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Soil Biology & Biochemistry 36 (2004) 1025–1036

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Diversity of laccase genes from basidiomycetes in a forest soil

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Received 15 April 2003; received in revised form 20 February 2004; accepted 20 February 2004

Abstract

Fungal oxidative exo-enzymes lacking substrate specificity play a central role in the cycling of soil organic matter. Due to their broad ecological impact and available knowledge of their gene structure, laccases appeared to be appropriate markers to monitor fungi with this kind of oxidative potential in soils. A degenerate PCR-primer pair Cu1F/Cu2R, specific for basidiomycetes, was designed to assess directly the diversity of laccase genes in soils. PCR amplification of mycelial cultures and fruit-bodies of a wide spectrum of basidiomycetes, covering all functional groups (saprophytes, symbionts, and pathogens), produced multiple DNA fragments around 200 bp. A neighbor-joining tree analysis of the PCR-amplified laccase sequences showed a clear species-specificity, but also revealed that most fungal taxa possess several laccase genes showing a large sequence divergence. This sequence diversity precluded the systematic attribution of amplified laccase of unknown origin to specific taxa. Amplification of laccase sequences from DNA, extracted from a brown (moder) forest soil, showed a specific distribution of laccase genes and of the corresponding fungal species in the various soil horizons (O_h, A_h, B_v). The most organic O_h-horizon displayed the highest gene diversity. Saprophytic fungi appeared to be less widespread through the soil horizons and displayed a higher diversity of laccase genes than the mycorrhizal ones.
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Keywords: Laccases; Gene diversity; Soil DNA; Basidiomycetes; Degenerate PCR primers

1. Introduction

An important part of the potentially bio-available global carbon pool is bound as organic compounds in soils (Killham, 1994). Both, the formation and mineralization of such compounds result essentially from biological processes in which microorganisms are highly involved (Emmerling et al., 2002). With 10^4–10^6 colony-forming units per gram dry weight of soil and 37–184 g dry weight of mycelium per square meter, fungi represent a major component of the soil biomass (Thorn, 1997). Concerning the turnover of the soil organic matter (SOM), they play an important role due to the production of oxidative exo-enzymes lacking substrate specificity such as manganese peroxidases, lignin peroxidases and laccases (Chefetz et al., 1998; Daina et al., 2002; Tuomela et al., 2002). Although, measuring the presence, abundance and diversity of soil fungi with this kind of oxidative potential appears to be necessary, it is a challenging task since it has been estimated that only 10% of fungal species have been described due to culture limitations (Thorn, 1997).

Recently, the development of molecular methods has opened new perspectives for investigations of functions of fungal groups in soils without cultivation steps. After extraction of total soil DNA, fungal diversity assessment by specific PCR amplifications of ribosomal DNA regions followed by cloning/sequencing (Landeweert et al., 2003), denaturing/temperature gradient gel electrophoresis (D/TGGE) (Emmerling et al., 2002) or terminal restriction fragment length polymorphism (Lord et al., 2002; Dickie et al., 2002) have provided ecological information about the fungal community structure in soils. Application of molecular techniques to target genes could reveal specific corresponding functions of soil fungi. However, to our knowledge, monitoring of fungal genes involved in specific functions in soils has not yet been reported.

Fungal laccases (benzenediol:oxygen oxido-reductase, EC 1.10.3.2), that usually contain four relatively conserved regions that bind the copper atoms directly involved in their

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0038-0717/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2004.02.017

active site (Thurston, 1994), are good candidates for a molecular approach in soil fungal ecology. Indeed, as laccases are the most widespread exo-enzymes in fungi and as they present conserved regions at the protein and gene level, it is possible to design primers compatible with a wide range of fungi. Laccases catalyze the oxidation of phenolic compounds and aromatic amines coupled to the reduction of molecular oxygen to water (Thurston, 1994). Despite their relatively low oxido-reductive potential compared to manganese and lignin peroxidases, they can also oxidize non-phenolic components in the presence of appropriate radical mediators such as artificial substrates like 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and 1-hydrobenzotriazole (HBT) (Bourbonnais et al., 1995, 1998) or fungal metabolites naturally present in soils such as 3-hydroxyanthranilic acid (3HAA) (Eggert et al., 1996). Laccases are therefore able to completely degrade lignins (Eggert et al., 1997) and appear to be an important actor in SOM turnover. Recent studies showed the presence of laccase activity in forest litters (Criquet et al., 1999) and several novel laccase genes have been obtained from fungi isolated from soils (Farnet et al., 2002).

The present study sought to assess the laccase gene diversity of ectomycorrhizal (ECM) and saprophytic basidiomycetes in a forest soil using PCR.

2. Materials and methods

2.1. Soil sampling and organisms

A brown forest soil from 'Steigerwald' (49°52′26″N, 10°27′54″E), an experimental station of the Institute of Ecosystem Research (BITÖK) of the University of Bayreuth (Bavaria) localized in a mixed oak–beech forest, was investigated. This soil is characterized by a fine moder humus layer, a pH of 4.2 and a C/N ratio of 20 (Seiler, 1995). The turnover of organic matter in this soil type is rapid, with a low accumulation in the lower horizons. The organic horizon O_h (1–0 cm), corresponding to the fine humus layer, contains about 19.0% of organic carbon (C_{org}), while the organo-mineral A_h (0–6 cm) and the mineral B_v (6–34 cm) horizons present, respectively, 3.4 and 1.2% of C_{org}. Within a plot of $6 \times 6\ m^2$, five soil cores (12 cm of diameter) were randomly collected in September 2002 and divided into O_h, A_h and B_v horizons. Soil samples were kept below 10 °C during transportation. Fresh fruit-bodies from all fungal species present in the field site at this time were also collected. Pure cultures of some species were obtained from spores or fruit-bodies. For the non-cultivable species, small pieces of fruit-bodies were used for the molecular analyzes (Table 1a). Additionally, culture collection strains were used (Table 1b). All fungal strains were maintained at room temperature in Petri dishes containing 2% malt extract (ME) agar (20 g l^{-1} ME, 15 g l^{-1} agar, Merck, Darmstadt, Germany).

2.2. DNA extractions

2.2.1. DNA extraction from fresh fruit-bodies and mycelial cultures

Freshly collected fruit-bodies were carefully washed and the inner part of the stipe was cut into small pieces, freeze-dried and conserved at −20 °C. Mycelium cultures were obtained from some of these collected fruit-bodies. The fungal cultures were grown on ME agar for 1 week at room temperature. The mycelium was collected by scraping and immediately frozen in liquid nitrogen. For *Collybia fusipes*, a longer cultivation time (2 weeks) was required. The genomic DNA was directly isolated from frozen material after grinding using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) as recommended by the manufacturer.

2.2.2. DNA extraction from soil samples

DNA extraction from soil cores was performed within a few hours of collecting the samples. Roots and leaves were discarded and samples corresponding to the different horizons were homogenized. Genomic DNA was isolated from 0.5 g of soil using the FastDNA™ Spin kit for soil (Q-BIOgene, Heidelberg, Germany) with a modified protocol, where instead of letting the GLASSMILK settle (protocol, Step 7), the Silica–DNA pellets obtained after brief centrifugation were washed 10 times with a solution of guanidine thiocyanate (5.5 M, pH 7). The final pellets were resuspended in 1 ml of guanidine thiocyanate, transferred to the SPIN filter, and after 1 min of centrifugation at 14,000*g*, the DNA extraction proceeded according to the manufacturer's protocol (Step 8).

2.3. Design of degenerate primers specific for basidiomycetes

Fungal laccase proteins typically contain four copper atoms per sub-unit. Several structural and functional studies have shown that one cysteine and 10 histidine residues are involved in binding the atoms, and that the protein sequences around these domains are highly conserved (Thurston, 1994). To perform single and nested PCRs from parts of the basidiomycete laccase genes, six degenerate primers were designed for these four regions according to the process described by D'Souza et al. (1996). All primers used in the study were synthesized by Invitrogen Life Technologies (Karlsruhe, Germany). In preliminary studies, various pairs of primers (Fig. 1a) were tested on DNA extracts from mycelial cultures of the strain collection listed in Table 1b. This collection comprised several basidiomycetes and ascomycetes known to have laccase genes and for which the presence of the corresponding exo-enzyme activity was verified by a simple ABTS test (Matsumura et al., 1987). The single PCR performed with the primers 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′) gave better results, in terms of positive

Table 1
Fungal strains used in the study

Species	Material type	ABTS test	PCR products	Accession numbers
(a) From the field site				
Agaricales-Agaricaceae				
Macrolepiota procera	M	+	+	AJ542610; AJ542611
Macrolepiota rachodes	F	ND	0	
Agaricales-Amamitaceae				
Amanita citrina	F	ND	+	AJ542646; AJ542647
Limacella illinata	F	ND	0	
Agaricales-Coprinaceae				
Psathyrella cf. dicrani	F	ND	+	AJ542620
Psathyrella corrugis	M	0	+	AJ542617 to AJ542619
Psathyrella murcida	M	0	+	AJ542612; AJ542613
Agaricales-Cortinariaceae				
Cortinarius cf. alboviolaceus	F	ND	0	
Cortinarius delibutus	F	ND	0	
Cortinarius cf. flexipes	F	ND	+	AJ542638
Cortinarius sp	F	ND	0	
Hebeloma radicosum	M	+	+	AJ542631 to AJ542633
Hebeloma sp	F	ND	0	
Agaricales-Hygrophoraceae				
Hygrophorus cossus	F	ND	0	
Agaricales-Tricholomataceae				
Clitocybe candidans	M	0	0	
Clitocybe clavipes	M	+	+	AJ542635
Clitocybe fuligineipes	M	+	+	AJ542634
Clitocybe nebularis	F	ND	+	AJ542626; AJ542627
Clitocybe odora	M	0	0	
Collybia butyracea	M	+	+	AJ542637
Collybia peronata	M	0	+	AJ542636
Laccaria amethystine	F	ND	0	
Laccaria laccata	F	ND	0	
Lepista flaccida	M	0	+	AJ542595; AJ542596
Lepista nuda	M	+	+	AJ420347; AJ542593; AJ542594
Marasmius alliaceus	M	0	+	AJ542603 to AJ542606
Megacollybia platyphylla	M	+	+	AJ542645
Mycena cinerella	M	+	+	AJ542607 to AJ542609
Mycena crocata	M	+	+	AJ542585 to AJ542592
Mycena galopus	M	+	+	AJ542639 to AJ542642
Mycena pura	F	ND	+	AJ542630
Mycena rosea	F	ND	+	AJ542628; AJ542629
Mycena zephirus	M	0	+	AJ542600 to AJ542602
Agaricales-Russulaceae				
Lactarius blennius	F	ND	+	AJ542649
Lactarius subdulcis	M	+	+	AJ542650
Russula atropurpurea	F	ND	+	AJ542624
Russula mairei	F	ND	+	AJ542625
Russula nigricans	F	ND	+	AJ420340; AJ542621; AJ542622
Russula ochroleuca	F	ND	+	AJ542623
Agaricales-Strophariaceae				
Stropharia squamosa	M	+	+	AJ542597 to AJ542599
Aphyllophorales-Clavariaceae				
Macrotyphula juncea	M	+	+	AJ542614 to AJ542616
Aphyllophorales-Clavulinaceae				
Clavinula cristata	F	ND	0	
Boletales-Boletaceae				

(*continued on next page*)

Table 1 (*continued*)

Species	Material type	ABTS test	PCR products	Accession numbers
Strobilomyces floccopus	F	ND	0	
Xerocomus chrysenteron	M	0	+	AJ542643; AJ542644
Boletales-Paxillaceae				
Paxillus involutus	M	0	+	AJ542648
Lycoperdales-Lycoperdaceae				
Lycoperdon perlatum	F	ND	0	
Sclerodermatales-Sclerodermataceae				
Scleroderma areolatum	F	ND	0	
Pezizales-Helvellaceae*				
Helvella crispa	F	ND	0	
(b) From collections				
Agaricales-Polyporaceae				
Lemtimula edodes	M	+	+	AJ420171; AJ420172
Pleurotus ostreatus	M	+	+	AJ420179 to AJ420181
Pleurotus cornucopiae	M	+	+	AJ420173; AJ420174; AJ420336; AJ420337
Agaricales-Strophariaceae				
Hypholoma sp.	M	+	+	AJ420177; AJ420178
Agaricales-Tricholomataceae				
Collybia fusipes	M	+	+	AJ420169; AJ420170
Aphyllophorales-Ganodermataceae				
Ganoderma lucidum	M	+	+	AJ420176; AJ185275
Aphyllophorales-Polyporaceae				
Pycnoporus cinnabarinus	M	+	+	AJ420334; AJ420335; AJ420182
Trametes versicolor	M	+	+	L78077
Auriculariales-Sebacinaceae				
Piriformospora indica	M	+	+	AJ542651
Diaporthales-Cryphonectria-Endothia complex*				
Cryphonectria parasitica	M	+	0	
Eurotiales-Trichocomaceae*				
Aspergillus nidulans	M	+	0	
Mycosphaerellaceae-mitosporic Mycosphaerellaceae*				
Cladosporium sp. *Pezizales-Morchellaceae**	M	+	0	
Morchella esculenta	M	+	0	
Verpa digitaliformis	M	+	0	
Pezizomycotina-Dothideomycetes*				
Cenococcum geophilum	M	+	0	
Sordariales-Lasiosphaeriaceae*				
Podospora anserina	M	+	0	
Sordariales-Sordariaceae*				
Neurospora crassa	M	+	0	

(M) indicates the mycelium cultures and (F) the fruit-bodies. The sign (+) represents a positive result, (0) a negative one, and (ND) a not done experiment. The arrows (*) indicate the ascomycete families tested.

amplification and specificity for basidiomycetes (Fig. 1b) and was thus used in the following investigations.

2.4. PCR amplification

Genomic DNA isolated from fungal material or from soil samples was used as a template in single PCR. For the amplifications, 3 μl of the DNA extracts were added to a 50 μl reaction mixture containing 5 μl of 10 × *Taq* buffer with $MgCl_2$ (Q-BIOgene, Heidelberg, Germany), 4 μl of dNTPs (10 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, Heidelberg, Germany). The reaction mixtures were overlaid with two drops of

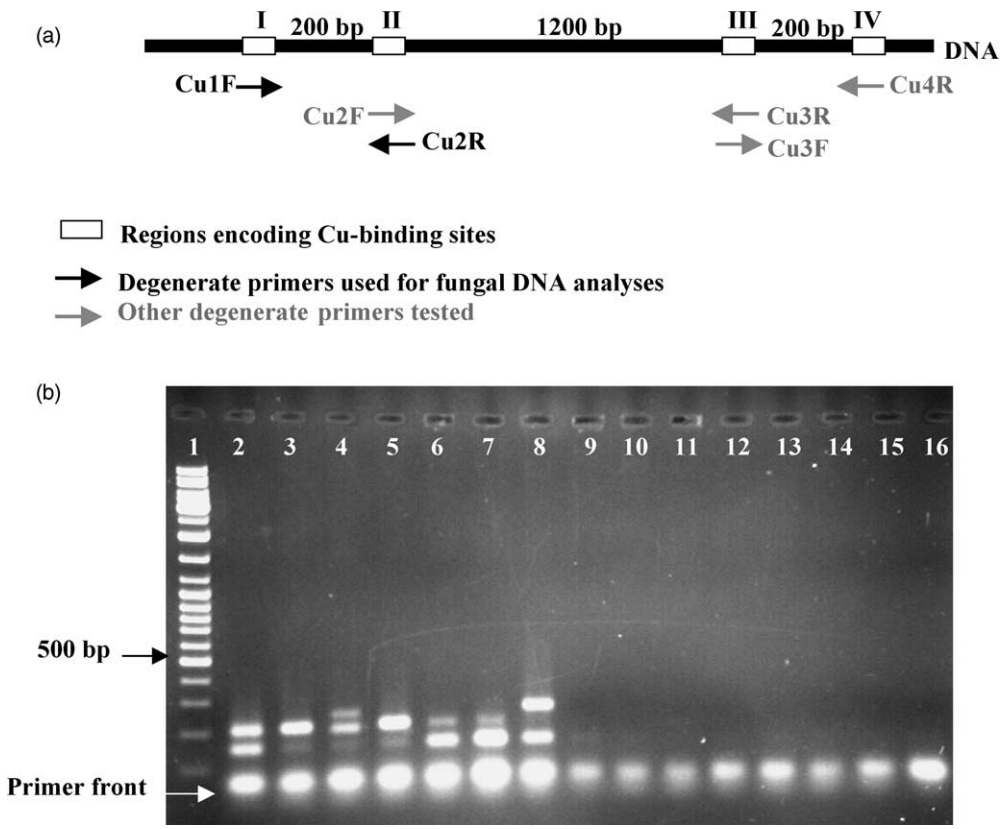

Fig. 1. Amplification of basidiomycete-laccase gene fragments from nucleic acids isolated from mycelial cultures or soil samples. (a) General structure of the fungal laccase gene and primer combinations tested. Only the pair Cu1F/Cu2R gave satisfying results and was used to amplify a 200 bp fragment between regions I and II in a single PCR. (b) Specificity of the primers Cu1F/Cu2R for basidiomycetes. Two percent agarose gel electrophoresis of fragments of laccase genes amplified by PCR on basidiomycetes (2–8) and ascomycetes (9–15) mycelial cultures. Lane 1, 100 bp DNA ladder mix (MBI fermentas); 2, *P. cinnabarinus*; 3, *G. lucidum*; 4, *P. ostreatus*; 5, *T. versicolor*; 6, *L. edodes*; 7, *Hypholoma* sp.; 8, *C. fusipes*; 9, *Neurospora crassa*; 10, *Aspergillus nidulans*; 11, *Cladosporium sp.*; 12, *C. parasitica*; 13, *P. anserina*; 14, *M. esculenta*; 15, *C. geophilum*; and 16, negative control without DNA.

sterile oil and PCRs were 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. Additionally, amplification of the internal transcribed spacer (ITS) in the nuclear ribosomal DNA region was performed with each DNA extract to estimate its quality and, especially in the case of amplifications from soil extracts, to detect the presence of possible inhibitory substances. Amplification of the laccase sequences from DNA of *Pycnoporus cinnabarinus* was also carried out in each series of samples as a positive control to detect PCR failures.

Seven microliters of each amplification product were 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, St Leon–Rot, Germany) was run in a separate lane. The gels were stained with ethidium bromide and the DNA bands visualized and photographed under UV light.

2.5. DNA sequencing and sequence analysis

PCR products were directly cloned into the pCR 4-TOPO vector, using the TOPO TA Cloning kit for sequencing (Invitrogen Life Technologies, Karlsruhe, Germany) as described by the manufacturer. The plasmid DNA, containing the PCR product, was extracted from 1.5 ml of *Escherichia coli* TOP10 chemically competent cells using the Perfectprep plasmid mini kit (Eppendorf, Hamburg, Germany). Cloned products were then sequenced in both directions with M13 reverse (5′-CAGGAAACAGCTATGAC-3′) and M13 forward (5′-GTAAAACGACGGCCAG-3′) fluorescent primers and the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, UK) on a DNA sequencer Long Reader 4200 (LI-COR, Nebraska, USA).

The nucleotide sequences obtained for the soil samples have been deposited at EMBL under the accession numbers

AJ420334; AJ420350; AJ420351; AJ540285 to AJ540297; AJ540236 to AJ540278; AJ540280 to AJ540284. The accession numbers of the sequences generated with the culture collection and the fungi collected from the field are indexed in Table 1.

Reverse, complement, or inverse sequences were generated by the IUPAC program (http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html) and sequences matches done using the GenBank DNA database and the Gapped BlastN (NCBI) search algorithm (Altschul et al., 1997). The coding regions and protein sequences were deduced after alignment of each DNA sequence with known laccase mRNA (e.g. *Trametes versicolor*; Accession No. L78077). The deduced coding regions of the 154 sequences issued from the present survey and the 19 additional laccase sequences retrieved from GenBank were aligned using the program MultAlin (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page = /NPSA/npsa_multalinan.html; IBCP, Lyon, France; Corpet, 1988). The resulting multiple alignments was optimized visually. Only unambiguous alignments were used in the phylogenetic and distance analyzes. The aligned sequences were exported to a NEXUS file and analyzed using PAUP*4.0b10 (Swofford, 1998). The final alignments of the laccase regions have a total of 100 unambiguously aligned sites and 94 characters were parsimony-informative. The final alignments of the deduced laccase coding regions are available on request. The final trees were constructed using the neighbor-joining (NJ) method. The approach selected for the analysis of the molecular data set was the Kimura two-parameter distance method (Kimura, 1980). All characters were treated with equal weight and the bootstrapping was performed with 1000 replications. Final results were compared to the 50% majority-rule consensus trees using maximum parsimony (MP) analysis. The MP trees were performed using the heuristic search option with 50 random sequence additions and tree bissection reconnection (TBR) branch-swapping (Swofford, 1998). Trees were drawn with CorelDraw 11. Laccase sequences of the ascomycetes *Colletotrichum lagenarium* (GenBank Accession No. AB055709), *Gaeumannomyces graminis* (GenBank Accession No. AJ437319) and *Botryotinia fuckeliana* (GenBank Accession No. AF243855) were used as out-groups to root the trees.

3. Results

3.1. Amplification of laccase genes from basidiomycetes

From the primer combinations tested on culture material, only the primer pair Cu1F/Cu2R consistently gave PCR products. According to available nucleotide sequences of laccase genes from basidiomycetes, the PCR performed with this primer pair was expected to produce fragments of approximately 200 bp (Fig. 1a). For all strains of basidiomycetes tested (Table 1b), agarose gel electrophoresis of the single-PCR products obtained with the primer pair Cu1F/Cu2R showed DNA fragments with the expected-size ranging from 140 to 300 bp (Fig. 1b). *P. cinnabarinus*, *Ganoderma lucidum, T. versicolor*, *Lentinula edodes* and *Hypholoma* sp. gave two fragments of 142 and 200 bp. *Pleurotus ostreatus* displayed an additional 240 bp band (Fig. 1b), whereas the phytopathogenic fungus *C. fusipes* showed two fragments of 142 and 250 bp. The absence of PCR product for all ascomycetes tested (Fig. 1b) confirmed the specificity of the primers for basidiomycetes.

After sequencing fragments of different size, the nucleotide sequences obtained were compared to the GenBank nucleotide database and showed that all of the PCR products correspond to regions of basidiomycete laccase genes. This result was supported by the high similarity levels revealed by the alignment of the predicted amino acid sequences (data not shown). The 200 bp gene fragments amplified from mycelial cultures of *T. versicolor* and *G. lucidum*, respectively, displayed 100% similarity with regions of the previously described laccase genes of *T. versicolor* (*lcc1*; X84683) and *G. lucidum* (AF185275). The 200 bp amplification fragment of the *Pleurotus cornucopiae* strain (AJ420173) presented 94% similarity with the *lcc4* laccase gene of *Trametes villosa* (L78077). Other amplified products (140–300 bp) corresponded to novel sequences and lacked high similarity to known laccase genes.

The alignment of the new nucleotide sequences obtained with known laccase genes and protein-coding regions (cDNA) retrieved from GenBank was used to identify the position of introns and exons. Introns appeared to be localized between positions: (1) 45 and 155 for *C. fusipes* (AJ420169; AJ420170), *L. edodes* (AJ420171), *P. cornucopiae* (AJ420174) and *P. ostreatus* (AJ420180), and (2) 121 and 190 for *Hypholoma* sp. (AJ420177), *P. cinnabarinus* (AJ420175), *P. cornucopiae* (AJ420173) and *G. lucidum* (AJ420176). In contrast, no intron was detected in the amplified laccase sequences from *L. edodes* (AJ420172) and *Hypholoma* sp. (AJ420178).

The sequencing of several clones from each amplification product revealed that a single PCR band could comprise fragments of different laccase genes, which is reflected by differences in the deduced protein sequences. Sequences of at least two different laccase genes were obtained with the single PCR fragment of *P. cornucopiae* (Fig. 2).

3.2. Sequence analysis of PCR products obtained from soil DNA

The primer pair Cu1F/Cu2R was used to amplify laccase-gene sequences from basidiomycetes present in the brown (moder) forest soil from the experimental station of Steigerwald (Bavaria). The fragments amplified from the DNA extracted from the various soil horizons were cloned into plasmids and subsequently amplified from bacterial clones. Although, they showed a significant size

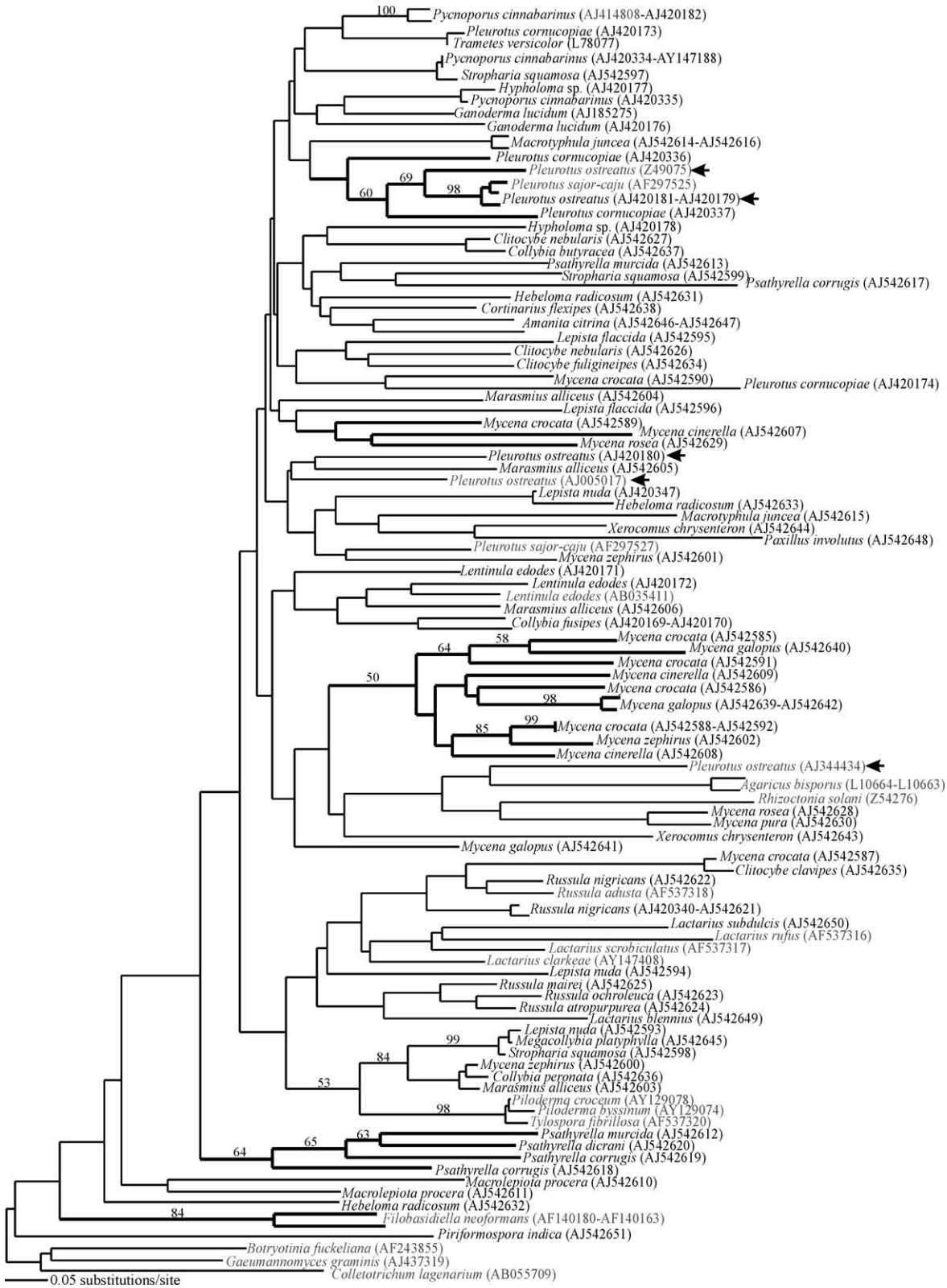

Fig. 2. Neighbor-joining tree representing the genetic distances between the 90 laccase-gene fragments obtained by PCR from the fruit-bodies collected from the Steigerwald forest site and from the fungal collection strains (both in black fonts), and the 19 sequences retrieved from GenBank (in grey characters). The tree was rooted using the laccase sequences of the ascomycetes *B. fuckeliana* (AF243855), *C. lagenarium* (AB055709) and *G. graminis* (AJ437319). Bootstrapping was performed with 1000 replications in a Kimura 2-parameter analysis. The bold branches indicate the sequences that are genus specific. The arrows indicate the position of the diverse *P. ostreatus* laccase-gene sequences.

heterogeneity it was possible to distinguish two main groups of fragment sizes, i.e. one around 140 bp and the other around 200 bp. Some longer fragments around 300, 350 and 380 bp were also amplified by single PCR from the most mineral horizons of this forest soil. As a sequence analysis of these longer fragments revealed no similarity with known laccase genes, they were discarded (Section 4). Five soil cores of the brown forest soil of Steigerwald were analyzed.

For each core, the PCR products obtained were cloned into bacteria and 25 clones (i.e. potential basidiomycete laccase sequences) for the O_h horizon and 15 clones for both A_h and B_v-horizons, respectively, were sequenced. A preliminary test had revealed that the sequencing of this number of clones allowed the diversity of the laccase-gene fragments in the respective horizons to be characterized (data not shown). Sixty-four different sequences showing a high similarity of their deduced protein sequences with known basidiomycete-laccase proteins were obtained.

3.3. Soil sequences versus fruit-body/fruit-body isolates sequences

Fruit-bodies of basidiomycetes growing at the experimental station of Steigerwald were harvested concomitantly with the soil samples to identify fungal species carrying the laccase genes detected in soil and to compare the above and the below ground diversity of laccase genes in basidiomycetes. When possible, cultured strains were isolated from the fruit-bodies and used for the molecular analyses. Based on the classifications of Singer (1986) and Jülich (1984), 15 fungal families, belonging to six orders, were considered. Of 48 species collected, 47 were basidiomycetes related to the Agaricales, Aphyllophorales, Boletales, Sclerodermatales and Lycoperdales and one was an ascomycete belonging to the Pezizales (Table 1a).

Two NJ trees were constructed from the deduced protein-coding regions of the amplified laccase-gene fragments. The first NJ tree shows principally the 90 sequences obtained from the culture strains and the fruit-bodies at the field site (Fig. 2). The second NJ tree shows the 64 laccase-gene fragments amplified from the soil DNA extracts and the reference laccase-gene sequences obtained from identified fungi (Fig. 3). The first tree (Fig. 2) shows the laccase-gene fragments to be species specific, as no sequence was shared by two different basidiomycete species. Additionally, 1–8 different laccase-gene sequences per species were detected with the highest gene diversity seen in the saprophytic fungus *Mycena crocata*. In contrast, the ECM species appeared to have fewer (1–3) different laccase-genes (e.g. *Hebeloma radicosum*). For most fungal taxa, the different laccase genes belonging to a single species were not grouped in a common clade (e.g. *P. ostreatus* marked by arrows in Fig. 2). This indicates that these fungi possess genes, belonging to different families of laccases, derived from different ancestral laccase genes. However, some of the clades in Fig. 2 (clade lineage marked in bold) group saprophytic fungi sequences within a genus, suggesting that they are derived from a common ancestral gene. 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). The second NJ tree showed that of the 64 sequences obtained from the soil DNA samples, only 17 sequences could be related to laccases of the collected fruit-bodies, 12 sequences originated from five different species of saprophytes and five sequences from two ECM fungi (Fig. 3). The majority of the mycorrhizal laccase sequences obtained appeared to be grouped in a few clades containing exclusively mycorrhizal species that for the most part belonged to the same fungal family (clade lineage indexed in bold in Fig. 3).

3.4. Vertical variation of the laccase-gene diversity

The diversity of the laccase sequences was measured for each soil horizon of each analyzed sample (Table 2) by calculating the Shannon–Wiener indexes (Shannon, 1948). The diversity of the laccase sequences was higher in the O_h horizon and decreased in the A_h and B_v horizons (Table 2). Most laccase sequences (65.7%) were exclusively located in one of the three horizons, i.e. respectively, 40.7% in the O_h, 9.4% in the A_h and 15.6% in the B_v horizons. Only 15.6% of the detected genes were found simultaneously in the first two horizons (O_h, A_h) and 7.8% in the lower horizons (A_h, B_v). Lost 10.9% of the laccase genes detected were present in all horizons and appeared to belong to ECM fungi of the family Russulaceae. These results show a high soil horizon specificity of the laccase genes. However, the ECM fungi of the family Russulaceae found in each of the three soil horizons appeared to be more widely distributed vertically than the other ECM species and to saprophytes that were mainly limited to the upper layers, A_h and especially O_h (Fig. 3).

4. Discussion

In the present study, we have designed six degenerate primers and used them to amplify specifically, fragments of laccase genes from basidiomycetes. Laccase sequences described in the literature show that introns may occur in the annealing site of some of the primers we have designed (Giardina et al., 1995). This could explain the negative results obtained with many of the primer combinations tested (data not shown). Only the newly designed primer pair Cu1F/Cu2R gave fragments of the expected length (Eggert et al., 1998; D'Souza et al., 1996) for all basidiomycetes. As expected, this primer pair failed to amplify ascomycetes. Comparison between the sequences generated with Cu1F/Cu2R and available mRNA sequences in database showed the presence of introns within most of the obtained laccase-gene fragments, confirming the findings of D'Souza et al. (1996). A relatively high diversity of

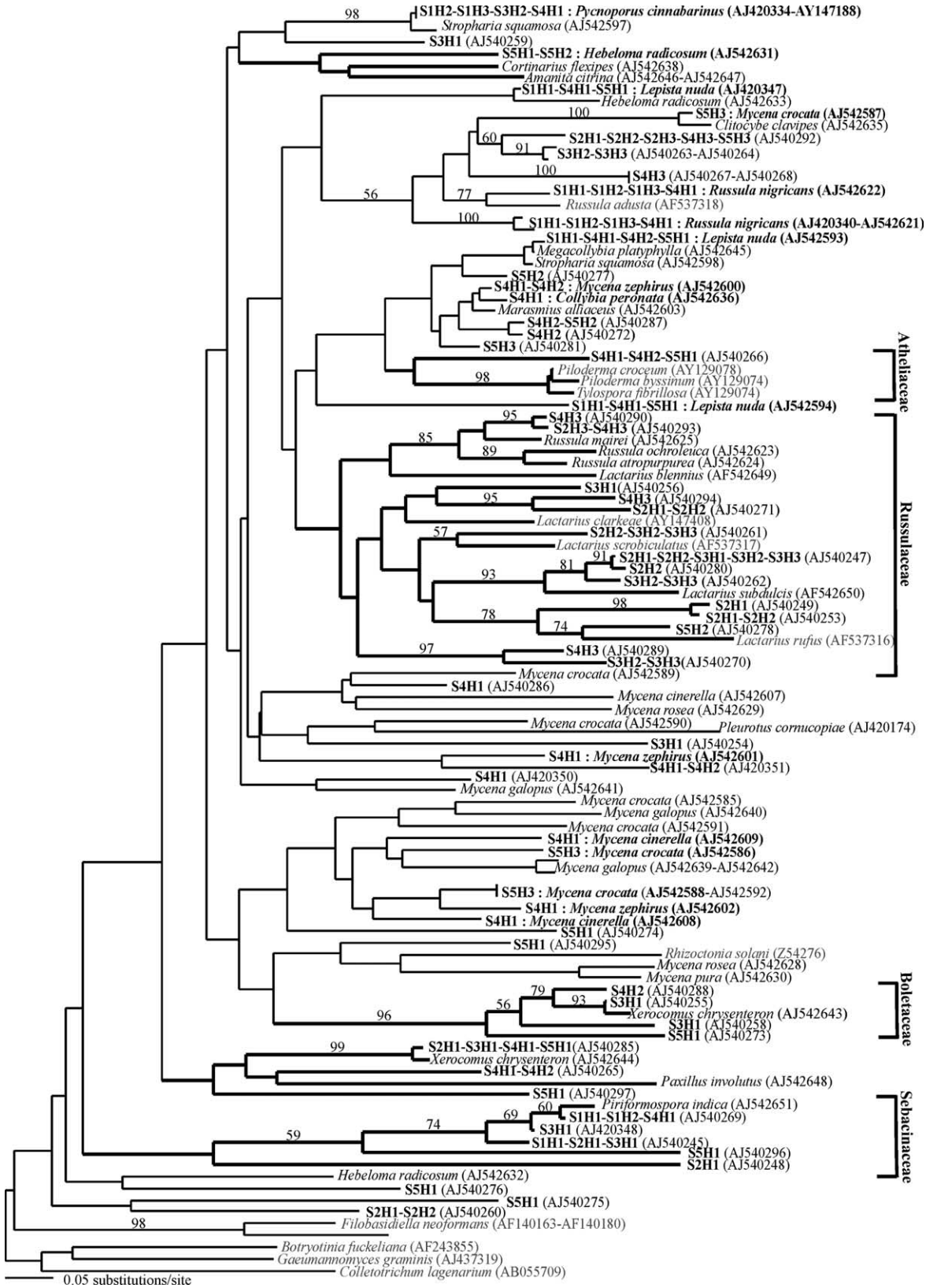

Fig. 3. Neighbor-joining tree representing the genetic distances between the 64 laccase-gene fragments amplified by PCR from the soil samples (in bold type) and the closely related sequences obtained from identified fungi (in black fonts) or retrieved from GenBank (in grey characters). S1–S5 correspond to the replicate numbers and H1–H3 to the horizons (1 for O_h, 2 for A_h and 3 for B_v). The NJ tree was rooted using sequences of the ascomycetes *B. fuckeliana* (AF243855), *C. lagenarium* (AB055709) and *G. graminis* (AJ437319). Bootstrapping was performed with 1000 replications in Kimura 2-parameter analysis. The potential mycorrhizal specific clades are presented in bold.

Table 2
Diversity of basidiomycete-laccase gene fragments in three horizons of a brown forest soil in Steigerwald (Germany)

Sample No.	O_h horizon	A_h horizon	B_v horizon
Sample 1	1.88	1.66	1.32
Sample 2	2.22	1.83	0.63
Sample 3	2.34	1.94	1.72
Sample 4	2.89	1.92	1.69
Sample 5	2.46	1.94	1.54

The diversity of the laccase sequences was measured for each soil horizon of each analyzed sample by the calculation of Shannon–Wiener indexes (Shannon, 1948).

laccase genes among the collection strains was found and most taxa presented at least two highly distinctive laccase gene sequences, with the differences not only evident as introns, but also in the deduced coding regions (i.e. the exons). However, the predicted amino acid sequences of all PCR products showed that despite their polymorphism they all corresponded to laccase-gene sequences.

Fruit-bodies from the experimental station of Steigerwald were analyzed to complete the laccase sequences obtained from the collection strains used to test the primers. In total, 14 basidiomycete families from four orders, covering all functional groups (saprophytes, symbionts, and pathogens) and including *Piriformospora indica* a primitive basidiomycete were amplified. This confirmed that the Cu1F/Cu2R primer pair could be used to screen laccase genes across a spectrum of soil basidiomycetes. Noteworthy is that for some of the mycelium isolated from the fruit-bodies (Table 1), laccase genes were found by PCR although no expression was detected with the ABTS test. This colorimetric enzymatic test has more an indicative value, as some cultural conditions could induce repression of the laccase gene expression (Schlosser et al., 1997). However, such enzymatic tests have suggested a laccase activity in some ECM fungi (Muenzenberger et al., 1997; Gramss et al., 1998). More recently, Burke and Cairney (2002) discussed the presence of polyphenol oxidases such as laccases in ECM fungi, and the presence of laccase genes in some ECM species has already been shown (Chen et al., 2003). Our analyses of fruit-bodies confirm the presence of laccase genes in ECM fungi such as *Lactarius* and *Russula*, and also in additional taxa (*Amanita*, *Cortinarius*, *Hebeloma*, *Paxillus* and *Xerocomus*). The discovery of laccase genes in additional ECM fungi is consistent with the fact that some of these fungi are directly involved in the attack of organic polymers in which nitrogen and phosphate resources are sequestered (Read and Perez-Moreno, 2003).

The NJ tree, constructed from the deduced DNA coding-regions (Fig. 2), showed a clear species-specificity for laccase genes, but also that most taxa possess multiple laccase sequences. This supports the report of different laccase gene families by Mansur et al. (1997) and Wahleithner et al. (1996). Even if there was a limited congruence between laccase gene diversity and fungal genus, some clades in which the sequences and the fungal taxon (genus or family) were congruent (Figs. 2 and 3) were found. This allowed us to attribute several new sequences detected in soils to these fungal groups. For example, the unidentified sequences grouping with the Russulaceae sequences (Fig. 3) are likely to originate from species of this taxon. However, the available knowledge on laccase diversity in fungi is still too scarce to assign reliably sequences to taxa. One limitation is the lack of detailed knowledge on the numbers of laccase gene families found in fungi (see below).

The NJ analysis shown in Fig. 2 suggests that the saprophytic fungi are more diverse in their laccase genes (up to eight per species) than the mycorrhizal fungi (1–3 per species). This difference in the enzymatic spectrum of these two functional groups of fungi might reflect their respective nutritional pathways. For soil saprophytes, the SOM is the unique carbon source and a high diversity of laccases might allow access to a wide range of soil substrates. For the ECM fungi, which receive most of their carbon as simple sugars from the host plant (Smith and Read, 1997), a wide diversity of laccases might be less essential. This interpretation is also supported by the fact that in the NJ trees, clusters of deduced laccase coding-regions were generally found at the genus level in saprophytes and at the family level in ECM.

After their evaluation on defined fungal material, the degenerate PCR-primers Cu1F/Cu2R were used to detect basidiomycete laccase genes in different horizons of a brown forest soil. To our knowledge, the present study represents the first attempt to characterize fungal laccase genes in soil DNA extracts. These primers allowed us to detect the presence of basidiomycetes with laccase genes and to explore their specific distribution in soil horizons. Except for some long fragments, all amplification products could be unequivocally attributed to basidiomycete laccases based on their deduced protein sequences. The unexpected long fragments (300–380 bp) displayed no similarity with any laccase genes previously reported. Interestingly, they were essentially restricted to the A_h and B_v horizons that have a low content of organic matter. At this time, it is not possible to know whether they correspond to PCR artefacts produced in mineral substrates with reduced concentration of laccase genes, or whether they represent sequences from unknown copper proteins. Such enzymes could belong to a new family of laccase as suggested by our amplification of longer laccase fragments (289 bp) from *Mycena cincerella* (AJ42607). However, as this putative new laccase family with longer gene fragments would be characterized by a high number of introns, it will be difficult to deduce the coding-region making it difficult to confirm definitively the affiliation of these fragments obtained from soil DNA extracts. Nonetheless, atypical PCR fragments did not hamper the monitoring of basidiomycete laccase genes in soils, as they were easily differentiated by their size.

DNA amplification from the soil samples revealed a higher diversity of laccase genes in the O_h-horizon (C_{org} of

19%) where the degradation of organic matter is expected to be higher. The species distribution of soil fungi is known to vary vertically between successive horizons (Zvyagintsev, 1994; Dickie et al., 2002). With 66% of the sequences exclusively located in one of the three horizons as opposed to 10% common to all three, the survey indicates a specific distribution of laccase genes and probably of the corresponding fungal species in soil forest horizons. Only ECM fungi in the Russulaceae had a wide vertical distribution, while the other ECM families (Atheliaceae, Sebacinaceae, Boletaceae) and the saprophytes were mainly limited to the A_h and especially the O_h layer (Fig. 3). This observation partially confirms the findings of Tedersoo et al. (2003) who found a domination of Atheliaceae (*Piloderma*, *Tylospora*) and Sebacinaceae in the organic horizon of a Podzol soil, whereas Euagarics (*Cortinarius*, *Hebeloma*, *Inocybe*) and ascomycetes (Pezizales and Helotiales) preferred the mineral horizons. In contrast to this distribution, we found *Hebeloma* species in the O_h and A_h, but not in the B_v horizon of our brown forest soil. The domination of the ascomycete species found by Tedersoo et al. (2003) in the mineral part of the podzol might, however, explain the reduced laccase diversity we found in the B_v horizon, as the primers used are unable to amplify ascomycete laccases.

Of the 64 sequences found in the forest soil, only 17 corresponded to laccase sequences of fruit-bodies growing at the experimental station, and only seven of the 48 fungal species collected as fruit-bodies were detected in soil using the laccase sequence analyses. Thus, the fruit-body population does not reflect the below ground genetic diversity of the soil fungal community, as reported by Dahlberg et al. (1997) and Jonsson et al. (1999). Erratic fruit-body production and the existence of hypogeous fruit-bodies (Guinberteau and Courtecuisse, 1997) may explain the lack of correspondence between soil sequences and fruit-body sequences.

Direct amplification of gene fragments from soil DNA extracts, and analyses of the corresponding coding regions, provide useful information on fungal species having potential laccase activities. However, the laccase genes amplified are not necessarily expressed in soil. To investigate the function of fungal laccases in the carbon cycle in soils and especially in the formation, stabilization and degradation of the organic matter, laccase transcripts need to be monitored in parallel with gene detection. Future work will focus on the development of approaches for analyzing extracted soil mRNA.

Acknowledgements

This work was financially supported by the Deutsche Forschungsgemeinschaft (SPP 1090, grant Bu941/1-1). We thank Dr H. Dörfelt (University of Jena, Germany), Dr B. Marçais (INRA of Nancy, France) and M. Lomberh (Institute of Botany of Kiev, Ukraine) for providing some of the fungal strains. Christine Delaruelle and Dr Annegret Kolher (INRA of Nancy, France) for their help with the laccase gene sequencing. We are indebted to Prof. Dr A. Hartmann and D. Selesi (GSF, Neuherberg, Germany) for their help in the first DNA extraction from soils, and to H. Boyle (Staatliches Museum für Naturkunde, Görlitz, Germany) who kindly revised the manuscript.

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