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Diversity of an ectomycorrhizal fungal community studied by a root tip and total soil DNA approach

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Abstract Molecular methods based on soil DNA extracts are increasingly being used to study the fungal diversity of ectomycorrhizal (EM) fungal communities in soil. Contrary to EM root tip identification, the use of molecular methods enables identification of extramatrical mycelia in soil. To compare fungal diversity as determined by root tip identification and mycelial identification, six soil samples were analysed. Root tips were extracted from the six samples and after amplification, the basidiomycete diversity on the root tips was analysed by denaturing gradient gel electrophoresis (DGGE). The soil from the six samples was sieved, total soil DNA was extracted and after amplification, the basidiomycete diversity in the soil fractions was analysed by DGGE. Fourteen different bands were excised from the DGGE gel and sequenced; fungal taxon names could be assigned to eight bands. Out of a total of 14 fungal taxa detected in soil, 11 fungal taxa were found on root tips, of which seven were EM fungal taxa. To examine whether the sieving treatment would affect EM species diversity, two different sieve mesh sizes were used and in addition, the organic soil fraction was analysed separately. DGGE analysis showed no differences in banding pattern for the different soil fractions. The organic fraction gave the highest DGGE band intensities. This work demonstrates that there is a high correspondence between basidiomycete diversity detected by molecular analysis of root tips and soil samples, irrespective of the soil fraction being analysed.

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Introduction

Ectomycorrhizal (EM) fungi live in symbiosis with mostly trees and woody perennials. About 6,000 species of EM fungi are known to exist, forming species-rich, highly dynamic and complex communities below ground (Taylor 2002). The high species richness and the apparent non-random distribution of species complicate sampling of EM communities to assess species richness. Many EM fungal species can be classified by morphological identification of colonised EM root tips and often the number or percentage of colonised root tips is used as a measure of species abundance (Taylor 2002). For identification of EM fungi, root tips are typically extracted from soil and sorted by morphological criteria. A carefully selected, small portion of these sorted root tips is subsequently used for molecular identification to species level. Often, not more than 2–5% of the total number of sampled root tips is used for identification by molecular methods (e.g. Jonsson et al. 1999; Rosling et al. 2003) and conspicuous types are sometimes considered characteristic enough to be identified without molecular methods (Dahlberg et al. 1997). Problems related to the sorting of fungal morphotypes and root tip sampling issues have been extensively discussed by Horton and Bruns (2001) and Taylor (2002). Potential problems with root tip sampling would be overcome if all sampled EM root tips were included in the molecular analyses. Single root tip DNA extractions are, however, not realistic when analysing thousands of root tips and therefore extracted root tips are sometimes pooled prior to DNA extraction (Zhou and Hogetsu 2002). Detection of a fungal species then depends on its initial quantity and on its DNA extraction and amplification efficiency.

Studies on fungal species diversity are increasingly conducted with molecular techniques and based on the

mycelial presence of fungi in soil or other substrates. Recently, molecular methods based on total soil DNA extracts were used to detect and identify EM mycelium in natural soil (Chen and Cairney 2002; Dickie et al. 2002; Landeweert et al. 2003). These molecular methods enable the identification of fungi in soil without the extraction or cultivation of the organisms themselves. The use of soil DNA extracts to identify EM fungi does not involve morphological sorting of EM root tips and sample bias related to root tip sorting does therefore not occur.

Now that studies of the diversity of EM mycelia are becoming more common, it would be useful to compare fungal diversity determined by molecular mycelia identification to fungal diversity determined by EM root tip identification. To make sure that detected differences in species diversity are not induced by the methods used, the identification methods should be kept as similar as possible in both approaches and root tip sorting should not be applied, as it might induce sampling bias. In addition, the method used to extract root tips from the soil that will be used to detect mycelium should be chosen after careful consideration. Other studies have removed root tips from soil samples by visually checking for the presence of root tips under the dissecting microscope or by sieving the soil before extracting total soil DNA (Chen and Cairney 2002; Dickie et al. 2002; Landeweert et al. 2003). Several sieve mesh sizes have been used to separate soil from EM root tips but it is unknown whether and how these different sieve mesh sizes influence the outcome of the study. EM root tips or even fragments of hyphal mantles that end up in the soil fraction will co-amplify and likely increase the number of species recorded in soil DNA extracts.

In the present study the species diversity of an EM fungal community in a *Pinus sylvestris* stand in the Netherlands was studied by molecular EM root tip identification as well as by molecular EM mycelia identification. To avoid methodological differences between the two approaches, the same molecular identification techniques were applied to the root tip samples and the mycelial soil samples and the EM root tips were not grouped in morphotypes. DNA was extracted from pooled root tips and soil and internal transcribed spacer (ITS) fragments were amplified with basidiomycete-specific primers. Denaturing gradient gel electrophoresis (DGGE) was used to analyse band diversity from the root tip and soil samples. A selection of the DGGE bands was excised from the gel and sequenced in order to identify the fungal taxa present. Furthermore, DGGE band diversity was analysed in two different fractions of sieved soil and, separately, in the organic soil fraction. The aim of this study was to determine whether the detected basidiomycete diversity would differ according to the root tip or soil DNA approach used and whether the diversity would depend on the soil fraction analysed.

Materials and methods

Field site and soil sampling

In June 2002 soil cores were taken in a forest dominated by Scots pine (*Pinus sylvestris* L.) in a drift sand area in the central part of The Netherlands (Hulshorsterzand, 52°21′N, 5°44′E). In 1994 the litter layer had been removed from this stand and in 1998 sporocarp production of EM fungi had been monitored (Schmidt 1999). In 2002, 8 years after sod-cutting, a very thin litter layer had redeveloped. Six 2- to 3-year-old pine seedlings were selected in an area of 15 m^2 and soil cores (6 cm diameter) were taken that included the seedling. The soil was sampled to a depth of 10 cm and after transport to the laboratory samples were frozen at −70°C until processing. After defrosting, roots and soil were separated on a 2-mm sieve. Root tips were removed from the sieve and were pooled per sample in a micro-centrifuge tube, cleaned by several distilled water washes and ground with a tube mortar in sodium-phosphate buffer. From the six sieved soil samples (2-mm fraction), sub samples of 50 g were air dried and sieved again through a 1-mm and 0.3-mm sieve. From three sieved soil samples, the 1-mm soil fraction was further separated into a light organic fraction and a heavy sand fraction by careful shaking. Of these three soil samples, DNA was extracted from the 1-mm total soil fraction, 1-mm organic fraction, 1-mm sand fraction and the 0.3-mm total fraction. A selection of shrivelled, dry and clearly dead root tips was collected from the 1-mm fraction of one soil sample, cleaned by several distilled water washes and ground with a tube mortar in sodium-phosphate buffer.

DNA extraction and amplification

DNA was extracted from 0.5 g of all soil fractions and 50 μl of the root tip suspensions, using a Fast DNA spin kit for soil (BIO101; Qbiogene, Vista, Calif.) according to the manufacturer's instructions. For the DGGE procedure, ITS sequences were amplified on a PCR Express thermocycler (Thermo Hybaid, Ashford, UK) with the primers ITS1F and ITS4B-GC (Gardes and Bruns 1993). ITS4B-GC is similar to ITS4B and includes a GC clamp on the 5′ end to stabilise the melting behaviour of the DNA fragments. The PCR reaction mix consisted of 1 μl of 50× diluted template DNA, 39 μl sterile Ultrapure water, 5 μl of 10× PCR buffer 2 (Roche, Basel), 200 μM of each deoxynucleoside triphosphate, 200 nM of each primer and 0.5 μl Expand enzyme mix (Roche). The following thermocycling pattern was used: 94°C for 3 min (one cycle); 94°C for 1 min, 50°C for 1 min and 72°C for 3 min (35 cycles); and 72°C for 10 min (one cycle).

Denaturing gradient gel electrophoresis

The presence of successfully amplified PCR products (ITS1F-ITS4B primer pair) was confirmed by analysing 2 μl of PCR product on a 1% agarose gel, stained with SYBRGold (Molecular Probes, Leiden, the Netherlands) and visualised using a Syngene CCD camera system (Genesnap 4.00.00, copyright Synoptics 1993–2000, Syngene, Cambridge, UK). Ten microlitres of the obtained PCR products of the root tip and 1 mm soil samples and 5 μl of the obtained PCR products of the different soil fractions were subsequently analysed by DGGE. Gel electrophoresis was performed on an 8% acrylamide gel containing a linear denaturant gradient from 20% to 60% of ureum and formamide. The 100% solution contained 4 ml acrylamide, 0.3 ml 50× TAE buffer, 6 ml formamide and 6.3 g ureum, supplemented with distilled water to a final volume of 15 ml. Gels were run overnight at a constant temperature of 60°C, at 80 V for 18 h. After completion of the electrophoresis, gels were stained with SYBRGold and documented on a Syngene CCD camera system.

Sequencing of DGGE bands

Bands were excised from the DGGE gel and allowed to stand in 50 μl distilled water for 30 min. After mixing, 1 μl of this solution was added to the PCR mix, containing 39 μl sterile Ultrapure water, 5 μl of 10× PCR buffer 2 (Roche), 200 μM of each deoxynucleoside triphosphate, 200 nM of each primer (ITS1F-ITS4B primer pair) and 0.5 μl Expand enzyme mix (Roche). DNA from the DGGE bands was re-amplified whereby the following thermocycling pattern was used: 94°C for 3 min (one cycle); 94°C for 1 min, 50°C for 1 min and 72°C for 3 min (35 cycles); and 72°C for 10 min (one cycle). Obtained PCR products were analysed on a 1% agarose gel. Bands of the right size (0.7 kb) were purified with the QIAquick purification kit (Qiagen, Valencia, Calif.) according to the manufacturer's protocol. The 0.7-kb fragments were sequenced in one direction on an ABI377 DNA sequencer by cycle sequencing using the dye terminator system and the ITS1F primer. To identify the obtained sequences, all sequences were compared to sequences from the GenBank database, making use of the Blast program from the National Center for Biotechnology Information (NCBI). Subsequently, alignments were made with all obtained sequences and a selection of the best matching sequences obtained from GenBank, using ClustalW (DNA Star program). Finally, phylogenetic trees were constructed by neighbour-joining using the Treecon program (version 1.36, Y. van der Peer). Based on the clusters that were formed sequences were sorted into groups and assigned taxon names. The obtained nucleotide sequences have been submitted to NCBI and have been given accession nos. AY288092–AY288099.

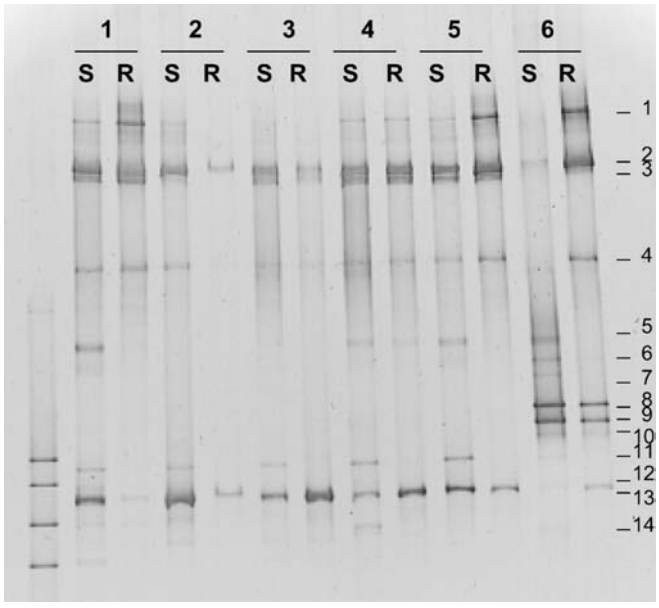

Fig. 1 Denaturing gradient gel electrophoresis (DGGE) of amplified internal transcribed spacer (ITS)-rDNA fragments representing the basidiomycete community in soils (*S*) and on root tips (*R*) of six samples (*1–6*). *Bands 1*, *2* , *4* , *5* , *8* , *9* and *13* could be matched to database sequences and were identified as ectomycorrhizal (EM) fungal taxa. *Band 11* was identified as a non-mycorrhizal fungal taxon

Results

Soil samples versus root tip samples

DNA extracted from dry, dead root tips could not be sufficiently amplified and produced a hardly visible product on a 1% agarose gel (data not shown). DNA was successfully extracted from the six root tip and soil samples (1-mm fraction) and fungal DNA was amplified with the ITS1F-ITS4B primer pair. The DGGE banding pattern revealed a total of 14 clear, different bands (Fig. 1). Out of this total of 14 bands, 11 bands were detected in the root tip as well as the soil samples, whereas three bands were exclusively found in the soil. Band no. 11 was found in every soil sample, but in none of the root tip samples. For five out of six samples (root tips as well as soil) the banding patterns were quite similar, whereas one sample (sample 6) revealed another, different banding pattern. Two bands (band nos. 8 and 9) were exclusively found in sample 6. Sequencing of re-amplified bands was not in all cases successful and out of 39 sequencing reactions, 19 sequences were obtained that could be used for sequence analysis (Table 1). Sequence

Table 1 Ectomycorrhizal fungal taxa detected on root tips and/or in soil (*x*) after denaturing gradient gel electrophoresis analysis, with their best Blast match

Band	Identity	Blast match	Similarity (%)	Soil	Root	No. of bands sequenced[a]	No. of sequences[b]
1	*Rhizopogon* sp. 1	AY254859	98%	x	x	5	3
2	*Rhizopogon* sp. 2	AY254859	98%	x	x	5	4
3	Unknown 1			x	x	0	0
4	*Rhizopogon* sp. 3	AY254859	99%	x	x	6	1
5	*Cortinarius* sp.	AY254860	98%	x	x	4	1
6	Unknown 2			x	x	1	0
7	Unknown 3			x	x	0	0
8	Uncultured *Clavulina* sp. 1	AY254863	98%	x	x	2	2
9	Uncultured *Clavulina* sp. 2	AY254863	98%	x	x	2	2
10	Unknown 4			x	x	0	0
11	*Cryptococcus* sp.	AY254865	97%	x		5	3
12	Unknown 5			x		0	0
13	*Lactarius* sp.	AY254871	99%	x	x	9	3
14	Unknown 6			x		0	0
Total						39	19

[a] No. of excised bands (from different lanes) that were sequenced
[b] No. of obtained sequences that could be used for sequence analyses

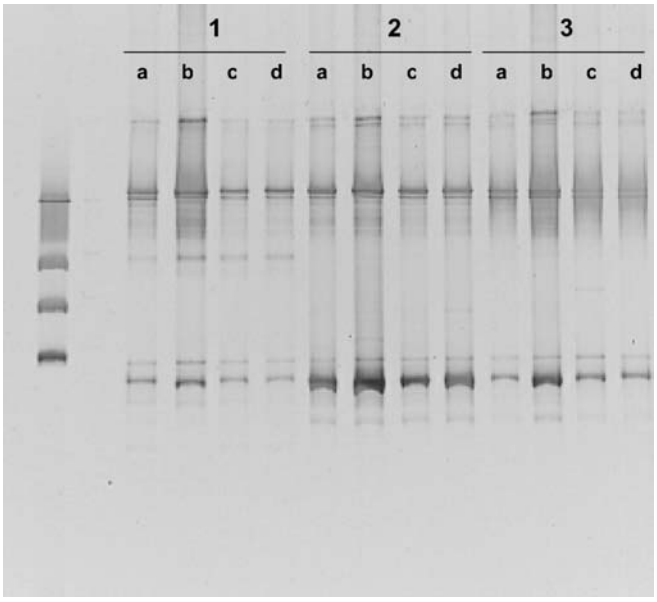

Fig. 2 DGGE of amplified ITS-rDNA fragments representing the basidiomycete community in four different soil fractions of three soil samples (*1–3*). *Lanes a* 1-mm total soil fraction, *lanes b* 1-mm organic soil fraction, *lanes c* 1-mm sand fraction, *lanes d* 0.3-mm total soil fraction. For abbreviations, see Fig. 1

analysis through Blast matching and phylogenetic tree construction revealed several *Rhizopogon* spp., a *Cortinarius* sp., a *Lactarius* sp. and an uncultured *Clavulina* sp. on the root tips as well as in the soil (Table 1). Sequences obtained from six bands did not satisfactorily match with anything in the database and remained unknown. Two of these unknowns were exclusively detected in the soil.

Different soil fractions

DNA was successfully extracted from three soil samples that had passed through a 1-mm and 0.3-mm sieve and also from the separated 0.3-mm organic and sand fraction. DNA was successfully amplified with the ITS1F-ITS4B primer pair. DGGE analysis revealed that within each sample, all fractions showed a similar banding pattern with varying band intensities between soil fractions (Fig. 2). In all three samples, the 1-mm organic fraction had the highest band intensity.

Discussion

Fungal community analysis by DGGE showed that fungal diversity detected in soil was a little higher (14 bands) than the fungal diversity detected on root tips (11 bands). Out of a total of 14 bands that were distinguished, eight were assigned a taxon name, including seven taxa that were identified as EM fungal taxa. Sequence analysis revealed that three different bands could be placed within the *Rhizopogon* clade, as they matched well with the sequence of a *R. luteolus* fruitbody (AY254879) found at a site nearby (Smit et al. 2003). An even better match was found with a *Rhizopogon* sequence (AY254859) obtained directly from soil from the same nearby site (Smit et al. 2003). It is clear, however, that the taxonomy of *R. luteolus* and *R. corsicus* has not yet fully been sorted out (Johannesson and Martin 1999). One obtained sequence was found to match well with a sequence obtained from a *Cortinarius fusisporus* fruitbody (AY254877) and a *Cortinarius* sequence (AY254860) obtained directly from soil from a nearby site (Smit et al. 2003). One sequence matched well with the sequence of a *Lactarius hepaticus* fruitbody and a sequence of an uncultured *Lactarius* sp. from the site nearby (Smit et al. 2003). Two bands matched best with an uncultured *Clavulina* sp. (AY254863) sequence (Smit et al. 2003), belonging to the Cantharellales. One band matched best with an uncultured cf. *Cryptococcus* (AY254865) sequence, obtained from a soil sample from the nearby site (Smit et al. 2003). In general, all detected EM fungal taxa had been reported in 1998 as fruitbodies and were now, in all cases, detected in soil as well as on root tips. Only the *Cryptococcus* sp. was found exclusively in the soil. Other studies on soil DNA extracts using fungal-specific primers have found several non-mycorrhizal fungal species in soil (Chen and Cairney 2002; Landeweert et al. 2003; Smit et al. 2003) and also detected *Cryptococcus* sp. (Landeweert et al. 2003; Smit et al. 2003).

No fungal species were exclusively detected on root tips, indicating that the mycelia of most species occur in high enough quantities in soil to be detected by the molecular methods. When root tips are not analysed, EM fungal species that do not form an extensive amount of extramatrical hyphae may remain undetected when using the soil DNA approach. However, *Russula* sp. and *Lactarius* sp., both having smooth hyphal mantles and lacking large amounts of emanating hyphae (Agerer 2001), have been detected in several molecular studies on soil fungal diversity (Chen and Cairney 2002; Dickie et al. 2002; Landeweert et al. 2003; Smit et al. 2003).

Except for differences in hyphal presence, the difference in the number of detected fungal species in the soil and root tip samples could also have been caused by the fact that the soil samples contained other fungal material than just hyphae. The use of total DNA extracts to identify fungi does not distinguish between DNA extracted from hyphae, spores or sclerotia. Not many fungi belonging to the Basidiomycota form sclerotia and co-extraction of DNA from sclerotia should therefore not pose a problem when analysing an EM fungal community. Spores, however, may persist on and in the soil, especially in autumn. The samples analysed in this study originated from a sod-cut forest plot, where fruitbodies of >20 EM fungal species had been collected earlier (Schmidt 1999). Fourteen basidiomycete taxa were detected in the soil DNA extracts from the topsoil of this plot. If extensive co-extraction of DNA from spores had occurred in this particular case, it would be reasonable to assume that >14 fungal species would have been

detected. Although co-extraction of DNA originating from spores does not seem to be of importance in this study, it might have been if, for example, soil cores would have been taken in autumn when many species sporulate.

Detection of single fungal species in soil through total soil DNA and PCR application has proven a useful method to rapidly and accurately identify fungal pathogens in soils and pre-symptomatic crops (Cullen et al. 2001). In contrast to the detection of fungal pathogens, accurate detection of EM mycelium in soil involves total removal of (fragments of) EM root tips. Separation of root tips from soil samples is often done by sieving the samples (Guidot et al. 2002; Landeweert et al. 2003) or by the removal of root tips with a pair of forceps under a dissecting microscope (Chen and Cairney 2002; Dickie et al. 2002; Landeweert et al. 2003). In some cases complete hyphal strands and mycelium have been extracted from soil (Zhou et al. 2001; H. Wallander, personal communication). To determine whether species richness depends on the soil fraction used, DNA was extracted from several soil fractions in this study. The 1-mm and the 0.3-mm sand fraction gave equal DGGE banding patterns, indicating that these two sieve mesh sizes were equally effective in separating root tips from sand. Although sieving with a 1-mm sieve proved sufficient in this study, careful examination is needed in each individual case to guarantee that no root tips or mantle fragments remain behind in the soil being extracted. While remaining root tip fragments may not be a huge concern when using mycelial data for identification purposes, it could lead to a distorted view when using molecular data to quantify fungal presence in soil.

Analysis of the 1-mm and 0.3-mm soil fractions showed that the highest band intensities were obtained for the 1-mm organic fraction. This 1-mm organic fraction consisted of fragments of dry and dark organic matter, including fragments of shrivelled EM root tips. Extracted DNA from a carefully rinsed selection of these shrivelled, dry root tips could not be amplified and it is therefore unlikely that DNA from the dead root tips caused high band intensities. It has been shown before that DGGE band intensities can provide an indication of fungal abundance in the template DNA mixture (Brüggemann et al. 2000) and in this case, providing a nutrient-rich substrate, the dead root tips might have been covered by fungal hyphae. It has been shown before that patches of organic matter are preferentially colonised by hyphae (Bending and Read 1995) and it can therefore be assumed that the intense DGGE band intensities from the organic matter fraction reflected DNA extracted from active mycelia.

The present study demonstrated that species diversities determined by the root tip or total soil DNA approach show high correspondence. The diversity obtained with the total soil DNA approach was even more comprehensive than the diversity obtained with the root tip analysis. In this study, total soil DNA analysis was therefore as robust as root tip analysis, indicating its potential for future diversity studies. Sampling of EM root tips is typically inadequate when attempting to obtain a true picture of species richness (Horton and Bruns 2001) and the use of soil DNA extracts can reduce several sample biases related to root tip sampling. The use of molecular identification methods to study EM hyphae in soil will become more common in the near future, as these techniques permit in situ testing of hypotheses on resource partitioning and niche differentiation of individual fungi (Dickie et al. 2002). Methods based on soil DNA extracts might be a good alternative when analysing species diversity of an EM fungal community, but it depends on the nature of the study whether the answers needed are best provided by a root tip or mycelial approach.

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