-0e (Fig. 3). While only few RFLP types of DC-2 co-occurred in other categories, a considerable fraction of the RFLP types found in fresh litter (DC-1) was also recorded from the phyllosphere (DC-0) and at the mineral soil interface (DC-3).

Sequence analyses (supporting information, Table S1) revealed five taxa (represented by 7 ITS1 genotypes, 22 RFLP types, and 255 clones) as being most frequent (Table 3). *Phaeosphaeria* sp. almost exclusively occurred in DC-0, being present in all samples there. By contrast, yeasts with relation to the *Erythrobasidium*-clade sensu Nagahama et al. (2006) and ‘*Galactomyces* sp.’, were found in all degradation categories including living leaves. Both taxa were most frequent in freshly fallen leaves. ‘*Mycota* sp.’ also invaded the leaves, but is rare in DC-1 and absent from the subsequent degradation categories. *Mycena* spp. were mostly restricted to DC-2. In DC-3, as in DC-0e, none of the taxa was particularly frequent.

Fig. 2 Similarities among samples according to the RFLP type composition. Similarities were calculated from the presence or absence of RFLP types in each sample and visualized by non-metric multidimensional scaling (NMDS). Affiliation of the samples to the different degradation categories is indicated: DC-0: non surface-sterilized living leaves; DC-0e: surface-sterilized living leaves; DC-1: freshly fallen leaves; DC-2: initially decomposed leaves; DC-3: largely decomposed litter

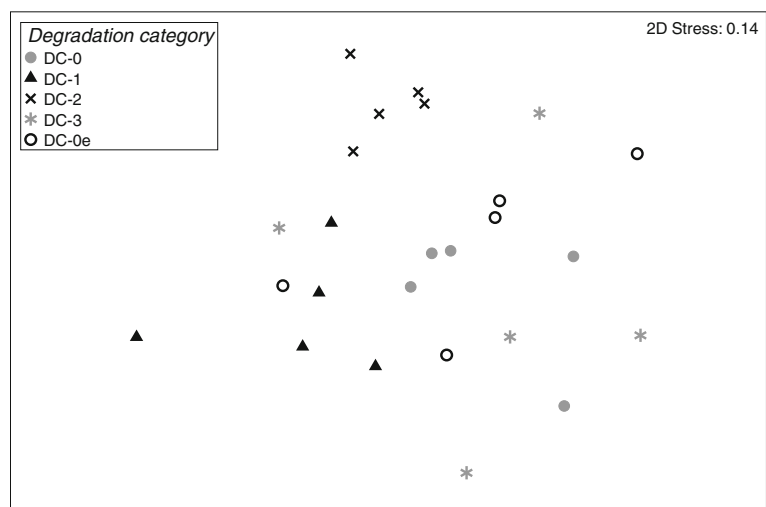
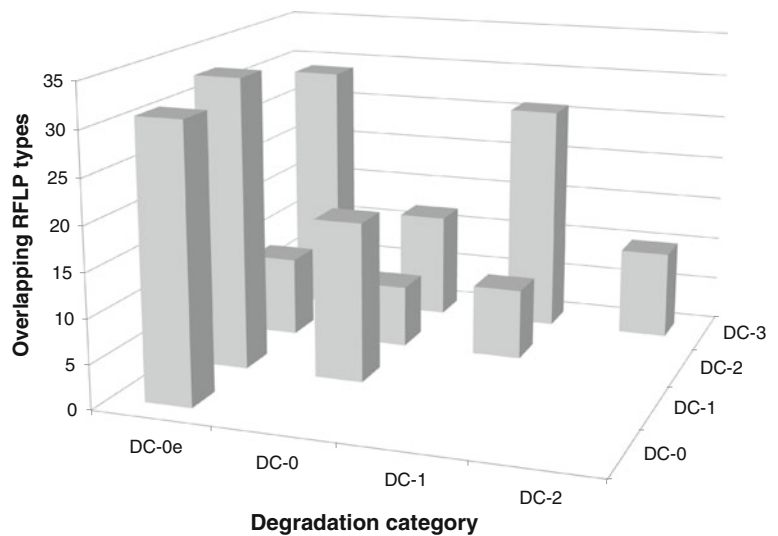


Fig. 3 Numbers of RFLP types shared between different degradation categories. DC-0: non surface-sterilized living leaves; DC-0e: surface-sterilized living leaves; DC-1: freshly fallen leaves; DC-2: initially decomposed leaves; DC-3: largely decomposed litter



Discussion

Methodology

A direct ‘environmental PCR’ approach was chosen for this study, because traditional cultivation techniques usually recover only part of the total diversity (Borneman and Hartin 2000; Bridge and Spooner 2001; van Elsas et al. 2002; Collado et al. 2007; Unterseher 2011). Massive parallel sequencing approaches (e.g., 454 pyrosequencing) seem best suited for fast automated analysis of DNA extracts from complex fungal communities, such as soil or phyllosphere fungi (Buée et al. 2009; Jumpponen and Jones 2010). However, these methods are still cost-intensive and even thorough data analyses do not guarantee the detection of rare species (Tedersoo et al. 2010) or a reliable estimation of species richness (Dickie 2010; Unterseher et al. 2011). Because the latter issue is due to the unsolved question of handling singletons and PCR bias from amplifications of complex metagenomic DNA preparations, as also applied in this study, we refrained from estimating the total diversity present in the samples. Nevertheless, the actually detected diversity is discussed, because these data are considered to be comparable among identically treated samples. Another limitation for the evaluation of sequences from environmental samples is their validation and taxonomic assignment. These mostly rely on reference sequences in public databases, which again are well known to have deficiencies (e.g., Bridge et al. 2003; Peršoh et al. 2012). Furthermore, reference sequences are available for only approximately 30 % of the known fungi (Brock et al. 2009) and

single-locus identification is not feasible for many taxa (e.g., Taylor et al. 2000; Schubert et al. 2007). To address the ecological questions at hand, cost-benefit analysis has therefore favoured the use of a fingerprinting rather than a sequence-based approach. However, DNA sequence analyses were additionally conducted, to validate the results from the RFLP analysis.

The multiple sequences obtained from each RFLP type mostly represented a single ITS1 genotype. However, several different RFLP types were derived from identical genotypes, a phenomenon known as ‘star activity’ (e.g., Polisky et al. 1975; Staab et al. 2009). Where a genotype is represented by different RFLP types in different groups, this may falsely indicate differences between groups. Because this does not apply to the data presented here, this inconsistency statistically weakens rather than boosts differences. More critical are the eight RFLP types representing multiple ITS1 genotypes. However, the inclusion of only RFLP types verified by the sequence analyses in the statistical analysis resulted in almost identical confidence values as did the inclusion of all RFLP types (Table 2). Altogether, the results clearly demonstrate that RFLP analysis of clone libraries is well-suited, in terms of reproducibility and cost, for distinguishing between complex fungal communities. Validation and identification by subsequent sequence analysis may be restricted to the key players, as revealed by statistical analysis of the RFLP type distribution pattern.

Such an approach based on environmental PCR, however, heavily depends on the properties of the

Table 3 Frequency of the most abundant taxa in the different degradation categories. The number of ITS1 genotypes, RFLP types and clones represented by each taxon is given in parentheses. Open circles indicate the absence, closed circles the

presence of the taxon in each sample. DC-0: non surface-sterilized living leaves; DC-0e: surface-sterilized living leaves; DC-1: freshly fallen leaves; DC-2: initially decomposed leaves; DC-3: largely decomposed litter

Degradation category	<i>Phaeosphaeria</i> sp. (1/8/67)	<i>Erythrobasidium</i> clade (2/2/94)	<i>Galactomyces</i> sp. (1/4/32)	Mycota sp. (1/1/9)	<i>Mycena</i> spp. (2/7/53)
DC-0e	○ ○ ○ ○ ○	● ○ ○ ○ ○	● ○ ○ ○ ○	● ● ○ ○ ○	○ ○ ○ ○ ○
DC-0	● ● ● ● ●	● ● ● ○ ○	● ○ ○ ○ ○	● ● ○ ○ ○	● ○ ○ ○ ○
DC-1	○ ○ ○ ○ ○	● ● ● ● ●	● ● ● ○ ○	● ○ ○ ○ ○	○ ○ ○ ○ ○
DC-2	○ ○ ○ ○ ○	● ● ● ○ ○	● ○ ○ ○ ○	○ ○ ○ ○ ○	● ● ● ● ●
DC-3	● ○ ○ ○ ○	● ● ○ ○ ○	● ○ ○ ○ ○	○ ○ ○ ○ ○	○ ○ ○ ○ ○

applied primers. The commonly used primers (ITS1F, ITS4) match perfectly with less than 75 % of the sequence diversity in all fungal groups, except for the Glomeromycota (supporting information, Table S2). For higher coverage, application of these primers has to rely on PCR conditions compensating for mismatches. This again is critical, because several sequences of non-target groups are also discriminated by a single mismatch. For many arthropods, which may be abundant in soil samples, this is even located in the 5'-half of the ITS1 primer sequence, being of minor relevance for primer specificity. Because ITS is not used as a barcoding region for arthropods, only relatively few reference sequences are available. This complicates their identification as non-fungal sequences, especially if only relatively short reads (i.e. the ITS1 region) are generated by massive parallel sequencing. The newly developed primers cover about 90 % of the known fungal sequence diversity and ensure selectivity for fungi by the three specific forward orientated primers. Only a considerable fraction of annelid DNA may be amplified under non-stringent PCR conditions.

Nevertheless, a certain degree of sequence variation occurs at every rRNA site, considering all fungal lineages. Because of the resulting different hybridization kinetics, the abundances of the products from environmental PCRs do not necessarily reflect the abundances in the template DNA (Manter and Vivanco 2007; Polz and Cavanaugh 1998). For statistical analyses, the abundance data resulting from the RFLP analysis were therefore transformed to a presence/absence matrix, reflecting the frequency of RFLP types in each degradation category.

Discussion of results

In this study, we investigated a vertical profile through the organic layers covering the mineral soil. The profile represents a chronological sequence of leaf degradation, starting with the living leaves. To address the hypothesized shifts in community structure over time, fungal diversity and community composition are discussed in the following section for each leaf-degradation stage, against the background of the functional role of the detected fungi in litter decomposition.

Before leaf fall, the phyllosphere fungal community is already undergoing successional development throughout the year (Kaneko and Kaneko 2004; Unterseher et al. 2007; Osono 2008; Hashizume et al. 2010). Because the leaves were sampled at one point in time, the phyllosphere samples represent a specific successional stage. The most fundamental event in this succession appears to be the onset of leaf senescence, being accompanied by high levels of sugar content in the leaves (Parrott et al. 2005). These readily available carbohydrates provide an optimal source of nutrition not just for endophytic but also for ubiquitous fungi colonizing the leaf surface, which are mostly incapable of degrading more recalcitrant substances (Osono and Takeda 1999). In conjunction with the decreasing defence capabilities of senescing leaves (Lee and Collins 2001; Zeier 2005), the conditions during autumn therefore favour the accumulation of primarily non-endophytic fungal taxa, i.e. 'primary saprobes', within the leaves (Frankland 1998; Saunders and Kohn 2009). However, ubiquitous fungi such as these colonize various substrates providing readily available sugars, which include fallen leaves (Moorhead and Sinsabaugh 2006). To assess

the most diverse assemblage of ‘true’ endophytic fungi, and to avoid states of high abundance of ubiquitous fungi in the phyllosphere samples, leaf material was collected during late summer, just before the onset of senescence. However, it should be noted that the fungal community in the phyllosphere may differ in leaves which fall later.

Epi- and endophytic fungal communities of leaves differed in all earlier studies, when analysed simultaneously (Santamaria and Bayman 2005; Lorenzi et al. 2006; Osono 2008; Kharwar et al. 2010). This also applied to the beech leaves in the present study, because the endophytic fungal community significantly differed from the overall phyllosphere community (Table 2). Because the former is part of the latter, these communities still largely overlap ($R=0.30$), but the most frequent RFLP types of the phyllosphere were not present inside the leaves (Table 3). Compared to the phyllosphere as a whole, samples from the endophytic fungal communities were less similar to each other (Fig. 2), indicating a patchy distribution pattern, as documented earlier for endophytic fungi (e.g., Espinosa-Garcia and Langenheim 1990; Johnston 1998). In the phyllosphere as a whole, this pattern is masked by one frequently occurring and evenly distributed fungal ITS1 genotype, assigned to the genus *Phaeosphaeria*. This is surprising, because *Phaeosphaeria* spp. are known to be phytopathogens, but the fungus was not detected in the inner leaf tissue. Furthermore, *Phaeosphaeria* is mainly associated with monocotyledonous plants and ferns (Câmara et al. 2002), and is so far not known from beech. Even if the records from the leaf surfaces were only due to the presence of inactive diaspores originating from a nearby population, their absence in the top litter layer samples would still be puzzling. As a working hypothesis, we suggest that feeding activity of the soil fauna rapidly eliminates epifoliar fungal material after leaf fall.

The distinctiveness of the fungal assemblage in the freshly fallen leaves from that of the phyllosphere, but not from the endophytic fungal community (Table 2), indicates that predominantly epiphyllous fungi are absent from freshly fallen litter. They seem to play a minor role during the whole decomposition process, because the phyllosphere fungal community significantly differs from all ground communities. Leaf-endophytic fungi, however, are known to become saprobes during leaf senescence and participate in litter decomposition (e.g., Promputtha et al. 2007; Korkama-Rajala et al. 2008).

Galactomyces sp. and taxa of the *Erythrobasidium*-clade are capable of using a broad range of sugars, but show no or limited growth on more complex substrates (Kurtzman et al. 2010). They obviously represent endophytic ‘sugar fungi’, known to rapidly increase in biomass after leaf decay (cf. Table 3), by using the readily available sugars as an energy source (Wildman and Parkinson 1979; Stone et al. 2004; Šnajdr et al. 2011). On the other hand, several endophytic fungi could not be traced in the leaf litter. The lower overall fungal diversity in freshly fallen than within living leaves is in agreement with a meta-study of Osono (2006), who recognized that only a fraction (67 %) of the endophytic fungi is also found in litter samples. However, the author argued that the majority of these fungi are actually present in fresh litter for a short period of time, but quickly sporulate and decay after leaf fall.

Fungal diversity further decreased downwards from the freshly fallen to the initially decomposed leaves (DC-2). However, this tendency cannot be generalized, because increasing fungal diversity with advancing litter degradation was reported for pine forest sites (Lindahl et al. 2007). Since similar methods were applied in both studies, the difference is probably caused by one or more of the various environmental factors characterizing the sampling sites (e.g., litter quality, plant community, soil properties, and climate). Generally, readily available sugars and easily degradable substances such as cellulose are already depleted in initially decomposed litter, and fungi capable of degrading structurally complex and recalcitrant compounds dominate the decomposer community (Berg and McClaugherty 2008; Šnajdr et al. 2011). The most frequent RFLP types found here were assignable to *Mycena* (Table 3), a genus of well-known lignin degraders (e.g., Lindeberg 1946; Miyamoto et al. 2000; Žifčáková et al. 2011). Interestingly, Lindahl et al. (2007) found a species of *Mycena* to be rather frequent already in freshly fallen pine needles. Assuming a similar functional role (i.e. degradation of polyphenolic compounds) for *Mycena* spp., this indicates substantial differences in the initial degradation process between the beech and the pine forest site. ‘Secondary sugar fungi’, i.e. fungi occurring for the first time in advanced decompositional stages and living on sugars released by the activity of lignolytic fungi, were not detected in DC-2 even though they would be expected (Osono and Takeda 2001). This functional group may either have been overlooked due to its low frequency in conjunction with high diversity, or because

it was outcompeted by species of the *Erythrobasidium*-clade which are still rather frequent in this layer. Otherwise, the community showed only marginal overlap with the assemblage in the fresh litter (Table 2). However, the observation that the layers below and above DC-2 have three times more RFLP types in common than they share with DC-2 (Fig. 3), makes the absence of the corresponding fungi in DC-2 unlikely. The distinctiveness of DC-2 is therefore likely to be caused by a high abundance of few dominating species (Fig. 1), reducing relative abundance of rarer species below the limit at which they can be detected.

The fairly decomposed litter at the mineral soil interface is considered to be colonized by the most diverse soil fungal community (Bills et al. 2004). Indeed, the number of RFLP types in DC-3 was twice as high as in the overlying DC-2, and the high number of singletons indicates that only a fraction of the total diversity was detected (Fig. 1). The low similarity among the fungal communities in the samples of DC-3 (Fig. 2) indicated a patchy distribution pattern of the fungi, corresponding to the patchy distribution of utilizable organic matter and local activity of the soil fauna (Coleman et al. 2004). Furthermore, small-scale variation of soil parameters such as pH-value (Bringmark 1989) and nitrogen content (Troedsson and Tamm 1969) may affect fungal community composition. The patchy distribution patterns of the fungi in DC-3 and DC-0e were therefore certainly caused by different factors. Nevertheless, the two communities shared several RFLP types (Fig. 3) and were statistically indistinguishable (Table 2). This is the first clear evidence for a substantial overlap between endophytic fungi and the fungal community of highly decomposed litter. In particular, yeast-like ‘sugar fungi’ occurred at similar frequencies in decomposed litter and within living leaves (Table 3). Furthermore, a functional role in the late stages of litter decomposition is conceivable for certain endophytic fungi (e.g., *Xylaria* spp.) that are capable of degrading lignin (Osono and Takeda 1999). However, clarification of the role of the two highly dissimilar habitats of soil and leaf interior in the life cycle of the inhabiting fungi will be a challenge for future studies.

In summary, several endophyllous fungi were also present in freshly fallen leaves, which supports hypothesis 2. While the phyllosphere and the initially decomposed litter were significantly distinct, fungal assemblages in highly decomposed litter, freshly

fallen and within living leaves largely overlapped. Therefore hypothesis 1, proposing structural differences in the fungal community along a vertical gradient, is partly rejected. While the phyllosphere community significantly differed from all ground communities, it was, like the endophyllous community, least distinguishable from the highly decomposed litter. This disagrees with the hypothesized decreasing similarity between the phyllosphere community and the fungal decomposer communities of increasingly decomposed leaves (hypothesis 3).

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