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Fungal communities in bulk soil and stone compartments of different forest and soil types as revealed by a barcoding ITS rDNA and a functional laccase encoding gene marker

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ABSTRACT

The soil fungal diversity and community partitioning between the bulk soil and stone compartments was investigated using PCR based approaches targeting the barcoding internal transcribed spacer (ITS) of rDNA and the laccase encoding functional gene as genetic markers. Soil samples were collected from the B-horizon of spruce and beech forests at the Hainich Biodiversity Exploratory, central Germany. The targeted markers were amplified from the respective DNA extracts using general fungal primers and basidiomycete laccase gene specific primers, cloned and sequenced. Differences in the fungal community composition between the two forest types and the soil compartments were indicated by both markers. When the effects of ecological factors were considered, the two markers produced different patterns of results. The ITS rDNA marker revealed communities principally influenced by forest type, while those detected with the functional marker were mainly affected by soil pH. The fungal communities detected by the functional marker in particular, differed significantly between soils and stones, indicating that laccase-producing fungi are specifically adapted to degrade organic matter in soils rather than weathering of stones. The study underlines the fact that coherent and complementary results may be obtained with both genetic markers used.

1. Introduction

With several tons of dry weight per hectare, fungi represent a significant proportion of soil biomass and biodiversity (Giri et al., 2005b). Several main functional groups can be distinguished among soil fungi, such as the mycorrhizal fungi that mediate adaptation of the vegetation to the soil habitat and saprotrophic fungi, which are involved in element cycles and the formation and transformation of soil organic matter (Giri et al., 2005a). Fungi are ecologically important for lignin and polyphenol degradation because of their ability to produce oxidative enzymes, such as lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), laccase (EC 1.10.3.2) and laccase-like multicopper oxidase (LMCO) (Baldrian, 2006; Chen et al., 2001; Leonowicz et al., 2001). A further fungal group has now been recognized and shown to break down rock, thus contributing to soil formation (Hoffland et al., 2003; Jongmans and van Breemen, 1997; Landeweert et al., 2001; van Breemen et al., 2000; Wollenzien et al., 1995). Stones are common in soils and, therefore, studies comparing the fungal communities found in bulk soil and on stones have the potential to broaden our knowledge of distribution of the fungal communities of the heterogeneous soil environment.

Because of the difficulty in isolating and cultivating most soil fungi, molecular approaches have provided valuable methods for the assessment of their diversity. Techniques based on PCR can target either ribosomal DNA markers (used to resolve the diversity among wide taxonomic units) or functional markers (for the resolution of diversity within groups with a given enzymatic potential) (Anderson and Cairney, 2004; Kelly, 2003). The rDNA fragments, including 18S, 25/28S and the internal transcribed spacer (ITS) region surrounding the 5.8S, have often been used as markers to assess fungal diversity in soils (Manter and Vivanco, 2007; O'Brien et al., 2005; Pritsch et al., 2000; Robinson et al., 2009; Toberman et al., 2008). Because of its high variability and resolution at the species level, the ITS region has become a commonly used marker for the identification of fungal species or for separating operational taxonomic units (OTUs) (O'Brien et al.,

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2005), fungal barcoding (Seifert, 2009) and diversity studies using environmental samples (Martin and Rygiewicz, 2005). In contrast, the use of genes encoding specific enzymes allows the detection of functional groups involved in a particular biogeochemical process (Chen et al., 2001; Zak et al., 2006).

Ecological studies targeting laccase or LMCO encoding genes have shown that soil fungi with this enzymatic potential occur widely, supporting their theoretical role in the primary attack of recalcitrant soil phenolic polymers (e.g. lignin), which is a bottleneck in the soil carbon cycle (Baldrian, 2006; Kellner et al., 2007; Lauber et al., 2009; Luis et al., 2004; Theuerl and Buscot, 2010). Studies of soil diversity have shown that fungi have a heterogeneous spatial distribution horizontally and vertically (Luis et al., 2005). Therefore, in most studies the soil horizons are separated during sampling. However, most procedures involve sources of heterogeneity within each horizon, such as plant debris and stones, being discarded, although these may constitute distinct habitats and therefore, harbour specific parts of the soil biodiversity reservoir.

The aim of the present study was to compare fungal diversity in the bulk soil and stone compartments of spruce and beech forest soils, by using ITS rDNA as a barcode and the functional gene encoding laccase as a molecular marker. In contrast to studies that employ a single genetic marker, we predicted that by using two we would gain a broader perspective of fungal diversity and communities in soils.

2. Materials and methods

2.1. Study site and sample collection

Soil and stone samples were collected from a forest site in the Hainich, the largest closed forest area in Germany, located to the west of Thuringia near the border with Hessen (latitude: 51.2167/N 51°13′0″; longitude: 10.45/E 10°27′0″). The Hainich area is one of three biodiversity exploration areas under investigation by a large interdisciplinary consortium funded by the German Science Foundation (see http://www.biodiversity-exploratories.de). Environmental samples were collected in April 2008 from eight 20 m × 20 m plots, representing a range of four managed forest types, i.e. spruce monoculture, beech forest of a single age class (forest with trees of the same age), beech forest with selected cutting (large timber trees are removed to improve the forest stand) and near natural beech forest. The chemical parameters of the study site soils are given in Table 1.

From each of the eight plots, five soil core samples (8.3 cm in diameter), were taken right down to the parent rock material. The litter and organic soil layers were excluded. As stones (with diameter ≥1 cm) were mainly found in the mineral B-horizon only soil samples of the B-horizon were used for further analyses. For each plot the respective samples of the B-horizons of the cores were combined into a composite sample, mixed and divided into subsamples, whereby stones were separated from the bulk soil. Then all soil and stone samples were sealed in plastic bags, transported in iceboxes to the laboratory and stored at −20 °C whilst awaiting molecular analysis.

Table 1
Forest type, soil type and soil parameters of the study sites. Sites are designated by location (H-Hainich, E-Experimental plot, W-Wald (English - forest)). Soil cores were taken down to the parent rock material.

Plot	Forest type	Soil type	C_{org} [g/kg]	N [g/kg]	pH
HEW1	Spruce forest	Cambisol	27.27	2.70	7.46
HEW3		Luvisol	10.33	1.10	6.25
HEW5	Beech age class	Luvisol	26.26	1.95	7.19
HEW6	forest		5.15	0.68	4.84
HEW7	Beech selection	Luvisol	6.63	0.75	6.21
HEW9	cutting forest		9.75	1.09	5.32
HEW10	Near natural beech	Luvisol	15.04	1.53	6.80
HEW12	forest		5.58	0.69	5.21

2.2. DNA extraction and PCR amplification

Stone surfaces were scraped with a scalpel to obtain powdery samples for subsequent DNA extraction. Genomic DNA was isolated from 0.5 g samples of soil and stone using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories Inc. Carlsbad, CA, USA) following the method recommended by the manufacturer.

The ITS region of ribosomal DNA and the laccase gene were amplified. The ITS fragment of the rRNA gene cluster was amplified with the primer pair ITS1F [5′CTT-GGT-CAT-TTA-GAG-GAA-GTA-A 3′] (Gardes and Bruns, 1993) and ITS4 [5′ TCC-TCC-GCT-TAT-TGA-TAT-GC 3′] (White et al., 1990). The PCR was performed in a total volume of 50 μl reaction mix containing 1 μl DNA template, 0.2 μl Taq DNA polymerase, 5 μl 10xTaq polymerase reaction buffer, 0.8 μl dNTPs (10 mM each) and 1 μl 25 pmol of each of the two primers. The thermal profile involved a touchdown PCR with initial denaturation for 5 min at 94 °C followed by: (1) 10 cycles of 94 °C for 30 s, 60–50 °C 45 s (−1 °C per cycle) and 72 °C for 1 min; and (2) 35 cycles of 94 °C 30 s, 50 °C 45 s and 72 °C 1 min with a final extension step of 7 min. PCR amplification of the laccase gene was performed using the specific basidiomycete laccase degenerate primer pair described by Luis et al. (2004): 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′]. The PCR conditions were as described above except that the thermal profile included a hot start PCR with an initial denaturation step of 5 min at 94 °C before adding the Taq polymerase at 80 °C. The thermal profile was as follows: 38 cycles of the denaturation step of 45 s at 94 °C, hybridization at 52 °C for 40 s and elongation at 72 °C for 45 s with a final extension of 2 min at 72 °C.

A total of eight soil and eight stone samples were analyzed and each sample was amplified in triplicate. Products of PCR were analyzed using a 2% agarose gel and products of the expected size (about 600 bp for the ITS region and 140–290 bp for laccases) from the same sample were pooled and purified using a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany).

2.3. Cloning and sequencing

The purified products of the ITS region and laccase genes were ligated into a pCR4-Topo vector and transformed using TOP10 chemical competent *Escherichia coli* cells according to the instructions provided with the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany). Transformed cells were grown overnight at 37 °C on LB Agar plates with Ampicillin and X-Gal. White colonies were checked for inserts with M13F and M13R primers using a PCR program with an initial cycle of 10 min denaturation at 94 °C, followed by 32 cycles of denaturation (40 s at 94 °C), annealing (30 s at 54 °C), extension (40 s at 72 °C) and a final extension of 4 min at 72 °C. Aliquots of positive PCR products were treated with ExoSAP-IT (USB Europe GmbH, Staufen, Germany) for 15 min at 37 °C to remove unwanted dNTPs, residual single stranded primers and extraneous single stranded DNA produced in the PCR and then incubated at 80 °C for 15 min to inactivate the ExoSAP-IT. At least 48 clones per sample were sequenced. Sequencing was carried out on an ABI PRISM 3100 Genetic Analyzer using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) with M13F as the sequencing primer.