Microbial Ecology

Microbial Colonization of Beech and Spruce Litter—Influence of Decomposition Site and Plant Litter Species on the Diversity of Microbial Community

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Received: 21 December 2005 / Accepted: 21 December 2005 / Online publication: 12 May 2006

Abstract

The present study was conducted to investigate the effect of decomposition site and plant litter species on the colonizing microbial communities. For this, litter bag technique using beech and spruce litter was combined with RNA-based fingerprinting and cloning. Litter bags were incubated for 2 and 8 weeks in the A_h horizon of beech and beech–spruce mixed forest sites. Although sugars and starch were rapidly lost, lignin content increased by more than 40% for beech and more than doubled for spruce litter at both soil sites at the end of the experiment. Denaturing gradient gel electrophoresis analysis of 16S and 18S rRNA RT–PCR products was used for screening of differences between bacterial and fungal communities colonizing the two litter types. Development of the microbial community over time was observed to be specific for each litter type and decomposition site. RT–PCR products from both litter types incubated in beech–spruce mixed forest site were also cloned to identify the bacterial and fungal colonizers. The 16S rRNA clone libraries of beech litter were dominated by γ-proteobacterial members, whereas spruce libraries were mainly composed of α-, β-, and γ-proteobacterial members. Ascomycota members dominated the 18S rRNA clone libraries. Clones similar to Zygomycota were absent from spruce, whereas those similar to Basidiomycota and Glomeromycota were absent from beech libraries. Selective effects of litter quality were observed after 8 weeks. The study provides an insight into the bacterial and fungal communities colonizing beech and spruce litter, and the importance of litter quality and decomposition site as key factors in their development and succession.

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Introduction

In order to better assess the potential of forest soils to sequester C assimilated from the atmosphere, it is necessary to understand the mechanisms of input, decomposition, and stabilization of organic material within the soil. Surface litter forms a prominent contribution to C stored within the forest ecosystem, which is between 3.5 and 4 times that stored in the soil [23], and both its quantity and quality are likely to change in response to the changing environment. Therefore, litter fall and its decomposition constitute an important aspect of nutrient cycling and energy transfer in most ecosystems. Plant litter is a mixture of labile (e.g., sugars, hemicellulose, and cellulose) and recalcitrant (e.g., lignin) substrates. Decomposition of plant litter material is a complex suite of biotic processes strongly affecting the mineralization, immobilization, and cycling of mineral nutrients, and is a principal pathway for the return of nutrients to the soil [25]. A decrease in decomposability of plant residues in the course of decomposition is a general concept. The decomposing material becomes enriched in recalcitrant chemical compounds, due to direct chemical changes in the substrate itself, resulting in a succession of microorganisms that are able to assimilate specific substrates [4]. During the early stages of decomposition, rates of decay are determined by easily decomposable carbohy-

drates, whereas at later stages lignin exerts the major control on decomposition rate.

Quality of plant litter, with respect to decomposition, has been defined therefore as its relative ease of mineralization by decomposing organisms [27]. In small-scale experiments of short duration, using defined cohorts of plant litters and more precise analytical methods, the effect of litter colonization by key fungal species during microbial succession becomes apparent [10, 16]. Under laboratory conditions, where much of environmental variability in the field can be factored out, forest soil material from different soil sites and associated microbial communities emerge as an important variable in rates of litter decomposition [9, 28]. However, still little is known about the microbial communities colonizing leaf litter *in situ* and how they are affected by litter quality. Furthermore, the dynamics of microbes with the ongoing decomposition is still an open question and needs to be investigated in order to understand the decomposition process of litter. Past studies on decomposer organisms or decomposer activity have focused on either litter quality [16, 33] or decomposition site specificity [12, 13]. There are relatively few studies that compare the relative importance of site and litter quality on the colonizing organisms under natural conditions [5, 28].

We selected beech and spruce litter, and beech forest and beech–spruce mixed forest to carry out our decomposition experiments because of the importance of these plants in Germany. Beech covers much of the afforested land in Germany today; however, for economic reasons beech has been replaced by conifers, especially spruce. In contrast to beech, spruce monocultures have a buildup of thick organic horizons, while the mechanisms responsible for the accumulation of organic matter remain little understood. We hypothesized that differences in decomposition rates between beech leaves and spruce needles is related to differences in the composition of the microbial decomposer communities colonizing the two litter types.

Therefore, the aim of the present study was to determine the effect of decomposition site and plant species on the colonization of litter by bacteria and fungi. For this, litter bag technique, which involves the incubation of litter bags filled with the leaf material under investigation (beech and spruce litter in the present study) in soil for selected time points (early time points in the present study), was combined with the molecular analysis of microbial community structure of bacteria and fungi that had colonized the initial litter material. To identify the active bacterial and fungal groups colonizing beech and spruce litter and their succession over time, 16S and 18S RT–PCR products from both litter types incubated in beech–spruce mixed forest site were cloned and sequenced.

Methods

Plant Litter and Decomposition in Litter Bags. Three-year-old beech and spruce plants were grown in the greenhouse for 40 days under controlled conditions in a sandy loam soil, taken from a beech–spruce mixed forest (A_h horizon, 20% clay, 32% silt, 48% sand). Ten plants each of beech and spruce were planted in containers of size 60 × 40 × 45 cm, containing 50 kg soil each. During the growth period, temperature was maintained at 25°C with a photoperiod of 14 h day^{-1}. Plants were watered every other day with 500 mL water per container. Leaf litter was removed by hand from the plants at the end of the experiment, dried at 70°C for 96 h, and stored in sealed bags at room temperature.

Nylon litter bags (10 × 10 cm; mesh size 40 μm) were filled with 4 g dried litter. From each litter type, 20 bags were filled. Ten bags of each litter type were buried into the A_h horizon of two different sites: beech forest (B) and beech–spruce mixed forest (BS) in August 2002. Soil from the two sites was classified as sandy loam and was comparable to the soil used in the greenhouse experiment for plant growth. After 2 weeks, five bags each of both beech and spruce litter were removed from each of the two sites. The remaining bags were removed after 8 weeks. The bags were shock frozen.

Biochemical Characterization of Litter. For sugar and starch measurements, dried, undegraded litter and frozen, decomposed litter were freeze-dried and milled from each bag separately. Sugars were extracted with hot water (80°C) from 20 mg sample in three steps (once with 1 mL water and twice with 0.5 mL water). The protocol involved incubation with water for 10 min at 80°C followed by centrifugation at 10,000 × *g* for 5 min. The supernatant was collected from the three steps and pooled. From the remaining pellet starch was enzymatically extracted using heat-stable amylase (1250 U mL^{-1}; Sigma, St. Louis, MO, USA) for 30 min at 60°C and amyloglucosidase (3 U mL^{-1}; Sigma) overnight at 37°C and measured as glucose. Sugars and starch were analyzed using HPLC with a Bio-Rad Aminex HPX-87C column (at 85°C, 0.6 mL flow) with water as mobile phase. For detection, a refractive index detector (RI 1755; Bio-Rad, Munich, Germany) was used. All samples were analyzed twice.

Cellulose determination was adopted from Updegraff [31]. The test was miniaturized using 2-mL Eppendorf screw-cap vials and a starting amount of 0.1 g plant material. The residue of acetic/nitric acid treatment was gravimetrically quantified after lyophilization.

Lignin was determined according to procedure described by Bruce and West [6], with 60 mg of finely powdered 1:1 mixtures of plant tissue and diatomaceous earth. The thioglycolic acid conjugate was dissolved in

oxygen-free 0.5 N NaOH and photometrically quantified. Organosolv lignin (Sigma-Aldrich, Taufkirchen, Germany) was used as a reference.

RNA Extraction and RT–PCR Amplification for Bacteria and Fungi. RNA extraction and cDNA synthesis from undegraded and decomposed litter was performed as described by Aneja *et al.* [2]. Each bag was processed separately for molecular analysis. Primer pair 968f-GC and 1401r [20], with an annealing temperature of 54°C, was used in specific RT–PCR reaction targeting bacteria. For fungi, primer pair NS1 and NS2-GC at an annealing temperature of 52°C [34] was used in the reaction. One microliter of synthesized cDNA was used in 48 µl PCR reaction, which consisted of 5 µl of 10× reaction buffer, 5 µl of 3% BSA, 2.5 µl of dimethysulfoxide, 3 µl of 25 mM $MgCl_2$ for bacteria (for fungi, 5 µl was added), 5 µl of 2 mM deoxyribonucleoside triphosphate mixture (dNTP), 1 µl each of 10 µM primer pair. The reaction involved hot start at 95°C for 10 min, followed by addition of 2.5 U *Pfu* DNA Polymerase (Stratagene, Amsterdam, The Netherlands) for bacteria and *Taq* DNA Polymerase (Invitrogen, Karlsruhe, Germany) for fungi. The cycling parameters were 94°C for 1 min, annealing for 1 min and 72°C for 1 min, for 30 cycles, followed by a final extension at 72°C for 10 min. RT–PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). DNase-treated nucleic acids, without being reverse-transcribed, were used as controls in RT–PCR to check for residual DNA in RNA preparations (data not shown).

Denaturing Gradient Gel Electrophoresis. Denaturing gradient gel electrophoresis (DGGE) for bacterial RT–PCR products was performed using 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 37:1) with a 48–58% denaturant. For analysis of fungal communities, gradient was reduced to 35–45%. A 100% denaturant is defined as 7 M urea plus 40% formamide[1]. Appropriate volumes containing about 2 µg purified RT–PCR products, measured by absorbance at 260 nm, were loaded. The gels were electrophoresed at 60°C at 50 V for 17 h using D-Gene system (Bio-Rad) and silver-stained using the protocol described by Heukeshoven and Dernick [21].

Image Analysis. Dried DGGE gels were scanned using HP Scanjet 7,400c. The obtained profiles were analyzed by clustering via the unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) using GelCompar II Software (Applied Maths, Kortrijk, Belgium). Only within-gel comparisons, taking into account only the presence and absence of bands, were performed for the profiles. The position tolerance was set at 1% and background subtraction was applied. Cophenetic correlation, which is a parameter to express the consistence of a cluster, was calculated using the same software.

Cloning of RT–PCR Products and Sequencing. Purified RT–PCR products for 16S and 18S rRNA from beech and spruce litter incubated in BS site were cloned for the two time points. Approximately 100 ng of purified bacterial RT–PCR product was cloned into pCR®-Blunt II-TOPO® vector of Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) as described by the manufacturer. This ligation mix was incubated for 30 min at room temperature. The same amount of purified fungal RT–PCR product was cloned into pCR®2.1 vector of TA Cloning Kit (Invitrogen) following the manufacturer's instructions. Ligations were incubated at 14°C overnight.

Two microliters of ligation mix was transformed into chemically competent One Shot® DH5α™-T1^R cells provided in the kit following manufacturer's instructions. Colonies were inoculated in LB medium (supplemented with 50 µg mL^{-1} kanamycin). Plasmids were isolated using Qiagen Plasmid Mini Kit (Qiagen) and screened for inserts of correct size by *Eco*RI digestion (MBI Fermentas, Heidelberg, Germany) at 37°C for 1 h. Inserts were sequenced on ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the manufacturer's instructions. Rarefaction curves (number of groups observed vs the number of clones screened), were generated using the software Analytic Rarefaction provided on the Web http://www.uga.edu/strata/software/).

Accession Numbers. Nucleotide sequences determined in this study have been submitted to the GenBank database under accession numbers AY556485–AY556534, AY681354–AY681471, AY682123–AY682194, and AY683239–AY683321.

Results

Biochemical Characteristics of Undegraded and Decomposed Litter. Decomposition of both litter types showed similar kinetics, with high degradation rates in the first 2 weeks followed by reduced degradation rates. Overall, in the first 2 weeks there was a 30–40% reduction of the litter dry weight. Further reduction in litter biomass in the second phase (2–8 weeks) was only between 10 and 15% (data presented in [2]). Beech and spruce litter differed with respect to the various biochemical components measured. Beech litter was characterized by lower sugars and higher starch amounts compared to spruce (Table 1). During the course of decomposition, sugars and starch were rapidly lost from the litter material. Effects of decomposition site were

Table 1. Values of sugars and starch (expressed as % dry weight) in undegraded and decomposed beech and spruce litter

Site of decomposition			Beech forest				Beech–spruce mixed forest			
Decomposition period	Undegraded		2 weeks		8 weeks		2 weeks		8 weeks	
					Component					
Litter type	Sugars[a]	Starch	Sugars[a]	Starch	Sugars[a]	Starch	Sugars[a]	Starch	Sugars[a]	Starch
Beech	6.8	12	1.3	4.9	0.6	2.6	1.6	9.7	0.7	5.4
Spruce	9.2	7	2.7	1.8	0.4	2.7	4.3	3.5	0.8	0.6

Standard deviation between replicate samples was below 5%.
[a]Sugar levels mentioned are the sum of glucose, xylose, fructose, and sucrose.

evident by the rapid mineralization of sugars and starch from both litter types incubated in the beech forest (B) site compared to the beech–spruce mixed forest (BS) site for 2 weeks. After 8 weeks, sugar levels were comparable for both litter types in both the sites. Starch was much higher for beech litter decomposed for 8 weeks in the BS site compared to spruce. Among polymeric substances, cellulose amounts were comparable for the litter of the two species, whereas higher amounts of lignin were observed for beech litter (Table 2). Lignin content in undegraded litter was 25 and 12% on a dry weight basis for beech and spruce, respectively, and increased by more than 40% for beech and more than doubled for spruce litter at both sites. Cellulose content ranged between 20 and 30% on a dry weight basis in both litter types and remained at similar levels or slightly increased over the exposure time of 8 weeks. Effect of decomposition site was evident at the 2-weeks time point by the rapid increase in lignin concentrations of both litter types incubated in the B site. In contrast, for BS site the values were comparable to the values for undegraded litter.

Analysis of Colonizing Bacterial Communities. Undegraded letter was checked for any residual rRNA before incubating in the forest sites for decomposition experiment. No amplification of 16S and 18S rRNA could be obtained, indicating its complete degradation during drying (data not shown). During the incubation period in the two sites, dried, undegraded litter was colonized by soil bacteria and fungi, as confirmed by successful amplification of both 16S and 18S rRNA genes and transcripts. Bacterial communities of the two decomposition sites and the colonizing bacterial communities were analyzed using 16S rRNA as a molecular marker. All samples yielded the desired RT–PCR product of 473 bp, and no residual DNA was detected in PCR reactions with DNase-treated nucleic acids without being reverse-transcribed (data not shown). On resolving the purified RT–PCR products by DGGE, high reproducibility (>95% similarity) with replicate litter bags was obtained for both plant species and soils (data not shown). For each site, the positions of most of the major bands in the fingerprints were different, indicating the composition of the bacterial community to be dependent on the litter type and the site used for incubation (Fig. 1). After 2 weeks, approximately 15 bands were observed for beech and spruce litter incubated in B site (Fig. 1a). Higher number of bands was observed in the bacterial profiles from beech and spruce litter incubated in BS site (Fig. 1b) compared to B site. Similar results were obtained after 8 weeks of incubation of both litter types, with more bands in the fingerprints obtained from the BS site. A general increase in bacterial diversity (as evidenced by the increase in the number of bands) over time was observed for both litter types in both sites. Analysis of the DGGE profiles using GelCompar II revealed two clusters, one for each litter type, reflecting the selective effect of litter quality on the bacterial community. This was observed for both the decomposition sites. For both soils, the bacterial community profile of the site was approximately 65% similar to the bacterial community colonizing the beech and spruce litter.

16S rRNA RT–PCR products from beech and spruce litter incubated in the BS site were also cloned to identify

Table 2. Values of cellulose and lignin (expressed as % dry weight) in undegraded and decomposed beech and spruce litter

Site of decomposition			Beech forest				Beech–spruce mixed forest			
Decomposition period	Undegraded		2 weeks		8 weeks		2 weeks		8 weeks	
					Component					
Litter type	Cellulose	Lignin	Cellulose	Lignin	Cellulose	Lignin	Cellulose	Lignin	Cellulose	Lignin
Beech	24	25	26	34	23	46	25	26	29	36
Spruce	22	12	25	20	26	33	26	14	32	39

Standard deviation between replicate samples was below 5%.

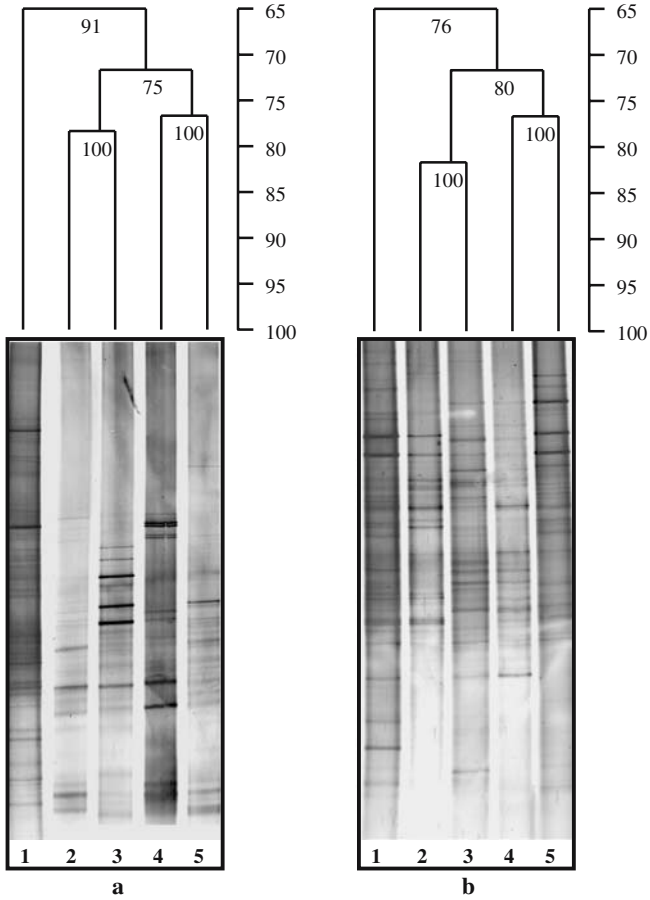

Figure 1. DGGE profiles and UPGMA tree showing the similarities between the patterns of 16S RT-PCR products of 2 and 8 weeks decomposed litter in beech forest site (a) and beech spruce mixed forest site (b). Scale represents % similarity. Numbers at branch points are the cophenetic correlation values which express the consistency of the cluster. *Lane 1*: soil from forest site. *Lane 2*: beech litter after 2 weeks decomposition. *Lane 3*: beech litter after 8 weeks decomposition. *Lane 4*: spruce litter after 2 weeks decomposition. *Lane 5*: spruce litter after 8 weeks decomposition.

the colonizing bacterial groups. This site was selected for detailed analysis as it is the natural site for decomposition of both litter types. Sequences were used to assign the clones to bacterial groups using the NCBI BLAST program. Sequences showing a match of 97% or higher to any member of a known bacterial group were assigned to that particular group, and rarefaction analysis (the number of different groups detected plotted vs the number of clones analyzed) was performed. Clones similar to sequences reported in earlier studies, but mentioned as "unclassified" in the NCBI database, have been grouped together as "unclassified" in the present study. A plateau, as expected for full coverage of library, was obtained after screening 40 clones for beech litter after 2 weeks (B2), 12 clones for beech litter after 8 weeks (B8), 42 clones for spruce litter after 2 weeks (S2), and 46 clones for spruce litter after 8 weeks (S8) (data not shown). Therefore, 50 clones each from B2, B8, S2, and S8 libraries encompass the total bacterial diversity with respect to bacterial groups within the limits of experimental protocols. Figure 2 shows the phylogenetic distribution of clones within each library. Majority of the clones were highly similar (>97% homology) to members of different subgroups of proteobacteria. In beech libraries, clones similar to γ-proteobacteria members were the most dominant and their proportion increased after 8 weeks. Clones similar to members of the α- and β-subdivisions of proteobacteria, Actinobacteria, and Acidobacteria were present in the 2 weeks library, but were absent from libraries after 8 weeks. In spruce libraries, most of the clones had sequences similar to members of α-, β-, γ-, and δ-proteobacteria. There was an increase in the proportion of clones similar to α- and γ-proteobacteria in the 8 weeks library. Also, a decrease in the proportion of clones similar to β- and δ-proteobacteria members was observed for spruce litter that had been decomposed for 8 weeks. Sequences similar to members of other groups such as Acidobacteria, Actinobacteria, and Gemmantimonadetes were also present, although in smaller proportions (<25%). Overall, as indicated earlier by the DGGE profiles, litter quality exerted a strong influence on the composition of the colonizing bacterial community structure. After a 2-week incubation, beech and spruce libraries had more bacterial groups in common as compared to 8 weeks libraries, indicating litter quality as the selecting parameter by the second time point.

Analysis of Colonizing Fungal Communities. 18S rRNA was targeted to characterize the fungal communities colonizing beech and spruce litter using RNA extracted from the corresponding litter bags. RT–PCR

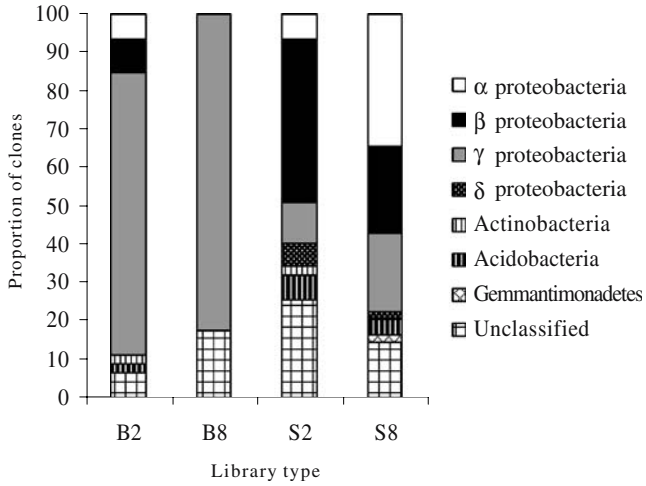

Figure 2. Relative distribution of clones to different bacterial groups. B: beech litter; S: spruce litter; numbers 2 and 8 stand for 2 and 8 weeks of decomposition of the litter, respectively.

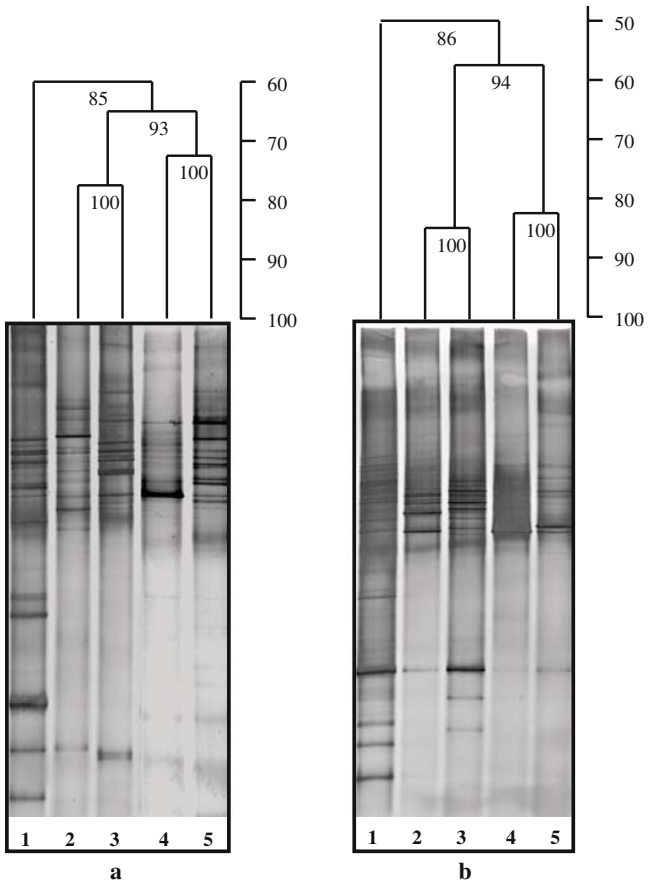

Figure 3. DGGE profiles and UPGMA tree showing the similarities between the patterns of 18S RT-PCR products of 2 and 8 weeks decomposed litter in beech forest site (a) and beech spruce mixed forest site (b). Scale represents % similarity. Numbers at branch points are the cophenetic correlation values expressing the consistency of the cluster. *Lane 1*: soil from forest site. *Lane 2*: beech litter after 2 weeks decomposition. *Lane 3*: beech litter after 8 weeks decomposition. *Lane 4*: spruce litter after 2 weeks decomposition. *Lane 5*: spruce litter after 8 weeks decomposition.

products were obtained for all the samples using NS1 and NS2 primers. On resolving the purified RT–PCR products from replicates by DGGE, results similar to those obtained for bacterial community profiles were observed. DGGE fingerprints derived from multiple nucleic acid extractions and subsequent processing, when carried out for the same sample or for replicates, were more than 95% similar to each other (data not shown). On comparison of the two forest sites, comparable number of detectable bands was obtained for both litter types. Comparison of the DGGE profiles revealed two major clusters (one for each litter type) (Fig. 3a, b). A similar trend was observed when bacterial 16S rRNA profiles were compared although the proportion of similarity varied.

For the identification of fungal colonizers, 18S RT–PCR products were cloned to generate libraries. As performed for bacterial sequences, fungal sequences were assigned to groups. Sequence showing a match of 94% or higher to any member of a known fungal group was assigned to that particular group. On performing rarefaction analysis, a plateau was obtained after screening 24 clones for beech litter after 2 weeks (B2), 26 clones for beech litter after 8 weeks (B8), 34 clones for spruce litter after 2 weeks (S2), and 32 clones for spruce litter after 8 weeks (S8) (data not shown). Therefore, 50 clones each from B2, B8, S2, and S8 represent the fungal diversity within the limitations of experimental protocols. Majority of the clones (97% in B2, 85% in B8, 98% in S2, and 70% in S8 libraries) had sequences similar (≥98%) to members of Ascomycota (data not shown). Sequences similar to members of other groups such as Zygomycota (present only in beech litter libraries with increased proportion in 8 weeks), Basidiomycota (present only in spruce litter library after 8 weeks), and Glomeromycota (present only in spruce litter library after 8 weeks) were present in lower proportions. Clones similar to sequences reported in earlier studies, but mentioned as "unclassified" in the NCBI database, have been grouped together as "unclassified" in the present study, and were present in B8 and S2 libraries at less than 5% proportions (data not shown). Within Ascomycota, a high proportion of clones was more than 98% similar to the 18S rRNA sequence of *Penicillium* (Fig. 4). There was a reduction in the proportion of such clones from 40% in B2 library to 15% in B8 library. However, clones similar to the 18S rRNA sequence of *Trichoderma* increased in proportion from 26% (2 weeks) to 35% (8 weeks) in beech libraries. Also, clones similar in sequence to *Myrothecium* were not present in the beech 2 weeks library but present in significant proportions after 8 weeks. In contrast to beech libraries, clones similar to the 18S rRNA sequence of *Penicillium* were observed in increased proportions after 8 weeks in spruce libraries. Moreover, sequences similar to *Trichoderma* constituted about 13% of the clones in

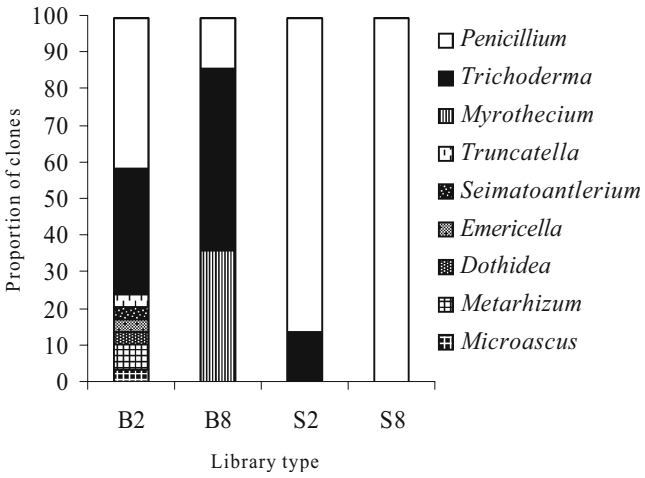

Figure 4. Relative distribution of clones similar to different genera of Ascomycota. For abbreviations, refer to Fig. 2.

the 2 weeks library, but were not detected in 8 weeks library.

Discussion

Leaf litter decomposition is a complex process involving a succession of biodegradative activities that precede an attack by lignocellulose degraders [14]. The one leaf characteristic that consistently predicts microbial colonization and leaf decomposition rates is lignin content [36]. Hence, besides the analysis of starch and sugar, lignin and cellulose were also taken into account. Green litter was used in the study to avoid age variability and differences in decomposition stages in natural litter, which would have additionally affected the process of decomposition in the litter bags. Although this variability occurs under natural conditions, its presence in the current study would have made it difficult to correlate the differences obtained after the experiment to age difference of litter types or to the factors under investigation. In the present study, there was a steady decrease in the amounts of sugars and starch during the course of decomposition. This was observed for both litter types incubated in the two decomposition sites. Lignin concentrations increased by more than 40% in beech litter and more than doubled in the case of spruce litter after 8 weeks of decomposition. Compared to lignin, substantially lower increases in cellulose amounts during the course of decomposition were observed. This is supported by other reports showing that during the initial weeks, the least recalcitrant components present, e.g., sugars, starch, and low-molecular-weight extractives, are rapidly decomposed followed by a slow decay of lignin [18]. As litter decomposition proceeds, concentration of lignin and lignin-like substances increase because they are more resistant to decay, and enzymes catalyze the polymerization of lignin-like substances and other forms of stabilized organic matter [29]. This could account for the observation of high degradation rates in the first 2 weeks followed by reduced degradation rates as reported by Aneja *et al.* [2]. Leaves were filled in litter bags of 40-μm mesh size, and so the observed processing can be primarily attributed to microbial and physical decomposition.

The observed changes during the course of decomposition were also reflected when the colonizing microbial communities were analyzed. The initial undegraded litter, during its incubation in the two sites, was colonized by soil bacteria and fungi. Succession of microbial community during the course of decomposition was evidenced by DGGE and also confirmed by cloning. A similar microbial succession during organic matter turnover in litter bags has also been shown earlier using cultivation-dependent techniques. Frankland [16] followed fungal succession on fallen leaves of *Quercus* (oak), *Betula* (birch), *Corylus* (hazel), and *Fraxinus* (ash) in a broad-leaved, U.K. woodland on mull humus. It was reported that the succession patterns were different for different litter types. In a related study, Cox *et al.* [10] used litter bags containing sterile Scots pine needles inoculated with two species of fungi (*Marasmius androsaceus* and *Trichoderma viride*) in the laboratory. These bags were then placed in the litter layer of a pine plantation for decomposition of litter. Following decomposition over the course of time, they showed that *M. androsaceus*, which can degrade lignocellulose, was initially displaced by other fungal colonizers and was not detected in the litter after 2–3 months, but was reisolated from the needles after 12 months.

Sequencing of 16S rRNA clone libraries revealed a dominance of sequences phylogenetically similar to proteobacteria for both beech and spruce litter. Clones similar in sequence to members of *Pseudomonas* dominated the bacterial libraries derived from beech litter. The proportion of such clones increased from 2 to 8 weeks. Furthermore, sequences similar to members of Actinobacteria and Acidobacteria were also present. In a related study, Wahbeh and Mahasneh [32], following the *in situ* degradation of seagrass *Halophila stipulacea*, showed that *Pseudomonas*, *Actinomyces*, and *Arthrobacter* were the most prominent genera involved in decomposition. All of the above-mentioned genera are capable of degrading structural carbohydrates. For spruce litter, there was an increase in the proportion of clones similar to α- and γ-proteobacteria members in the 8 weeks library. Members of α-proteobacteria have also earlier been shown to be key degraders of salt marsh grass, *Spartina alterniflora* [7]. The absence of sequences similar to α-, β-, and δ-proteobacterial members, which constitute more than 50% of the colonizing bacterial community in the spruce library after 8 weeks, from beech library after 8 weeks, highlight the selective nature of litter quality on the colonizing bacterial community. High levels of sugars present in spruce litter could be one of the reasons for the high diversity of typical "r-strategists" such as proteobacteria.

In the four 18S libraries, clones similar in sequence to members of Ascomycota constituted the major proportion. Previous studies of fungal community in southeastern U.S. salt marshes have identified several species of ascomycetous fungi as major decomposers of *S. alterniflora* blades, based on both traditional culture- and microscopy-based methods [26] as well as using molecular approaches [7, 8]. Among Ascomycota, major proportions of clones were similar in sequence to *Penicillium* and *Trichoderma*. These two genera are among the most studied filamentous fungi with respect to biopolymer (cellulose, hemicellulose, and lignin) degrading enzyme production [3]. In 18S rRNA libraries from spruce litter, clones similar to Basidiomycota members were absent from the 2 weeks library, but were present in

significant proportions in library after 8 weeks. A succession from Ascomycota to Basidiomycota occurs during the process of decomposition as the latter are able to synthesize enzymes required to degrade complex polymers [11]. Cox *et al.* [10] and Frankland [16] have earlier shown the effect of litter quality on the sequence of fungal succession during the course of decomposition using cultivation-dependent techniques. The absence of Zygomycota members from spruce libraries and Basidiomycota members from beech libraries reflects the selective nature of plant litter.

Fungi are believed to perform a critical role in conditioning leaf material before subsequent invertebrate use, whereas bacteria are thought to play a secondary role [15]. However, Gulis and Suberkropp [17] proposed a synergistic effect of bacteria and fungi. Bacteria are limited in what leaf components they can utilize because they are unable to access the interior sections of leaves, but these sections become available after fungal hyphae penetrate the leaf tissue. Although no attempts to analyze whether bacteria or fungi were the first colonizers were made in this study, this would be a topic of investigation in further experiments.

In this study, the decay parameters for litter are shown to be dependent on decomposition site, plant species, and time. By molecular techniques, it could be shown that the structure of the microbial community is dependent upon both the site of decomposition and litter species. During the first 2 weeks, members of the microbial community, probably "r-strategists" like microbes of the particular site, invade and degrade the easily accessible metabolites, e.g., sugars and starch. In the later stages, chemical differences between beech and spruce act as selection parameters, and there occurs a succession of colonizing microbial communities. However, it needs to be noted that in the present study, green litter was removed from the plants and so there was no reabsorption of nutrients by the plant prior to normal leaf fall resulting in litter rich in nutrients that are otherwise limiting. Also, as the litter was dried prior to filling in litter bags, the endophytic and epiphytic microbial community was rendered inactive. It must be considered that leaves in litter bags may face a slightly different microclimate and microhabitat compared to natural environment, so the results in the field may differ slightly from that in the litter bag. The mesh size of the bag allows bacteria and fungi, but other large invertebrates that play significant roles in leaf litter decay [30] are excluded from the study. Lemons *et al.* [22] demonstrated that when mesoinvertebrates were excluded using fine mesh and litter was placed in a microenvironment with a fungal endophyte, the difference between endophyte-infected and endophyte-free litter was strongest. Although soil-ingesting animals play an important role in both aggregate turnover and in litter breakdown [24], microorganisms mainly carry out litter decomposition in forest soils [19, 30]. The litter bag method may underestimate actual decomposition, but reflects trends and allows comparison among species and sites [35]. Therefore, additional long-term decomposition experiments with natural litter are required for better understanding of the dynamics of the involved microbial community during litter colonization. To correlate colonization and decomposition directly, studies combining the techniques of fluorescence *in situ* hybridization (FISH), FISH–microautoradiography (FISH–MAR), and *in situ* PCR are needed to provide greater insights into the bacterial and fungal groups involved in decomposition and their enzymatic activities *in situ*.

Acknowledgment

The study was supported by a research grant from Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany (SFB 607).

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