Microbial Ecology

Patchiness and Spatial Distribution of Laccase Genes of Ectomycorrhizal, Saprotrophic, and Unknown Basidiomycetes in the Upper Horizons of a Mixed Forest Cambisol

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Abstract

Decomposition of plant litter by the soil microbial community is an important process of controlling nutrient cycling and soil humus formation. Fungal laccases are key players in litter-associated polyphenol degradation, but little is known about the diversity and spatial distribution of fungal species with laccase genes in soils. Diversity of basidiomycete laccase genes was assessed in a cambisolic forest soil, and the spatial distribution of the sequences was mapped in a 100-m^2 plot by using polymerase chain reaction (PCR) on soil DNA extracts. Diversity of laccase sequences was higher in the organic horizon and decreased with the depth. A total of 167 different sequences sharing 44–96% oligonucleotide similarity was found in 13 soil cores harvested in the 100-m^2 plot. Dissimilarity in laccase sequence content was 67% between adjacent cores; 45.5%, 35.5% and 19% of laccase sequences were attributed to ectomycorrhizal, unknown and saprotrophic basidiomycetes, respectively. Most dominant sequences were attributed to the extramatrical hyphae of known ectomycorrhizal taxa (e.g., Russulaceae) and restricted to small patches (<0.77 m^2) in a specific soil horizon. Soil fungi with laccase genes occupied different niches and showed strikingly variable distribution patterns. The distribution of laccase sequences, and corresponding fungi, likely reflected a part of the oxidative potential in soils.

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Introduction

Decomposition of litter, humification, and mineralization of soil organic matter (SOM) are essential processes of the terrestrial carbon cycle and soil formation. Soil microorganisms through the secretion of numerous extracellular enzymes play a key role in these processes [7, 45], and their high diversity contributes to multiple degradative functions in the rhizosphere and soil organic horizons [16]. Secreted oxidoreductases, such as tyrosinases, Mn peroxidases, lignin peroxidases, and laccases, are involved in decomposition of lignin and phenols [6, 11, 12]. Laccase genes have been detected within various functional groups of fungi (saprotrophs, symbionts, and pathogens) but not within all species (e.g., *Phanerochaete*, see Martinez *et al.* [37]). These enzymes are known to be mainly produced by saprotrophic fungi [19, 24, 50, 53]. Ectomycorrhizal (ECM) fungi have substantially diverged from their ancestral saprotrophs [21], and their ability to degrade plant cell wall polymers appears to be limited [6]. Recent reports, however, showed that many ECM fungi have retained genes for phenoloxidases [9, 32] and other degradative exoenzymes [30]. This contention is supported by evidence that some ECM fungi can partly degrade cell wall components [5].

Laccases contribute to several developmental and metabolic processes including lignin and polyphenols degradation, morphogenesis of multihyphal structures (fruit body and rhizomorph formation), and pathogenesis [6, 24]. As the extraradical hyphal network of ECM is actively involved in the uptake of soil nutrients [5], laccases expressed in this compartment likely play a role in nutrient acquisition. To gain more insights into the ecological roles of fungal communities in soil, it is essential to obtain information on the spatial distribution of the extraradical

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mycelium and its catabolic and assimilative activities [2, 28]. Direct DNA extraction from soils, polymerase chain reaction (PCR) amplification of ribosomal DNA regions followed by sequencing [28], or terminal restriction fragment length polymorphism [13, 48] have been used to assess the genetic diversity of fungal communities and the spatial distribution of prominent species in forest soils. PCR amplification of protein-coding regions from soil DNA allowed the identification of fungal species having the potential to secrete degradative enzymes, such as laccases [32, 33] and chitinases [30].

In the present study, we have used a PCR-based approach for characterizing the spatial distribution of basidiomycete laccase genes in a Dystric Cambisol, which gave indications about the horizontal and vertical extension of corresponding fungal mycelia. The spatial distribution of dominant laccase gene sequences belonging to ectomycorrhizal and saprotrophic fungi within organic and mineral soil horizons was compared. The study is a step in developing molecular approaches to assign functional activities to fungal groups with different trophic strategies in forest ecosystems.

Materials and Methods

Study Site. Soil samples were collected at the "Steinkreuz" site (49°52′26″N, 10°27′54″E), an experimental station of the Institute of Ecosystem Research (BITÖK; University of Bayreuth), located at 460 m above sea level (a.s.l.) in Northern Bavaria (Germany). The site is covered by a 100-year-old mixed stand of European beech (*Fagus sylvatica* L.) and European oak (*Quercus robur* L.) with a sparse undergrowth. The soil is a Dystric Cambisol [17] characterized by a fine moder humus layer and pH of 4.2 for the O_h, 3.2 for the A_h, and 3.9 for the B_v horizons. The turnover of organic matter in such soil is rapid, with a low accumulation in the lower horizons, as reflected by the decreasing values along the upper horizons (O_h, 354.3 g kg^{-1}; A_h, 70 g kg^{-1}; B_v, 6 g kg^{-1}) [26].

Soil Sampling. To characterize the spatial distribution of the laccase gene sequences within the soil profile of the "Steinkreuz" Cambisol, a complex and nonrandom sampling design was used. From an initial soil core taken in the center of a 10 × 10 m square plot, 12 samples were additionally collected at increasing intervals (0.3, 1, 3, and 6 m) along three diverging axes (i.e., three transects) forming 120° angles (for tree positions, see Fig. 4). Soil cores (12-cm diameter) were sampled in September 2002 and divided into O_h, A_h, and B_v horizons, giving a total of 39 soil samples (13 cores × 3 horizons). Immediately after their collection, all samples were sealed in plastic bags, transported back to the laboratory in ice chests, and stored at −80°C pending further analysis.

Soil DNA Extraction. From each soil sample, large wood debris, roots, and leaves were discarded, and the remaining soil was carefully mixed by handshaking for 1 min before DNA extraction. Genomic DNA was isolated from 0.5 g of soil using the FastDNA™ Spin kit for soil (Q-BIOgene, Heidelberg, Germany) and a modified protocol previously described [32].

PCR Amplification. Genomic DNA isolated from the different soil core horizons was used separately as template in PCR amplifications. PCR were performed using the specific basidiomycete laccase primer pair Cu1F [5′-CAT(C) TGG CAT(C) GGN TTT(C) TTT(C) CA-3′] and Cu2R [5′-G G(A)CT GTG GTA CCA GAA NGT NCC-3′] [32]. For the amplification, 3 μL of DNA template was added to a 50-μL reaction mixture containing 5 μL of 10× *Taq* buffer with $MgCl_2$ (Q-BIOgene), 4 μL of dNTPs (2 mM each; MBI Fermentas, St. Leon-Rot, Germany), 1 μL of each primer (60 μM), and 0.2 μL of *Taq* DNA polymerase (Q-BIOgene). The reaction mixtures were overlaid with two drops of sterile oil, and PCR was run on a Master cycler gradient system (Eppendorf, Hamburg, Germany) with an initial cycle of denaturation (3 min at 94°C) followed by 35 cycles with denaturation (30 s at 94°C), annealing (30 s at 50°C), and elongation (2 min at 72°C), and by a final elongation (10 min at 72°C). A control reaction without template was run to rule out the presence of contaminant DNA. Amplification of the laccase sequences from a DNA extract of *Pycnoporus cinnabarinus* was carried out as a positive control to detect technical PCR failures. Additionally, amplification of the internal transcribed spacer (ITS) in the nuclear ribosomal DNA region was performed with each extract to estimate its DNA quality and to detect the presence of possible PCR inhibitory substances contained in soil samples.

Seven microliters of each amplification product was loaded onto a 2% agarose gel (Applichem, Darmstadt, Germany) and electrophoresed in Tris–acetate–EDTA buffer for 45 min at 80 V cm^{-1}. The 100-bp DNA size ladder mix (MBI Fermentas) was run in a separate lane. The gels were stained with ethidium bromide, and the DNA bands were visualized and photographed under UV light.

Cloning and Identification of Potential Laccase PCR Products. PCR products were ligated into a pCR 4-TOPO vector, and ligation mixtures were transformed into *Escherichia coli* TOP10 chemically competent cells according to the manufacturer's instructions of the TOPO TA Cloning kit for sequencing (Invitrogen Life Technologies, Karlsruhe, Germany). The bacteria were plated out in three dilutions. For each cloned PCR product, about 40 colonies were picked and the respective bacterial clones were then amplified and analyzed on 2% agarose gels. As the size of the expected laccase gene

fragments is ~140 or ~200 bp [32], the bacterial clones containing fragments of these sizes were selected for plasmid extraction and DNA insert sequencing. For organic (O_h) and mineral horizons (A_h and B_v) of the Cambisol, 25 and 15 bacterial clones, respectively, with bands of the expected size were cultured overnight in 1.5 mL of Luria–Bertani medium (10 g L^{-1} bactopeptone, 5 g L^{-1} NaCl, and 5 g L^{-1} yeast extract) for further plasmid extraction and DNA sequencing. A preliminary rarefaction analysis showed that sequencing of these numbers of clones allowed to characterize most laccase gene fragments in these horizons. The rarefaction analysis was performed on three independent soil cores. A total of 50 clones were sequenced for each horizon (O_h, A_h, or B_v), and the clone number, for which no additional new laccase gene was found, was determined [32].

DNA Sequencing and Sequence Analysis. Plasmid DNA containing PCR products were extracted from 1.5 mL of *E. coli* suspensions by using the Perfectprep plasmid mini kit (Eppendorf). Cloned products were then sequenced in both directions with M13 reverse (5′-CAGGAAACAGCTATGAC-3′) and M13 forward (5′-GTAAAACGACGGCCAG-3′) primers. The sequencing reactions were run on automated multicapillary DNA sequencers, a CEQ 2000XL sequencer (Beckman Coulter, Fullerton, CA, USA) or a ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), using, respectively, the CEQ Dye-labeled Dideoxy-Terminator Cycle Sequencing kit (Beckman Coulter) or the BigDye Terminator v.3.1. Ready Reaction Cycle Sequencing kit (Applied Biosystems) as recommended by the manufacturer's instructions. During all experiments, each laccase gene was cloned and sequenced several times confirming that the high similarity of some sequences was not due to *Taq* polymerase errors.

Reverse, complement, or inverse sequences were generated by the IUPAC program (http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html), and search for sequence similarity was performed using the GenBank DNA database and Gapped BlastN (National Center for Biotechnology Information) search algorithm [1]. The position of the coding regions in the amplified laccase fragments was deduced from the alignment of each DNA sequence with *Trametes versicolor* laccase mRNA (GenBank accession no. L78077). Nucleotide sequences of the 167 laccase-related fragments from soil coding for fungal laccase genes have been deposited at European Molecular Biology Laboratory under the accession numbers AJ420340, AJ420344, AJ420346–AJ420348, AJ420350, AJ420351, AJ540236, AJ540244–AJ540298, AJ542586, AJ542588, AJ542593, AJ542594, AJ542600–AJ542602, AJ542605–AJ542609, AJ542611, AJ542621, AJ542622, AJ542625 AJ542631, AJ542632, AJ542636, AJ542650, AJ580829, AJ622909–AJ622935, AJ626647–AJ626654, AJ626656–AJ626658, AJ626660–AJ626669, AJ626671–AJ626677, AJ626680–AJ626690, AJ626692–AJ626696, AJ626728–AJ626733, AJ626735–AJ626741, and AY147188.

Laccase sequences were aligned using the program MultAlin (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_multalinan.html; Institut de Biologie et Chimie des Protéines, Lyon, France) [10]. The final alignment, available online (Treebase, http://www.treebase.org/treebase/index.html), was optimized visually, exported to a NEXUS file, and analyzed using PAUP*4.0b10 [46]. The approach used to construct the neighbor-joining (NJ) tree was the Kimura two-parameter distance method [27]. All characters were treated with equal weight, and the bootstrapping was performed with 1000 replications. The different types of laccase genes, their eventual affiliation to taxa, and their distribution were determined from the neighbor-joining (NJ) tree constructed. We described as "type" all detected laccase genes having the same DNA sequence. Final results were compared to a strict consensus tree using maximum parsimony (MP) analysis. The MP trees were performed using the heuristic search option (max. trees = 10,000) with simple stepwise sequence addition and tree bisection reconnection (TBR) branch swapping [46].

Data Analysis

Spatial Distribution of Basidiomycete Laccase Genes Within the Cambisolic Forest Soil. A quantitative matrix compiling the number of each laccase sequence type in each soil core horizon was built. From this matrix, three variables of interest were reported for each soil horizon: the richness (S), which corresponds to the total number of laccase sequence types in the sample, the diversity of laccase sequences measured by calculating the Shannon index (H) [44], and the evenness (E), which is a combination of the Shannon index and the richness coefficient [$E = H/\ln(S)$]. For further statistical analysis, a simplified quantitative matrix was used. For each soil core, the data of the three horizons were pooled and used to build a new matrix giving the number of each laccase gene type in each soil core. From this data set, a Jackknife analysis was performed using the program PC Ord for Microsoft Windows (version 4.27) [40]. This program generated a saturation curve used to estimate the total number of different laccase genes present in the soil plot. This simplified matrix was then transformed in a Sørensen-distance matrix by the program PC Ord. The Sørensen distance, measured as percent dissimilarity (PD), is a proportion coefficient given by the formula [$1 - 2W/(A + B)$], where W is the sum of shared abundances and A and B are the sums of abundances in individual sample units [4]. The Sørensen coefficient (or Bray–Curtis coefficient) was originally applied to presence–absence, but it works equally well with quantitative data,

and compared to Euclidean distance, it retains sensitivity in more heterogeneous data sets and gives less weight to outliers [39].

This Sørensen-distance matrix was compared to a topographic distance matrix, and a curve giving the dissimilarities (Sørensen distance) between the soil cores according to their topographic distances (values in centimeter) was generated. The effect of the spatial separation on the laccase sequence contents was also tested by regular Mantel test [35] on the Sørensen-distance matrix and the Euclidean matrix of spatial distance between the soil cores (Mantel test module in the program PC Ord). The Euclidean matrix was previously generated in PC Ord from the spatial distance matrix. The significance of the correlation between the two matrices was tested by Monte Carlo permutations (9999 permutations) of the dissimilarity matrix to generate a null distribution of correlation coefficients (z-values). For $P \leq 0.05$, the two matrices were significantly correlated if the observed value was greater than the randomly generated statistics.

Laccase Activity in the Cambisolic Forest Soil. The total laccase activity was measured for the three soil horizons (O_h, A_h, and B_v) collected in the "Steinkreuz" Cambisol. For each horizon, equivalent quantity of soil was sampled from the different collected cores and pooled to reach the 40 g needed for performing four technical replicates. For each assay, 5 g of soil was dissolved in 6 mL of a 50 mM phosphate buffer (pH 7.1), shaken, and sonicated for 3 min in an ultrasonic bath (Sonorex Super RK 1028 H; Bandelin Electronic, Berlin, Germany). For each analysis, a negative control was included. It corresponded to the same soil preparation preincubated for 15 min at 100°C. Total laccase activity in the extracts was measured spectrophotometrically (U-2000, Hitachi Ltd., Tokyo, Japan) using 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS; Sigma) as a substrate [38]. For kinetic measurements, 850 μL of 50 mM phosphate–citrate buffer (pH 4.5), 100 μL of ABTS (final concentration 0.3 mM), and 50 μL of soil extract were mixed in a 1-mL cuvette. The oxidation rate of the ABTS was measured for 5 min at 420 nm (ξ^M = 36,000 M^{-1} cm^{-1}). The results were expressed in units defined as μmol of product formed from the ABTS per minute (U) and per gram of dry matter (U g^{-1} DM). For dry weight determination, the soil material was dried at 100°C for 24 h.

Results

Characterization of Basidiomycete Laccase Sequences in the Cambisolic Forest Soil. Basidiomycete laccase sequences were amplified from DNA extracts of 39 soil samples (13 cores × 3 horizons) collected on a 100-m^2 plot from the "Steinkreuz" site. Among 715 amplified sequences, 432 showed a high similarity (≥70%) to known basidiomycete laccase proteins (data not shown). The remaining 283 sequences tended to show a relatively low similarity (≤40%) to hypothetical proteins of bacteria and cyanobacteria species and may be related to unknown Cu-containing proteins (see Discussion). The NJ analysis clustered the laccase sequences into 167 different types, which shared 44–96% oligonucleotide similarity (see Fig. S1 in Supplementary material). The topology and the branching of the main clades found with the NJ analysis were confirmed in the MP analysis run in parallel (data not shown).

Vertical Distribution of Laccase Sequences and Activity. The distribution of the different laccase sequence types in the soil profiles is presented in Table 1. Most of the detected basidiomycete laccase genes (41%) were restricted to the horizon O_h. The richness and the Shannon diversity indices were higher in the O_h horizon and decreased in the A_h and B_v horizons (Table 2). The evenness E coefficient was similar throughout the soil profiles.

The total laccase (ABTS) enzyme activity, measured for each horizon, showed a distribution profile similar to the gene diversity, i.e., a decreased activity with increasing depth. The ABTS oxidation measured in A_h and B_v was 3- and 15-fold lower than the activity found in O_h, respectively (Fig. 1).

Spatial Distribution of the Laccase Genes. The horizontal distribution of the laccase sequences was determined in the "Steinkreuz" plot by analyzing 13 soil cores collected along three radiating transects. The Jackknife analysis performed on this data set (i.e., 432 sequences) showed that the rarefaction of laccase gene types was not reached after the analysis of 13 soil cores (Fig. 2). The potential number of different laccase genes present in the soil plot was estimated to be in the range 281–365. The laccase sequence composition highly differed between soil cores (Fig. 3) as two adjacent soil cores, separated by 30 cm, shared only 33% of their laccase sequences. Despite this low similarity levels between adjacent cores, the diversity of laccase types between two soil cores increased with the topographic distance (Fig. 3). The maximal diversity (94.6%) was reached when 6 m separated two soil cores collected along one transect. The overall correlation between physical and genetic (i.e., laccase types) distance matrices was $r_M = 0.2$ [$P < 0.05$ (Mantel's test, 9999 permutations)] confirming that spatial distance between soil cores has an influence on the specific types of laccase genes found. These results indicated a highly heterogeneous spatial distribution of basidiomycete laccase genes in the analyzed forest soil.

Table 1. Distribution of the different basidiomycete laccase gene sequences detected in one or several (two or three) horizons of a Dystric Cambisol in the Steinkreuz forest site (Bavaria, Germany)

Horizon	*Number of different laccase gene sequences*	*Percentage*
O_h	68	40.7
A_h	34	20.3
B_v	23	13.8
O_h&A_h	17	10.2
A_h&B_v	14	8.4
O_h&A_h&B_v	11	6.6
Total	167	100.0

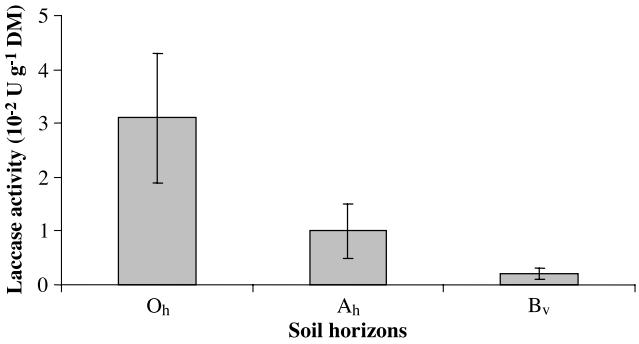

Figure 1. Total laccase (ABTS) activity measured in the three upper horizons of a Dystric Cambisol from Steinkreuz (Bavaria, Germany). The values represent the average of four replicates on samples collected in September 2002. *Bars* represent standard deviations.

Several laccase sequences could be attributed to fungal taxa at the family, genus, or species level. Among the 167 different laccase sequences detected, 59 belonged to unknown basidiomycete species, 76 to ECM, and 32 to saprotrophic taxa (Table 3). Laccases from Russulaceae and Tricholomataceae taxa were the most abundant. Laccase genes of the ECM Russulaceae appeared to have a wider vertical distribution than those of other ECM. Genes of saprotrophic fungi were mainly found in the upper layers A_h and O_h (Table 3).

The spatial distribution of the most abundant laccase sequences was mapped throughout the three soil transects (Fig. 4). The eight dominant laccase sequences (at least five occurrences in at least one core) belonged to an unknown fungus, *Lactarius subdulcis*, *Mycena zephirus*, three Russulaceae, and two Boletaceae species, respectively. The soil core S11 showed no dominance of any laccase gene sequence, whereas all other cores presented either one (S2, S8, S10) or several dominant sequences (S0, S1, S3–S7, S9). Spatial distribution of these dominant basidiomycete laccase gene sequences was patchy, and each sequence type was only detected within a relatively small area (<0.77 m^2).

Discussion

Our knowledge of diversity and functioning of soil fungal communities remains poor relative to that of soil bacterial communities [23]. Molecular methods have been mostly used to identify isolated fungi or to investigate discrete fungal units such as ectomycorrhizal root tips in various ecosystems [23]. Nevertheless, such studies provide little information regarding fungal mycelia in soils [36]. Approaches based on direct extraction of DNA from soils are beginning to provide significant advances on this topic [3, 20, 25, 28] and have the potential

Table 2. Diversity of basidiomycete laccase gene sequences in the three upper horizons of a Cambisol in the Steinkreuz forest site (Bavaria, Germany)

	O_h horizon			*A_h horizon*			*B_v horizon*		
Core sample	*S*	*E*	*H*	*S*	*E*	*H*	*S*	*E*	*H*
S0	20	0.96	2.90	8	0.92	1.92	7	0.87	1.69
S1	14	0.93	2.46	7	1.00	1.94	5	0.96	1.54
S2	9	0.98	2.16	6	1.00	1.79	4	0.96	1.33
S3	21	0.96	2.94	7	1.00	1.94	2	1.00	0.69
S4	11	0.97	2.34	7	1.00	1.94	6	0.96	1.72
S5	14	0.96	2.55	11	0.93	2.24	6	0.97	1.74
S6	12	0.91	2.26	9	0.89	1.95	9	0.98	2.16
S7	4	0.87	1.21	8	0.98	2.04	9	0.95	2.10
S8	13	0.73	1.88	6	0.93	1.66	4	0.95	1.33
S9	10	0.90	2.08	2	1.00	0.69	5	0.96	1.55
S10	8	0.98	2.04	6	0.96	1.73	4	0.92	1.27
S11	2	1.00	0.69	5	0.96	1.55	5	0.91	1.47
S12	11	0.92	2.22	7	0.94	1.83	2	0.91	0.63
Average	11	0.92	2.12	6	0.96	1.78	5	0.94	1.47

The diversity of the laccase gene sequences was estimated for each horizon of each analyzed soil core by the Shannon index (*H*) [44]. *S* and *E* represent the richness and the evenness of the samples, respectively.

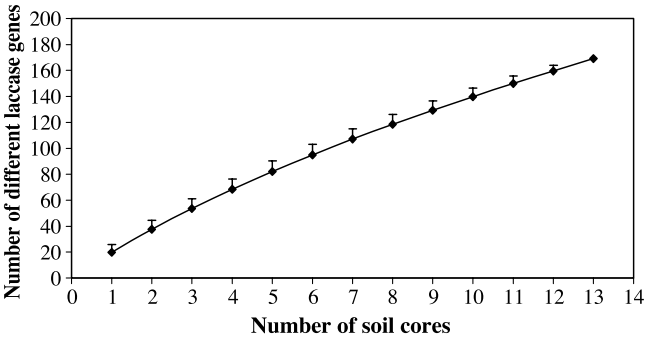

Figure 2. Number of different basidiomycete laccase genes identified by analyzing an increasing number of soil cores. The curve was generated from a quantitative matrix pointing out the number of each different laccase gene found in each soil core in a Jackknife analysis performed on the program PC Ord [40]. The values represent averages. *Bars* represent standard deviations.

to allow novel insights into genetic-functional diversity of soil fungal communities [2].

We used this kind of approach for detecting and quantifying multiple variants of basidiomycete laccase genes in three horizons of a cambisolic forest soil [32]. To identify these multiple variants, we used degenerate primers specific for basidiomycetes, which amplify a relative highly variable region. Until now, only the amplification between copper binding sites Cu1 and Cu2 allows the monitoring of a wide range of basidiomycete laccase gene variants from fungal culture and soil DNA extracts [32]. The present work confirmed the high diversity of the amplified region as the detected sequences shared 44–96% similarity (see final alignment in Treebase).

Compared to structural molecular markers in the rDNA region, genes encoding proteins provide information on functional potentials of the detected fungi. However, the sequence amount available for laccase genes is not as high as for rDNA, and most single fungal species shows several isoforms coded by different gene families [34, 51]. For these reasons, we could not relate about one third of our laccase sequences to any basidiomycete taxon. Most other sequences were, however, attributable to upper taxonomic levels (family or genus) as they clustered within genus or family-specific clades (Fig. S1). The limitation of fungal identification via their laccase sequences might progressively regress because of the rapid extension of the laccase gene database [41]. As each amplified sequence type is species specific [32], its detection reveals the presence of one species. Our approach was therefore adequate to trace diversity and spatial distribution of basidiomycete laccase genes in the investigated soil and to give indications about the extension of corresponding fungal mycelia.

From 715 DNA fragments amplified, only 432 could be unequivocally attributed to basidiomycete laccases based on their deduced protein sequences. Most sequences >210 bp displayed no similarity with any laccase genes previously reported. Interestingly, such fragments appeared essentially in the A_h and B_v horizons having low organic matter contents. At present, it is not possible to know whether they correspond to PCR artifacts produced in mineral substrates with reduced level of laccase genes, or whether they reflect the presence of unknown genes encoding Cu proteins. However, their low similarity (≤40%) to hypothetical proteins of bacteria might support the second hypothesis.

Within our 100-m^2 plot, 167 different laccase sequences were detected. They likely represented a subfraction of the actual basidiomycete laccase gene diversity as the Jackknife analysis suggested the potential number of different laccase sequences detectable to be ~300 (Fig. 2). Most identified sequences belonged to ECM Basidiomycetes and especially to Russulaceae. As ECM fungi have less types of laccase genes than saprotrophic ones [32], the effective dominance of ECM might be higher than the detected one. Laccase sequences of saprotrophic fungi were restricted to the upper horizons. The evenness (E) diversity index was close to 1.0 reflecting a diversity structure characterized by a few genes with wide occurrence and many genes occurring rarely and unlikely to be recorded in several soil cores. This high gene diversity level can be related to the high diversity of fungi in soils [29, 49] of which a considerable proportion present laccase activities [19, 24] and also to the multigenic structure of laccase genes [33, 34, 51].

Up to 74% of the 167 different laccase sequences occurred in only one horizon of the soil profile (Table 1). This supports earlier observations of a specialized distribution of fungi among soil horizons [13, 28]. The

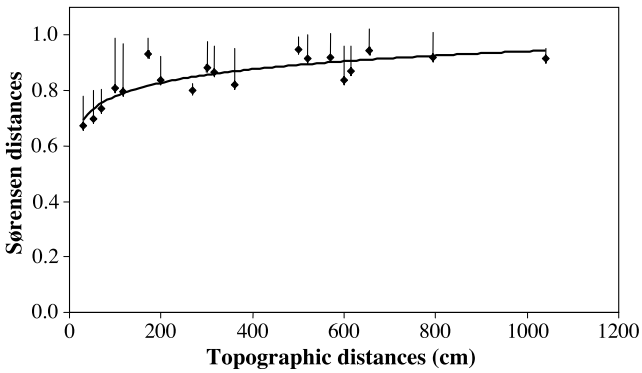

Figure 3. Dissimilarity in the diversity profiles of basidiomycete laccase genes (Sørensen distance) between soil cores taken at increasing distances in a mixed oak-beech forest in Steinkreuz (Bavaria, Germany). The values represent averages of the Bray–Curtis coefficients calculated with the program PC Ord [40] for cores separated by the same spatial distance. The curve equation is $y = 0.0704\ln(x) + 0.455$ with a correlation coefficient of $R^2 = 0.73$. *Bars* represent standard deviations.

Table 3. Distribution and frequency of the different basidiomycete laccase gene sequences and corresponding taxa detected in one or several (two or three) horizons of a Dystric Cambisol in the Steinkreuz forest site

Family	*Species*	*Number of different laccase gene sequences*	*Distribution of the different laccase gene sequences in the soil horizons*						*Occurrence of the different laccase gene sequences in soil cores*
			O_h	A_h	O_h&A_h	A_h&B_v	B_v	O_h&A_h&B_v	
Unknown basidiomycetes	–	59	30	16	3	3	5	2	1 to 5
Boletaceae	Unknown species[(a)]	16	5	5	2	1	1	2	1 to 7
Cortinariaceae	*Hebeloma radicosum*[(a)]	2	–	–	2	–	–	–	2
Russulaceae	Unknown species[(a)]	49	10	12	3	9	13	2	1 to 14
	Lactarius subdulcis[(a)]	1	–	–	–	–	–	1	1 to 10
	Russula nigricans[(a)]	3	–	–	–	–	–	3	1 to 4
	Russula mairei[(a)]	1	–	–	–	–	1	–	1
Sebacinaceae	Unknown species[(a)]	4	3	–	1	–	–	–	1 to 4
Agaricaceae	*Macrolepiota procera*[(b)]	1	–	–	–	–	1	–	1
Polyporaceae	*Pycnoporus cinnabarinus*[(b)]	1	–	–	–	1	–	–	1 to 3
Tricholomataceae	*Collybia peronata*[(b)]	1	1	–	–	–	–	–	1
	Mycena unknown species[(b)]	17	13	1	2	–	–	1	1 to 4
	Mycena cinerella[(b)]	3	1	–	2	–	–	–	1 to 2
	Mycena crocata[(b)]	2	–	–	–	–	2	–	1 to 2
	Mycena zephirus[(b)]	3	2	–	1	–	–	–	1 to 5
	Lepista nuda[(b)]	2	1	–	1	–	–	–	1
	Marasmius alliceus[(b)]	2	2	–	–	–	–	–	1

The letters (a) and (b) indicated the ECM and saprotrophic species, respectively.

highest laccase gene diversity occurred in the organic horizon (Table 2) and paralleled the profile of the total laccase (ABTS) enzyme activity (Fig. 1). Both factors are in accordance with the expectable higher degradation of the organic matter in the O_h. Their congruence suggests that a large diversity of laccase genes might indicate a high laccase activity. However, the variation of both factors was not in the same order of magnitude, and no simple correlation should be expected at this level. The proliferation of fungi producing phenoloxidases and laccase activities were also found to be linked in oak forest litter [11].

Along each transect, the laccase gene content dissimilarity was high even for adjacent cores (30 cm), but increased with the interval between cores. Comparison of the Sørensen and topographic distance matrices suggested that two soil samples were independents (>94% of dissimilarity), when separated by at least 6 m (Fig. 3). Tedersoo et al. [48] found high differentiation in ECM species composition even between soil cores distant of 5 cm. The spatial distribution of the eight most frequent sequences was patchy, and these sequences were only detected within relatively small areas (<0.77 m^2). Considering that each gene corresponds to a fungal species, this restricted extension may reflect changing microenvironmental conditions in soils [8] and/or hyphal competition [52]. Variations in ECM species composition within small areas were also found by Zhou and Hogetsu [54] and Tedersoo *et al.* [48].

Despite their wider spatial distribution, the detected genes of ECM fungi had not a uniform vertical repartition. Laccase genes of *L. subdulcis* were preferentially found in the organic horizon (O_h), and the ones of Russulaceae type 1 in the mineral part of the soil cores (B_v). As previously mentioned, several soil factors could explain this differential distribution such as pH, moisture, O_2 and CO_2 concentrations, quantity, and quality of organic matter [28]. However, the known ability of these taxa to degrade soil litter polymers is consistent with the gene distribution found in the present study. As shown by $\delta^{13}C$ and $\delta^{15}N$ signatures [22, 47], *Lactarius* belongs to a group of ECM fungi with high saprotrophic ability that preferentially acquires carbon and nitrogen from remnants of decayed wood and humic polymers. In contrast, *Russula* belongs to a group essentially acquiring carbon from their phytobiont [22, 47].

Saprotrophic fungi can generate qualitatively different habitat patches enhancing the development of highly diverse communities of soil organisms including ECM fungi [42]. The approximate diameter of such soil nutrient patches was found to be 20 cm in deciduous woodland [18]. The heterogeneous spatial distribution of the fungal laccase sequences likely reflects this patchiness of nutrient resources, suggesting that substrate preference could be one key factor impacting fungal distribution. Soil substrates may display rapid changes [14], and additional analyses of seasonal variations in the laccase sequence profiles at defined sites are therefore needed to confirm this contention.

The present study demonstrates that direct amplification of laccase gene sequences from soil DNA extracts

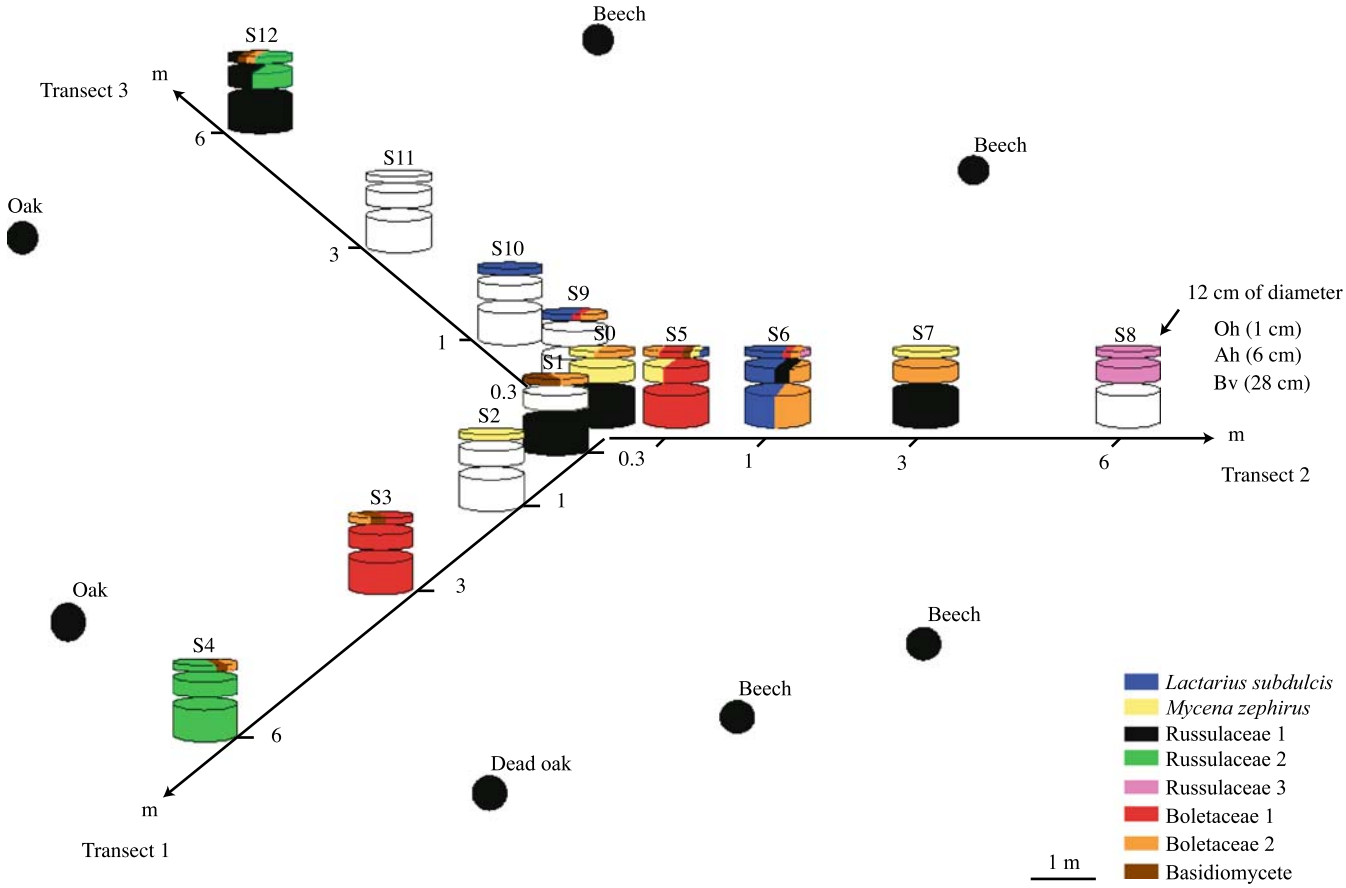

Figure 4. Vertical and horizontal 2D spatial distribution of eight dominant basidiomycete laccase genes through three transects in the "Steinkreuz" Cambisol (Bavaria, Germany). Each corresponding fungal taxon is indicated by a different color. *Black circles* indicate tree positions. A laccase gene was considered dominant when it was detected more than five times in at least one of the 13 soil cores. No dominant basidiomycete laccase gene was detected in *blank squares*.

allows identification and tracing the distribution of fungal species with a potential role in cycling of soil organic matter. The analyses revealed a short-scaled patchy distribution of saprotrophic and ECM fungi with laccase genes both in the vertical and horizontal dimensions. In some cases, the distribution of the laccase genes was congruent with the known strategy of the corresponding fungi to gain C and N resources. However, this kind of interpretation must be regarded cautiously as laccases are considered to play a role in soil organic matter cycling, but also in defense and developmental processes, such as basidiome pigmentation and rhizomorph formation [6, 15, 24]. Additionally, the detection of a gene gives no indication about its expression. We recently realized a first work on expression of laccase genes by semiquantitative RT–PCR on mRNA extracted from soils [31]. Actually, we improved the reliability of this technique for monitoring at scales similar to the one of this work. In future, information gathered from applying PCR-based methods to study expression dynamics of fungal genes in soils will allow following the behavior of multiple guilds, clades, or functional groups of fungi (i.e., saprotrophs vs ECM) involved in soil organic matter cycling over time or along physicochemical gradients of interest. Coupling this approach with the soil proteomics actually in progress [43] will provide valuable information on how the environment affects population dynamics and how the composition of functional units that may not reflect strict phylogenetic groupings affects biochemistry in forest soils.

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