Soil fungal communities along land use gradients of three German biodiversity exploratories: A comparison of classical cloning and sequencing approach with high throughput sequencing

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Abstract
Our research is carried out in the frame of the Biodiversity Exploratory initiative, an interdisciplinary consortium granted by the German Science Foundation (DFG). Our knowledge and view of soil microbial diversity is changed in the last decades due to the advance in the sequencing technology. We test several molecular techniques ranging from classical cloning and sequencing over screening of metagenomic fosmid libraries to high throughput 454 sequencing to investigate soil fungal diversity in forest and grassland ecosystems of the German Biodiversity Exploratories. The results and implications of these comparisons will be presented and discussed.

Key Words
Fungal diversity, metagenomic library, laccase gene

Introduction
The Biodiversity Exploratories is a research project comprising three research sites, called Exploratories, across Germany. The project provides the opportunity to investigate the relationship between biodiversity of different organism groups, the role of land use for biodiversity and the role of biodiversity in ecosystem processes. Each Exploratory comprises grassland and forest ecosystems each with 1000 plots, which are further rescaled to 100 experimental plots and 18 very intensive plots reflecting land use gradients and different land use intensity levels. The Biodiversity Exploratories is a platform for an interdisciplinary research including abiotic and biotic soil ecology projects. Within the biotic soil ecology project consortium our focus is to investigate soil fungi and fungal enzymes using PCR-based, metagenomic and high throughput sequencing techniques.

Among soil microorganisms, fungi play a major role in degradative processes and their ability to produce oxidative enzymes such as laccases (EC 1.10.3.2) and laccase-like multicopper oxidases (LMCO) contributes to high ecological importance especially for lignin and polyphenol degradation (Baldrian 2006; Chen *et al.,* 2001; Leonowicz *et al.,* 2001). Polymerase chain reaction (PCR) based approaches have recently been used to investigate soil fungal diversity and community composition of different ecosystems, which avoids limitations associated with traditional culture-based techniques (e.g. Kirk *et al.,* 2004). Actually two approaches coexist, the use of structural markers on the one hand and functional markers on the other hand. The internal transcribed spacer (ITS) region surrounding the 5.8S ribosomal DNA became the most commonly used structural marker for fungal species identification and diversity studies (Martin and Rygiewicz, 2005). In contrast to studies based on structural markers for the detection of the whole fungal community, the use of functional genes encoding enzymes that catalyze key steps in biochemical pathways allows detection of specific functional groups involved in a particular biogeochemical process (Chen *et al.,* 2001; Zak *et al.,* 2006). Ecological studies using fungal laccase or LMCO genes showed their wide occurrence and role in the primary attack of recalcitrant soil organic matter by breaking down crosslinks and aromatic ring structures (Baldrian, 2006; Kirk and Farrell, 1987). Due to the fact that fungal laccases are widespread exoenzymes with low substrate specificity and broad ecological impact, they have been frequently used as functional marker genes in monitoring diversity of fungi with oxidative potential in different soils (Lauber, 2009; Luis *et al.,* 2004). Despite several advantages of such approaches they also reflect certain limitations and our knowledge about key ecological questions could also be expanded using new molecular techniques like metagenomics and metatranscriptomics, which allows investigating different genes and their products within multispecies communities.

Our study presents one of the first comparison of soil fungal communities revealed by PCR based methods using primer pairs targeting the ITS rDNA fragment as structural marker and fungal laccase genes as functional marker. Additionally, we constructed soil metagenomic fosmid libraries and we will screen them for fungal laccase genes and will also employ a 454 deep sequencing of the fungal ITS rDNA. These datasets will be analysed with soil and vegetation metadata to assess the ecological implication of the fungal species and laccase gene diversity in the functioning of these ecosystems.

Methods

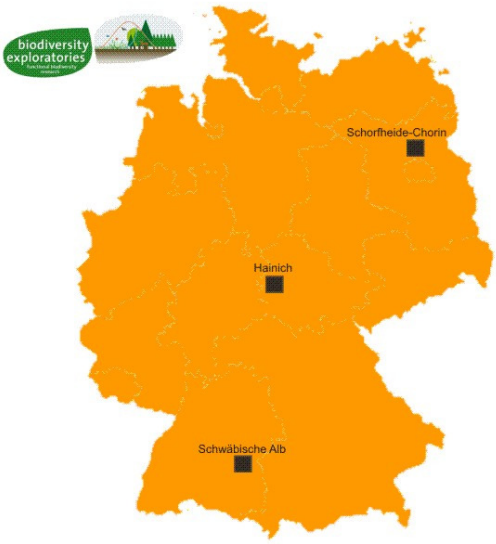
Study site and sample collection

Soil cores were taken in April 2008 from very intensive plots (VIP) of forests and grasslands of the three biodiversity exploratories (http://www.biodiversity-exploratories.de). From each of the plots soil cores (8.3 cm in diameter), excluding the organic soil layer, were taken down to the parent rock material. The respective mineral soil horizons of the cores were combined into a composite sample and stones were separated from the bulk soil. The management and soil types of the plots are given in Figure 1.

Plot	Management	Soil type
SEG1	fertilized meadow (low moor)	Histosol
SEG2		Histosol
SEG3		Histosol
SEG4	mowed pasture (low moor) without fertilization	Histosol
SEG5		Histosol
SEG6		Histosol
SEG7	pasture (low moor)	Histosol
SEG8		Histosol
SEG9		Histosol

Plot	Management	Soil type
HEG1	meadow mowed 3 times per year and fertilized	Cambisol
HEG2		Stagnosol
HEG3		Stagnosol
HEG4	mowed and fertilized pasture with cattle	Stagnosol
HEG5		Stagnosol
HEG6		Stagnosol
HEG7	pasture with cattle and without fertilization	Stagnosol
HEG8		Stagnosol
HEG9		Stagnosol

Plot	Management	Soil type
AEG1	meadow mowed 2-3 times per year and fertilized	Leptosol
AEG2		Leptosol
AEG3		Leptosol
AEG4	mowed and fertilized pasture with cattle and horses	Leptosol
AEG5		Leptosol
AEG6		Leptosol
AEG7	pasture with sheep and without fertilization	Leptosol
AEG8		Leptosol
AEG9		Leptosol



Plot	Management	Soil type
SEW1	Pine monoculture	Arenosol
SEW2		Arenosol
SEW3		Arenosol
SEW4	Pine-Beech forest	Arenosol
SEW5	Beech forest	Arenosol
SEW6		Arenosol
SEW7	Natural beech forest	Arenosol
SEW8		Albeluvisol
SEW9		Arenosol

Plot	Management	Soil type
HEW1	Spruce monoculture	Cambisol
HEW2		Cambisol
HEW3		Luvisol
HEW4	Beech age class forest	Leptosol
HEW5		Cambisol
HEW6		Luvisol
HEW7	Beech selection cutting forest	Luvisol
HEW8		Luvisol
HEW9		Luvisol
HEW10	Natural beech forest	Luvisol
HEW11		Luvisol
HEW12		Luvisol

Plot	Management	Soil type
AEW1	Spruce age class forest	Cambisol
AEW2		Cambisol
AEW3		Cambisol
AEW4	Beech age class forest	Cambisol
AEW5		Cambisol
AEW6		Cambisol
AEW7	Natural beech forest	Cambisol
AEW8		Cambisol
AEW9		Cambisol

Figure 1. The three Biodiversity Exploratories are located at: Schorfheide-Chorin 52° 57′ 0″ N, 13° 37′ 0″ E, Hainich 51° 1′ 17″ N, 10° 30′ 36″ E, Schwäbische Alb 48° 34′ 58.8″ N, 9° 30′ 5.4″ E. Tables show very intensive plots (VIP) and their corresponding management and soil types. Sites are designated by location (S-Schorfheide-Chorin, H-Hainich, A-Schwäbische Alb, E-Experimental plot, W- Forest and G-Grassland).

Classical PCR-cloning-sequencing

Genomic DNA was isolated from 0.5 g of soil and stone samples using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories Inc. Carlsbad, CA, USA) as recommended by the manufacturer. The ITS region of the ribosomal DNA and the laccase gene were amplified with the primer pairs ITS1F and ITS4 (Martin and Rygiewicz 2005) and Cu1F and Cu2R (Luis *et al.,* 2004). The purified products of ITS and laccase genes were ligated into a pCR4-Topo vector and transformed using TOP10 chemical competent *E. coli* cells according to the instructions provided with the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany). Colonies were checked for inserts with M13F and M13R primers. Sequencing was done on an ABI PRISM 3100 Genetic Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) with M13F as sequencing primer. Both ITS and laccase gene sequences were grouped into operational taxonomic units (OTU) using online tool FastGroup II (http://biome.sdsu.edu/fastgroup/fg_tools.htm) at 97% and 99% similarity, respectively, according to Yu *et al.* (2006). The resulting taxonomic units were checked for their identity against the National Centre for Biotechnology Information (NCBI, GenBank) database using the BLAST search algorithm for identification (Altschul *et al.,* 1997).

Metagenomic library construction and screening
Metagenomic libraries were constructed from soil genomic DNA extracts, sheared and gel extracted for fragment length of 35 – 40 Kbp genomic DNA. The DNA fragments were packed in pCC1FOS™ Fosmid vector and titrated to library pools following the CopyControl Fosmid Library Production protocol (http://www.epibio.com). Fosmid library pools are screened by PCR for fungal laccase genes using the primer pair Cu1F/Cu2R. For the isolation of positive clones from complex DNA libraries the method of Hrvatin and Piel (2007) is used.

High throughput amplicon sequencing
The ITS2 fragment of the ITS rDNA will be amplified using tagged fungal specific primers. PCR products will be cleaned and sequenced using the 454 titanium amplicon sequencing kit and the 454 Genome Sequencer (Roche Applied Science).

Results and discussions
Our preliminary data showed that the fungal diversity revealed with structural (primer pair ITS1F/ITS4) and functional marker genes (primer pair Cu1F/Cu2R) differs and considering the effect hierarchy (e.g. forest type, soil type, soil chemical parameters) the two approaches showed different patterns. Additionally we will focus on the interpretation of fungal communities in different habitats (bulk soil and stones). We will present the complete comparative analysis of the three approaches in revealing soil fungal communities. Correlation of the fungal diversity data with the different management levels and environmental variables will be presented and discussed in detail.

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