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Spatial distribution of the below-ground mycelia of an ectomycorrhizal fungus inferred from specific quantification of its DNA in soil samples

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Abstract

In natural forest ecosystems several ectomycorrhizal fungal species cohabit on host plant root systems. To evaluate the ecological and functional impact of each species, it is necessary to appreciate the distribution and abundance of its mycelia in the soil. We developed a competitive PCR (cPCR) method for the basidiomycete *Hebeloma cylindrosporum* that allows quantification of its DNA in complex DNA mixtures extracted directly from soil samples. The target sequence chosen for the cPCR analysis was a 533-bp fragment of the nuclear ribosomal intergenic spacer, amplified using two species-specific primers. The detection threshold of the cPCR protocol developed was 0.03 pg of genomic DNA. This method was applied to soil samples collected from beneath and at various distances from a group of fruit bodies in a *Pinus pinaster* forest stand. The results revealed that *H. cylindrosporum* below-ground biomass was concentrated directly underneath the fruit bodies or very close to them, while no DNA of this species could be detected in soil samples collected at more than 50 cm away. In the vicinity of fruit bodies, *H. cylindrosporum* soil DNA concentration varied considerably (between 10 and 0.07 ng g soil^{-1}) and decreased sharply with increased distance from the fruit bodies. This work demonstrates the potential of competitive quantitative PCR for the study of the distribution, abundance and persistence of the mycelia of an ectomycorrhizal fungal species in soil.

Keywords: Below-ground mycelium; Soil DNA; DNA quantification; Competitive polymerase chain reaction; Ectomycorrhizal fungus; *Hebeloma cylindrosporum*

1. Introduction

Although many fungal species can be grown in vitro, most of them cannot be isolated from complex environments such as soil due to the slow growth rate of their mycelia and the absence of truly selective media. This particularly applies to those species that do not form asexual spores including numerous saprophytic basidiomycetes, the ectomycorrhizal fungal symbionts and several soil-borne pathogens (e.g. *Rhizoctonia* sp.). These species are therefore untraceable over the mycelial stage of their life cycle. Their presence in the environment can only be detected through the fruit bodies that some of these species occasionally form, or through the symptoms they produce on compatible host plants (pathogenic species), or through the mycorrhizas they form on root systems.

If we refer to the symbiotic ectomycorrhizal (ECM) species, recent studies, based on the sampling of root tips and the subsequent identification of the fungal partner at the species level by combining morpho- and molecular typing methods, have greatly contributed to our understanding of the spatial composition and temporal dynamic of ECM fungal communities [1–9]. None of these studies, however, has so far taken into account the extramatrical mycelia, which explore the soil scavenging nutrients, a fraction of which is transferred to the host plant. Identification of mycelia at the species level and quantification of their biomass in soils are necessary in order to evaluate the ecological and functional impact of a given species in its natural environment. Specifically, species within a commu-

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nity have been ranked according to their abundance on root systems [2,3,5,7–9], and the same ranking would not necessarily be obtained if the total biomass of each species in the soil (mycorrhizas+extramatrical mycelium) was used as the classification criterion. It is indeed known that ECM species differ in the amount of extramatrical mycelium they produce. This ranges from species producing 'smooth' mycorrhizas from which no or few hyphae emanate, to species differentiating hyphal cords or rhizomorphs that explore the soil several centimeters away from the root system [10]. It can also be asked if the clumped distribution of mycorrhizas or fruit bodies of many of the species reflects a clumped distribution of their mycelia. Connected to this latter question is the survival potential in a truly saprophytic stage of mycelia of symbiotic ECM species. In the absence of specific detection tools for the mycelia in the soil, some of these questions have so far been tentatively addressed using either single species microcosms in the laboratory [11], or by considering the whole ECM community without any distinction between species [12].

Species-specific detection and biomass quantification of microorganisms present in complex environments (e.g. soil, sediments or activated sludge) without prior isolation have been achieved through PCR-based methods that identify DNA extracted directly from environmental samples. These methods have been widely applied in bacterial studies (e.g. [13–16]), but rarely for soil-borne fungal species. The use of soil DNA in mycology has essentially been restricted to the qualitative analysis of communities [17–22], or to the detection without quantification of specific pathogens or saprophytic species by PCR using species-specific primers [23–25]. This latter method can be made quantitative by including a known amount of a competitor sequence in the PCR mix [26]. A competitor sequence is a plasmid-cloned DNA fragment that is homologous to the genomic sequence to be quantified except in that it differs from the genomic sequence by, for example, an insertion or a deletion so that both PCR-amplified sequences can easily be distinguished in gel electrophoresis and quantified separately. As both sequences are amplified simultaneously with the same primer pair, the final amplification ratio of the two sequences is proportional to the initial (known) amount of competitor sequence and unknown (to be quantified) amount of target sequence added to the PCR mix. For the purposes of detecting a sequence in an environmental sample, competitive PCR (cPCR) presents an advantage over traditional 'single target PCR' in that 'false negatives' (absence of amplification product resulting from such as the presence of inhibitory molecules) can be immediately identified as the added competitor will not be amplified either. So far, cPCR has not been used to track mycelia of ECM species in forest soils. It has, however, been successfully used to quantify the growth of a genetically modified strain of the mycoparasitic fungus *Trichoderma virens* [27] and the growth of the nematophagous fungus *Verticillium chlamydosporium* [28] inoculated in a soil.

In this study, we developed a cPCR method for the detection and quantification of DNA of the ECM agaric fungus *Hebeloma cylindrosporum* growing naturally in soil, in order to delimit the boundaries of the territories occupied by its mycelia, and to estimate below-ground biomass distribution within these territories. In our previous studies we have defined the spatial structures and temporal dynamics of populations of this species based on the analysis of both fruit bodies and mycorrhizas [29–31]. We have shown that mycorrhizas of this species are not found more than 10–20 cm away from fruit bodies [31]. In this study, we specifically address whether the pattern of soil mycelium abundance matches the pattern observed for mycorrhizas. We discuss our results in terms of sampling design and consider the limitations of cPCR when used for the quantification of the biomass of filamentous fungi in soil.

2. Materials and methods

2.1. Soil characteristics and sample collection

Soil samples were collected in November 2000 in a 60-year-old *Pinus pinaster* forest stand located 50 km west of Bordeaux in south-west France (TVF site in [31]). This site is characterized by a sandy soil (98–99% non-calcareous sand, 0.5–3% organic matter, pH (in water) about 5.7) with no obvious horizons as a result of local forest management, which prevents litter and humus accumulation.

To quantify soil mycelial distribution around fruit bodies of *H. cylindrosporum*, a group of 24 fruit bodies (approximately 0.2 m^2) was selected and a regular, grid-based sampling design was used for the collection of soil cores either directly underneath the patch of fruit bodies or outside of it (Fig. 2A). Fruit bodies were mapped and distances between the center of each soil core and the fruit bodies were measured. As most of the fine roots were distributed in the first 10 cm of soil, we collected $10 \times 10 \times 10$ cm soil cores. Cores of soil were grossly sieved to remove pieces of long root and large organic debris, thoroughly homogenized by hand and for each homogenized core three 0.5 g sub-samples were taken and stored at −70°C until DNA extraction.

2.2. Soil DNA extraction and quantification

DNA was extracted from the 0.5 g soil samples using the commercial Fast DNA SPIN Kit for Soil (Bio101) whose principle is outlined below. Cells were mechanically broken for 1 min at room temperature in the presence of supplied buffer and glass beads in a bead-beater (1800 strokes min^{-1}, mikro-dismembrator U, Braun Biotech). Elimination of contaminants and DNA purification are based on the selective adsorption of DNA onto a silica-

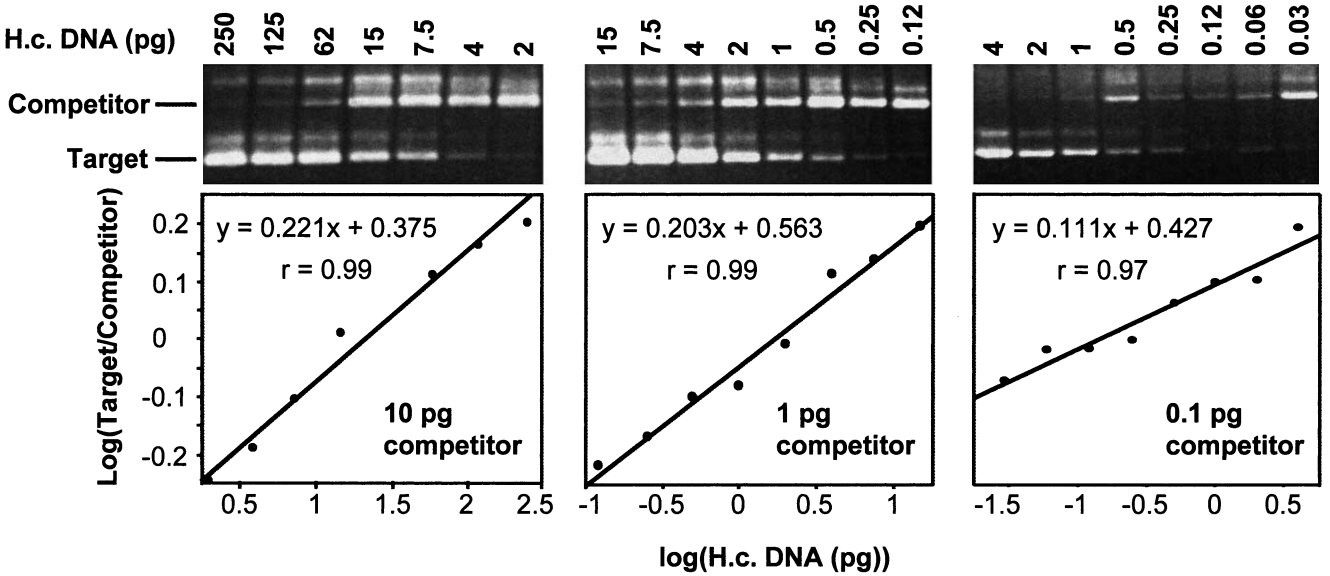


Fig. 1. Standard curves for the quantification of *H. cylindrosporum* DNA by cPCR constructed using 10, 1 or 0.1 pg of plasmid-cloned competitor and different amounts of purified *H. cylindrosporum* (H.c.) genomic DNA (from 0.03 to 250 pg). Top panel: Illustration of SYBR Gold-stained agarose gels of the different amplification products obtained after 35 PCR cycles. Each cPCR reaction was repeated three times and each point of the curves shown below corresponds to the log of the mean value of the ratios amplified target/amplified competitor obtained from the separate quantification by image analysis of the amplification products.

based binding matrix. DNA was eluted using 50 μl H_2O and a final 45 μl solution was routinely obtained and stored at −20°C. DNA concentration and integrity were estimated by running 2 μl of each extract in parallel with known amounts of calf thymus DNA in 0.8% agarose gels in 0.5×TBE (0.04 M Tris–HCl, 0.04 M boric acid, 1 mM Na_2EDTA, pH 8.0). Images of the ethidium bromide-stained gels were captured and digitized using a video camera and the gel Doc 1000 system (Bio-Rad). The concentration of high molecular mass DNA in the samples was estimated using the Molecular Analyst Software (Bio-Rad) by comparison to a standard curve constructed using the known amounts of calf thymus DNA run in parallel.

2.3. Competitor and primer specificity

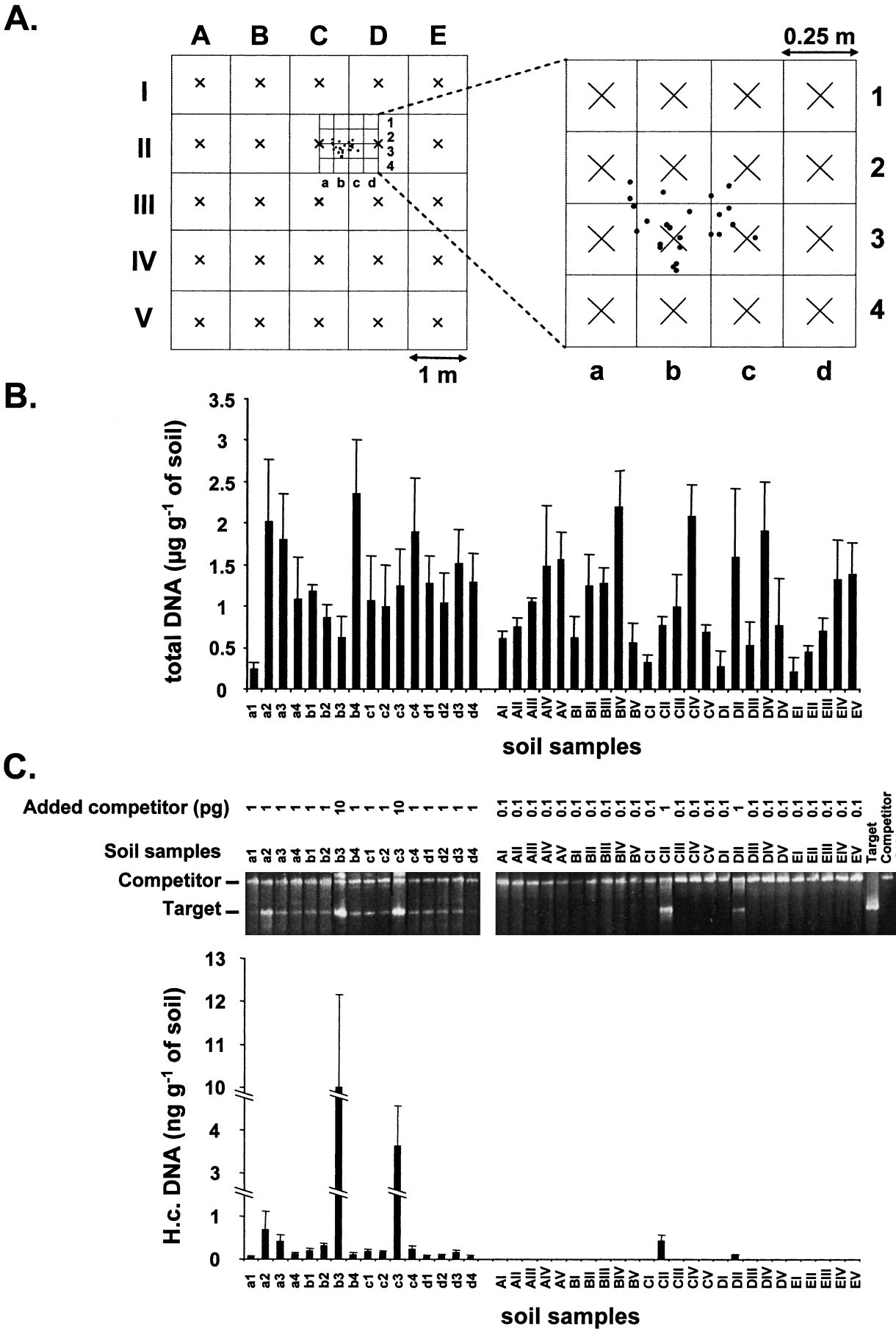
The target sequence chosen for the quantification of *H. cylindrosporum* DNA was a 533-bp fragment located within the nuclear rDNA untranscribed intergenic spacer 2 (IGS2), which spans between the 3′-end of the 5S and the 5′-end of the 18S rDNA genes. This fragment is included within the cloned IGS2-b fragment ([32], sequence accession No. AJ006147) and was amplified using primers IGS2A (5′-TTAACTAGCCCAACCCCACTTT-3′) [32] and IGS2B (5′-CCTTTCAGACTTTCCCTCCATA-3′).

To create a competitor, we inserted into the unique *Sac*I site of the cloned IGS2-b sequence a 225-bp *Sac*I-*Sac*I fragment originating from the *H. cylindrosporum nar1* gene [33]. Plasmids were propagated in *Escherichia coli* DH5α and plasmid DNA purified using commercial columns (Qiagen). The primer pair IGS2A+IGS2B amplifies both target and competitor which can easily be distinguished from each other in agarose gels owing to the 225-bp size difference between them (Fig. 1).

Because IGS2 has only been partially sequenced from one *H. cylindrosporum* strain [32] and from very few other fungal species, the specificity of the IGS2A+IGS2B primer pair towards *H. cylindrosporum* DNA was tested by PCR using genomic DNA extracted from (i) 24 *H. cylindrosporum* haploid strains representative of the 24 IGS RFLP alleles identified in this species [32], (ii) 10 different *Hebeloma* species representative of at least five of the eight clades defined in this genus by Aanen et al. [34] and (iii) 21 other saprophytic or ECM basidiomycete species whose fruit bodies were collected within the study site. Strains of *Hebeloma* are maintained in the culture collection of the laboratory. Mycelia were grown as previously described [29] and DNA was extracted from lyophilized mycelia or fruit bodies according to van Kan et al. [35]. PCR conditions were as described below for the quantification of *H. cylindrosporum* in soil DNA extracts, except that 20 ng of genomic DNA were used.

2.4. Competitive PCR and quantification

To minimize pipetting inaccuracies, PCR mixes were prepared using 6 μl of a 1/6 dilution of soil DNA extract and 6 μl of a 1/6 dilution of plasmid-cloned competitor solution at 10, 1 or 0.1 pg μl^{-1}. In addition to the target and competitor DNAs, the 25-μl PCR mixes contained 400 nM of each primer (IGS2A and IGS2B), 200 nM of each dNTP, 1.5 mM $MgCl_2$, 0.5% (w/v) bovine serum albumin, the buffer supplied by the manufacturer (Gib-


A.
A B C D E
I II III IV V
1 2 3 4
a b c d
1 m
0.25 m
1 2 3 4
a b c d
B.
total DNA (μg g-1 of soil)
3.5 3 2.5 2 1.5 1 0.5 0
a1 a2 a3 a4 b1 b2 b3 b4 c1 c2 c3 c4 d1 d2 d3 d4 AI AII AIII AIV AV BI BII BIII BIV BV CI CII CIII CIV CV DI DII DIII DIV DV EI EII EIII EIV EV
soil samples
C.
Added competitor (pg)
1 1 1 1 1 1 10 1 1 1 10 1 1 1 1 1
0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 1 0.1 0.1 0.1 1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1
Soil samples
a1 a2 a3 a4 b1 b2 b3 b4 c1 c2 c3 c4 d1 d2 d3 d4 AI AII AIII AIV AV BI BII BIII BIV BV CI CII CIII CIV CV DI DII DIII DIV DV EI EII EIII EIV EV Target Competitor -DNA
Competitor
Target
H.c. DNA (ng g-1 of soil)
13 12 11 10 4 3 1 0
a1 a2 a3 a4 b1 b2 b3 b4 c1 c2 c3 c4 d1 d2 d3 d4 AI AII AIII AIV AV BI BII BIII BIV BV CI CII CIII CIV CV DI DII DIII DIV DV EI EII EIII EIV EV
soil samples

co-BRL) and 1 U of *Taq* DNA polymerase. Amplification conditions were as follows: initial denaturation at 95°C for 3 min and then 35 cycles of 95°C for 2 min, 55°C for 1 min, and 72°C for 3 min. Reactions were completed with a 7-min extension step at 72°C. Control reaction mixtures without DNA were always run in parallel to ensure that there was no contaminating DNA in the solutions. The amplification products (25 μl) were separated by electrophoresis in 2.5% agarose gels (1.66% Multipurpose agarose and 0.83% Standard agarose (Q.BIOgene)) in 0.5×TBE buffer for 4 h at 120 V. The gels were stained for 1 h with SYBR Gold™ according to the manufacturer's instructions (Molecular Probes) and destained in water for 3 h. The relative amounts of amplified products (target and competitor DNA) were quantified by image analysis as described above for the soil DNA extracts. To determine the quantity of target DNA initially present in the PCR mix before amplification, the log value of the ratio amplified target/amplified competitor was reported on the standard curve corresponding to the quantity of competitor added to the PCR mix (Fig. 1).

2.5. Construction of the standard curves

Standard curves were constructed by amplifying different known amounts of target genomic DNA with a constant amount of competitor. A standard curve was obtained by plotting the log values of the amplification ratios of target DNA/competitor against the log values of the target DNA (pg genomic DNA) added to the PCR mix before amplification (Fig. 1). As preliminary experiments showed that the different soil samples contained very different amounts of target DNA, three different standard curves were constructed, each with one of the following amounts of plasmid-cloned competitor: 10, 1 and 0.1 pg. When combined, these three curves allowed the quantification of between 0.03 and 250 pg genomic DNA added to the cPCR reaction (Fig. 1). The *H. cylindrosporum* DNA used to construct the standard curves was extracted from freeze-dried mycelium of the haploid strain h1 by the method of Van Kan et al. [35], further purified using the Fast DNA SPIN Kit for Soil and quantified by optical density at 260 nm. Similar standard curves were obtained using, as target, *H. cylindrosporum*-purified genomic DNA mixed into a γ-ray-sterilized soil sample prior to purification by the Fast DNA SPIN Kit for Soil, thus showing that any co-extracted contaminating chemicals had no significant effect on the accuracy of the cPCR.

2.6. Other amplified sequences

As controls, additional DNA sequences were amplified from either fruit body or soil DNA. These sequences and the corresponding primers used for their amplification were (i) the fungal ribosomal ITS with primers 2234C (5′-GTTTCCGTAGGTGAACCTGC-3′) and 3126T (5′-ATATGCTTAAGTTCAGCGGGT-3′) [36], (ii) a 248-bp fragment of the *H. cylindrosporum* nitrite reductase gene (*nir1*, sequence accession No. AJ238664) with primers NIR1U (5′-GGGGTACCGGGCGGGAGGTCATCT-TC-3′) and NIR1L (5′-CGGAATTCCACACGGTTATA-GGCGAAG-3′) and (iii) a 945-bp fragment of the *nir1* gene with primers NIR1U and NIR2L (5′-GCTCTA-GAGTTGGTGAGAACCTGAACGC-3′). PCR protocols were identical to the one used for quantification by cPCR except that 37 cycles were performed on 1 μl of soil DNA solution or 20 ng of fruit body DNA.

2.7. Statistics

PCR reactions for the construction of standard curves were repeated three times. For each soil extract, cPCR amplifications were performed three times. Results are presented as mean values ± S.E.

3. Results

3.1. Amplified sequences and primer specificity

In preliminary experiments, different primer pairs amplifying DNA sequences of various lengths and present in one or multiple copies per genome, were tested on 16 soil DNA extracts containing *H. cylindrosporum* DNA (samples a1 to d4, Fig. 2). Reliable amplification of single copy genes was problematic. Reliable but low yield amplification of a 248-bp fragment of the *nir1* gene could be obtained with primers NIR1U/NIR1L while, under our PCR conditions, a 945-bp fragment of this gene could not be amplified from any of the 16 samples tested using primers NIR1U/NIR2L (data not shown).

In contrast to the single copy sequences, strong and reproducible amplifications were obtained for the 533-bp

←

Fig. 2. Quantification of *H. cylindrosporum* genomic DNA by cPCR in soil samples collected from beneath and around a group of fruit bodies. A: Location of the 41 10×10×10 cm soil samples (represented as crosses drawn to scale) and of the 24 fruit bodies (represented by dots). B: Amounts of total DNA extracted from each soil sample: mean values ± S.E. (three different 0.5-g sub-samples were extracted for each soil sample). C: Detection and quantification of *H. cylindrosporum* (H.c.) genomic DNA in the different samples. The SYBR Gold-stained gel illustrates one of the cPCR reactions carried out on the different soil extracts with different amounts of competitor DNA; lane '−DNA' contains a control PCR without added DNA. The quantities of *H. cylindrosporum* DNA initially present in the soil samples were determined from the ratio amplified target/amplified competitor according to the amounts of competitor DNA added to the cPCR mix. Mean values ± S.E. are given in the graph below (three different 0.5-g sub-samples were extracted for each soil sample and three cPCRs were performed for each soil extract).

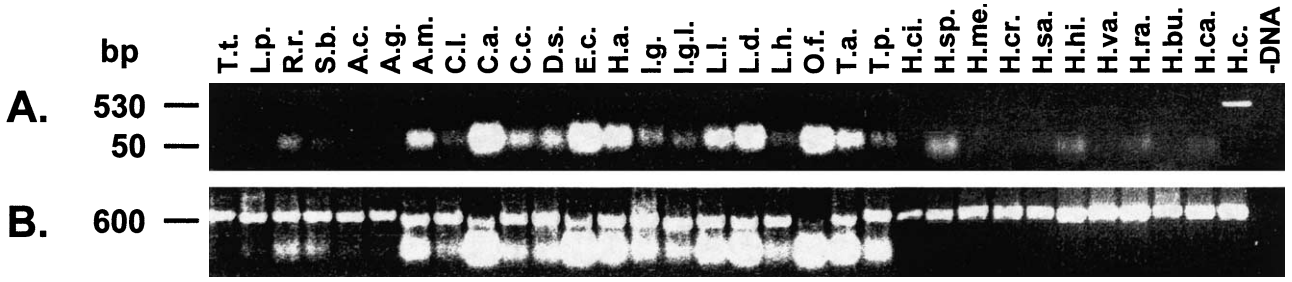


Fig. 3. Specificity of the PCR primers IGS2A/IGS2B towards *H. cylindrosporum* DNA. A: The primer pair IGS2A/IGS2B failed to amplify any sequence from the genomic DNA extracted from 31 basidiomycete species. The small, prominent bands present in some tracks are primer artefacts. B: Control positive amplifications of the ITS sequence using primers 2234C and 3126T. T.t., *Thelephora terrestris*; L.p., *Lycoperdon perlatum*; R.r., *Rhizopogon roseolus*; S.b., *Suillus bovinus*; A.c., *Amanita citrina*; A.g., *Amanita gemmata*; A.m., *Amanita muscaria*; C.l., *Cantharellus lutescens*; C.a., *Cortinarius albaviolaceus*; C.c., *Cortinarius cinnamomeolutescens*; D.s., *Dermocybe semisanguineus*; E.c., *Entoloma cetratum*; H.a., *Hygrophoropsis aurantiaca*; I.g., *Inocybe gausapata*; I.g.l., *Inocybe geophylla* var. *lilacina*; L.l., *Laccaria laccata*; L.d., *Lactarius deliciosus*; L.h., *Lactarius hepaticus*, O.f., *Omphalina fibula*; T.a., *Tricholoma auratum*; T.p., *Tricholoma pessundatum*; H.ci., *Hebeloma cistophilum*; H.sp., *Hebeloma spoliatum*; H.me., *Hebeloma mesophaeum*; H.cr., *Hebeloma crustuliniforme*; H.sa., *Hebeloma sarcophyllum*; H.hi., *Hebeloma hiemale*; H.va., *Hebeloma vaccinum*; H.ra., *Hebeloma radicosum*; H.bu., *Hebeloma bulbiferum*; H.ca., *Hebeloma cavipes*; H.c., *Hebeloma cylindrosporum*; −DNA, control reaction without added DNA.

multi-copy IGS2 fragment using primers IGS2A and IGS2B. Because the IGS sequence was known to be highly variable in *H. cylindrosporum* [32], we verified that primers IGS2A/IGS2B amplified a single 533-bp DNA fragment from the 24 known *H. cylindrosporum* IGS-RFLP alleles of unknown sequences (data not shown). Primer specificity towards *H. cylindrosporum* was tested using DNA extracted from 31 basidiomycete species, including 10 different *Hebeloma* species. In no case was an amplification product obtained, while all control amplifications of the ITS sequences using primers 2234C/3126T were positive (Fig. 3).

3.2. Optimization of the cPCR conditions

Two parameters that can affect quantification by cPCR were evaluated: the number of PCR cycles and the total amount of DNA present in the PCR mix. The amplification ratio of target over competitor DNA remained constant between 25 and 40 cycles; a higher ratio (i.e. preferential amplification of the smaller, target DNA fragment) was obtained with 45 cycles. Although the mean value of the ratio remained constant for 40 cycles, this number of cycles resulted in variable amplification yields between repeats. Therefore, 35 cycles was chosen for quantification as this gave the highest and most consistent amplification yields. To estimate the influence of the amount of foreign DNA on cPCR we added between 0.1 and 10 ng of calf thymus DNA to a PCR reaction containing 1 pg of competitor DNA and 1 μl of a soil DNA extract containing an unknown amount of *H. cylindrosporum* DNA. An amplification ratio of 0.73 was obtained whatever the amount of foreign DNA added. This result indicated that cPCR could be conducted on a fixed volume of soil DNA extract instead of on a fixed amount of DNA which would have necessitated making dilutions leading to a decrease in signal intensity.

Using the PCR conditions described above, bands additional to those from the competitor and target DNAs were resolved following electrophoresis (Fig. 1). Such bands, commonly observed in cPCR (e.g. [13]), are usually interpreted as being produced by heteroduplex DNA resulting from hybridizations between the homologous target and competitor sequences. The presence of such bands did not affect quantification because (i) both target and competitor were equally affected and (ii) we were able to separate them by electrophoresis from both target and competitor amplification products (Fig. 1). As a result, the different and overlapping standard curves used for quantification were linear over a wide range of target DNA concentrations (from 0.03 to 250 pg μl^{-1}, Fig. 1). The detection threshold of target DNA corresponded to about 0.03 pg of genomic DNA using 0.1 pg of competitor DNA. Reliable quantification could not be achieved when 0.01 pg of competitor DNA was used.

3.3. DNA extraction from soil samples and quantification of H. cylindrosporum DNA

The use of the Fast DNA SPIN Kit for soil (Bio101) enabled purification of high molecular mass, undegraded DNA from the 0.5-g samples collected in the field. The 41 soil cores analyzed (Fig. 2A) contained variable amounts of extractable DNA with a mean value of 1.12 μg g $soil^{-1}$ (minimum: 0.24 μg g^{-1}, maximum: 2.35 μg g^{-1}, S.E. = 88) (Fig. 2B). Similar mean values and range of variation in the amount of extractable DNA were obtained for soil cores collected from beneath or near the patch of fruit bodies (inner 1 m^2 sampling grid, 16 samples a1 to d4, Fig. 2A) and those collected farther away from the fruit bodies (outer 25 m^2 grid, 25 samples AI to EV, Fig. 2A).

H. cylindrosporum DNA was detected in the 18 soil samples collected less than 50 cm away from the fruit bodies (i.e. the 16 samples of the inner sampling grid plus samples CII and DII taken at the edges of this grid), but not in any of the 23 samples collected farther

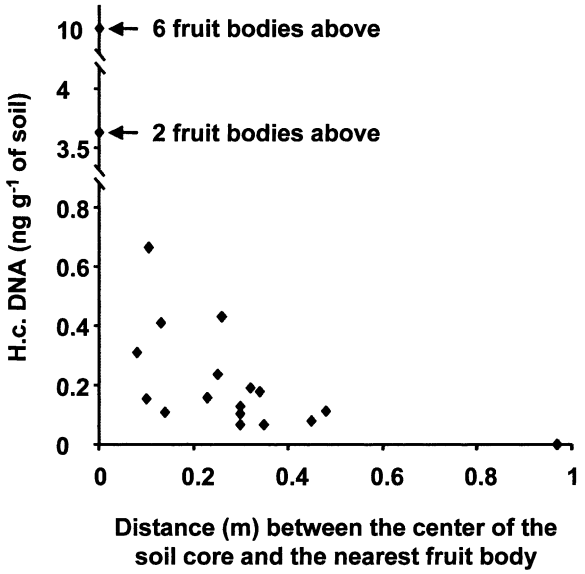


Fig. 4. Negative correlation between the amount of *H. cylindrosporum* (H.c.) DNA detected in the soil and the distance of the soil core to the nearest fruit body.

away (Fig. 2C). In these 23 samples, amplification of the target sequence could not be achieved even in the absence of added competitor. The successful amplification of the competitor molecule (initial amount 0.1 pg) when added to these DNA samples demonstrated that amplification was not being prevented by any inhibitory compounds present in the samples (Fig. 2C).

In the 18 soil samples in which target DNA was detected (represented by $18 \times 3 = 54$ different DNA extracts), quantification could be performed using 1 or even 10 pg of competitor (Fig. 2C). For some of the samples (e.g. sample a1 or d4), a more accurate quantification was nevertheless achieved using 0.1 pg of competitor. The amount of *H. cylindrosporum* DNA detected varied considerably (between 0.07 and 10 ng g soil^{-1}), with the two highest values corresponding to the two soil cores collected immediately underneath the fruit bodies (samples b3 and c3, Fig. 2A). There was a negative correlation between the concentration of *H. cylindrosporum* DNA in soil and the distance between the center of the soil sample and the nearest fruit body (Spearman rank coefficient $\rho = -0.7$, significance level of $P < 0.001$) (Fig. 4).

4. Discussion

4.1. cPCR enables the specific detection and quantification of the mycelium of an ECM fungus in a forest soil

Despite its role in plant nutrition and fruit body formation, the extramatrical mycelium of ECM fungi has never been quantified together with the corresponding mycorrhizas and fruit bodies. In this study, DNA was extracted from soil samples using a commercial extraction kit previously used and evaluated for the study of soil bacterial and fungal DNA [17,18,37]. This method enabled the reproducible isolation of *H. cylindrosporum* DNA from a forest soil. Although the soil we used in this study was sandy and contained a low level of organic matter (two factors that could explain its low DNA content), this extraction kit is known to perform well with more complex soils such as cultivated ones [18,37]. It might, however, be necessary to modify and improve the DNA extraction conditions for the more conventional forest soils that are characterized by a high content of organic matter [38–41].

When attempting to quantify low amounts of a specific DNA molecule of prokaryotic or eukaryotic origin in bulk soil DNA extracts, cPCR appeared much more sensitive when applied to a DNA sequence present in multiple copies per genome. This was the reason why a fragment of the nuclear rDNA repeat unit was selected in this study. Although the number of rDNA repeated units per haploid genome has not been quantified in the case of *H. cylindrosporum*, for the basidiomycete *Coprinus cinereus* it has been estimated to be in the range 60–90 [42], and to be about 120 in the basidiomycete *Schizophyllum commune* [43]. Similarly, insertion sequences (IS elements) have been used for cPCR quantification of bacterial cells [44]. In contrast to the IGS sequence, numerous fungal ITS1–5.8S–ITS2 sequences (which span between the 3′-end of the 18S and the 5′-end of the 26S rDNA genes) are now available in the databases. In the future, these sequences could be targetted to design primers for cPCR which could first be tested in silico for specificity. As intraspecific variations in the ITS sequences have, however, been reported, the specificity of these primers would still need to be tested experimentally on different isolates of the species studied. The cPCR protocol we developed had a detection threshold of about 0.03 pg µl^{-1} of genomic DNA added to the PCR mixture. This corresponds to about 3 pg of *H. cylindrosporum* DNA present in 1 g of soil (a 45-µl DNA solution was obtained from 0.5 g of soil). It may be possible to lower this threshold using, for example, high yield *Taq* DNA polymerases or more sensitive detection methods such as 'real time quantification of PCR products' (using, for example, the ABI Prism 7700 Sequence Detection System). However, in a study on *Mycobacterium tuberculosis*, comparison of the ABI 7700 system and cPCR for quantification of its DNA showed that the two PCR systems were comparable with respect to reproducibility and accuracy [44].

In similar studies carried out on bacteria, the DNA quantities measured are usually converted into cell numbers and the results expressed in terms of the number of cells per gram of soil [14,15]. If such a conversion factor can easily be obtained and appears meaningful in the case of unicellular microorganisms, this is not the case for filamentous organisms. Not only do many fungal species have variable numbers of nuclei per cell, but even for the strictly dikaryotic cells of field isolates of *H. cylindro-*

sporum, cell length and volume vary considerably within a thallus. In most studies, fungal biomass in complex environments is usually evaluated by measuring the total hyphal length, or by assaying specific molecules such as chitin or ergosterol (e.g. [45]). For the purposes of comparing the spatial variations of a single species biomass, expressing the results in terms of the amount of DNA per gram of soil may be sufficient. This was the main purpose of the work undertaken with *H. cylindrosporum*. However, if the purpose of the study is to compare the relative amounts of mycelia of several species occurring together in soil, the establishment of conversion factors between DNA and biomass would appear necessary.

4.2. The use of cPCR for the study of H. cylindrosporum mycelia abundance

When cPCR was used to estimate the abundance of *H. cylindrosporum* DNA underneath and around a patch of its fruit bodies, it revealed that the below-ground biomass of this species was concentrated directly underneath the fruit bodies or very close to them, while no DNA could be detected in soil samples collected more than 50 cm away. These results are in agreement with those obtained from the analysis of mycorrhizas [31]. However, compared to the analysis of individual mycorrhiza, the cPCR method presents the following advantages. Firstly, despite the initial methodological developments, once running, cPCR allows the rapid analysis of many different soil samples, compared to the analysis of mycorrhizas which necessitates the laborious sampling of mycorrhizal short roots under the microscope and their separate analysis. Secondly, for the purpose of quantifying the relative abundance of a mycorrhizal species in a soil sample, the use of cPCR is more straightforward compared to the estimation of the relative abundance of mycorrhizas formed by a single species. Thirdly, cPCR quantification takes into account the extraradical mycelium. In addition, the detection of soil mycelia by cPCR seems to be more sensitive than the detection of mycorrhizas. Indeed, in the present study we detected the mycelia of *H. cylindrosporum* at more than 40 cm away from the fruit bodies whereas, in a previous study, mycorrhizas could not be found at more than 10–20 cm distance [31]. In the case of *H. cylindrosporum*, which produces undifferentiated (not aggregated) hyphae emanating from the mycorrhizas, it is not possible at this stage to conclude whether the mycelium detected 50 cm away from the fruit bodies is connected to mycorrhizas located at less than 20 cm from the fruit bodies or, alternatively, if a low density of *H. cylindrosporum* mycorrhizas exists at a distance of 50 cm from the fruit bodies.

The results obtained on the spatial distribution of mycelia of *H. cylindrosporum* in soil further indicate that some ECM species have a discontinuous and aggregated distribution in forest stands. This patchy distribution of mycelia in forest stands could be a response to the heterogeneous nature of the environment, the mycelia proliferating only when they encounter nutrient-rich patches. Spatial heterogeneity had also been estimated from the analyses of mycorrhiza distribution which showed that most species typically occurred in less than 10% of the soil cores sampled in most forest stands (reviewed in [46]). The occurrence of the fungal mycelia at such a fine scale of below-ground patchiness makes a sampling design that gets a true picture of ECM species richness and abundance a challenge (see also [46]). According to our results on *H. cylindrosporum*, which show that the biomass of a species can vary by a factor of 10–100 between two soil samples collected at less than 50 cm from each other, the sampling design of ECM fungal mycelia could be optimized by either collecting soil cores far bigger than the 1-dm^3 ones used in the current study, or by pooling several small soil cores collected in a cell whose size could be of about 1 m^2 in the case of *H. cylindrosporum*.

4.3. Conclusions

This study represents the first report of quantification of the extramatrical mycelial DNA of an ECM fungus naturally growing in soil. The experimental scheme described should be applicable to any ECM species in return for an initial development (construction of the competitor and of standard curves) providing useful information on the distribution, abundance and persistence of different fungal mycelia in soil. The analysis of soil mycelia of the mycorrhizal fungi in parallel with the analysis of mycorrhizal root tips could enable better monitoring and ranking of species according to their total biomass. Compared to the number of mycorrhizas, mycelial biomass will more adequately depict the biological and ecological activity of each species in a community.

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References

[1] Bidartondo, M., Kretzer, A.M. and Bruns, T.D. (2000) High root concentration and uneven ectomycorrhizal diversity near *Sarcodes sanguinea* (Ericaceae): a cheater that stimulates its victims? Am. J. Bot. 87, 1783–1788.

[2] Dahlberg, A., Jonsson, L. and Nylund, J.R. (1997) Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in south Sweden. Can. J. Bot. 75, 1323–1335.

[3] Gardes, M. and Bruns, T.D. (1996) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. Can. J. Bot. 74, 1572–1583.
[4] Gehring, C.A., Theimer, T.C., Whitham, T.G. and Keim, P. (1998) Ectomycorrhizal fungal community structure of pinyon pines growing in two environmental extremes. Ecology 79, 1562–1572.
[5] Grogan, P., Baar, J. and Bruns, T.D. (2000) Below-ground ectomycorrhizal community structure in a recently burned bishop pine forest. J. Ecol. 88, 1051–1062.
[6] Jonsson, L., Dahlberg, A., Nilsson, M.C., Zackrisson, O. and Kårén, O. (1999) Ectomycorrhizal fungal communities in late-successional Swedish boreal forests, and their composition following wildfire. Mol. Ecol. 8, 205–215.
[7] Jonsson, T., Kokalj, S., Finlay, R. and Erland, S. (1999) Ectomycorrhizal community structure in a limed spruce forest. Mycol. Res. 103, 501–508.
[8] Stendell, E.R., Horton, T.R. and Bruns, T.D. (1999) Early effects of prescribed fire on the structure of the ectomycorrhizal fungus community in a Sierra Nevada ponderosa pine forest. Mycol. Res. 103, 1353–1359.
[9] Taylor, D.L. and Bruns, T.D. (1999) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. Mol. Ecol. 8, 1837–1850.
[10] Agerer, R. (2001) Exploration types of ectomycorrhizae. A proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. Mycorrhiza 11, 107–114.
[11] Brulé, C., Frey-Klett, P., Pierrat, J.C., Courrier, S., Gérard, F., Lemoine, M.C., Rousselet, J.L., Sommer, G. and Garbaye, J. (2001) Survival in the soil of the ectomycorrhizal fungus *Laccaria bicolor* and the effects of a mycorrhiza helper *Pseudomonas fluorescens*. Soil Biol. Biochem. 33, 1683–1694.
[12] Wallander, H., Nilsson, L.O., Hagerberg, D. and Bååth, E. (2001) Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. New Phytol. 151, 753–760.
[13] Lee, S.Y., Bollinger, J., Bezdicek, D. and Ogram, A. (1996) Estimation of the abundance of an uncultured soil bacterial strain by a competitive quantitative PCR method. Appl. Environ. Microbiol. 62, 3787–3793.
[14] Lleó, M.M., Tafi, M.C., Signoretto, C., Dal Cero, C. and Canepari, P. (1999) Competitive polymerase chain reaction for quantification of nonculturable *Enterococcus faecalis* cells in lake water. FEMS Microbiol. Ecol. 30, 345–353.
[15] Phillips, C.J., Paul, E.A. and Prosser, J.I. (2000) Quantitative analysis of ammonia oxidising bacteria using competitive PCR. FEMS Microbiol. Ecol. 32, 167–175.
[16] Van Elsas, J.D., Rosado, A.S., Wolters, A.C., Moore, E. and Karlson, U. (1998) Quantitative detection of *Sphingomonas chlorophenolica* in soil via competitive polymerase chain reaction. J. Appl. Microbiol. 85, 463–471.
[17] Borneman, J. and Hartin, R.J. (2000) PCR primers that amplify fungal rRNA genes from environmental samples. Appl. Environ. Microbiol. 66, 4356–4360.
[18] Lowell, J.L. and Klein, D.A. (2001) Comparative single-strand conformation polymorphism (SSCP) and microscopy-based analysis of nitrogen cultivation interactive effects on the fungal community of a semiarid steppe soil. FEMS Microbiol. Ecol. 36, 85–92.
[19] Pennanen, T., Paavolainen, L. and Hantula, J. (2001) Rapid PCR-based method for the direct analysis of fungal communities in complex environmental samples. Soil Biol. Biochem. 33, 697–699.
[20] Smit, E., Leeflang, P., Glandorf, B., Van Elsas, J.D. and Wernars, K. (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Appl. Environ. Microbiol. 65, 2614–2621.
[21] Van Elsas, J.D., Duarte, G.F., Keijzer-Wolters, A. and Smit, E. (2000) Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. J. Microbiol. Methods 43, 133–151.
[22] Viaud, M., Pasquier, A. and Brygoo, Y. (2000) Diversity of soil fungi studied by PCR-RFLP of ITS. Mycol. Res. 104, 1027–1032.
[23] Errampalli, D., Saunders, J. and Cullen, D. (2001) A PCR-based method for detection of potato pathogen, *Helminthosporium solani*, in silver scurf infected tuber tissue and soils. J. Microbiol. Methods 44, 59–68.
[24] Leclerc-Potvin, C., Balmas, V., Charest, P.M. and Jabaji-Hare, S. (1999) Development of reliable molecular markers to detect non-pathogenic binucleate *Rhizoctonia* isolates (AG-G) using PCR. Mycol. Res. 103, 1165–1172.
[25] Nechwatal, J., Schlenzig, A., Jung, T., Cooke, D.E.L., Duncan, J.M. and Obwald, W.F. (2001) A combination of baiting and PCR techniques for the detection of *Phytophthora quercina* and *P. citricola* in soil samples from oak stands. For. Pathol. 31, 85–97.
[26] Zimmermann, K. and Mannhalter, J.W. (1996) Technical aspects of quantitative competitive PCR. Biol. Tech. 21, 268–279.
[27] Baek, J.M. and Kenerley, C.M. (1998) Detection and enumeration of a genetically modified fungus in soil environments by quantitative competitive polymerase chain reaction. FEMS Microbiol. Ecol. 25, 419–428.
[28] Mauchline, T.H., Kerry, B.R. and Hirsch, P.R. (2002) Quantification in soil and the rhizosphere of the nematophagous fungus *Verticillium chlamydosporium* by competitive PCR and comparison with selective plating. Appl. Environ. Microbiol. 68, 1846–1853.
[29] Gryta, H., Debaud, J.C., Effosse, A., Gay, G. and Marmeisse, R. (1997) Fine-scale structure of populations of the ectomycorrhizal fungus *Hebeloma cylindrosporum* in coastal sand dune forest ecosystems. Mol. Ecol. 6, 353–364.
[30] Gryta, H., Debaud, J.C. and Marmeisse, R. (2000) Population dynamics of the symbiotic mushroom *Hebeloma cylindrosporum*: Mycelial persistence and inbreeding. Heredity 84, 294–302.
[31] Guidot, A., Debaud, J.C. and Marmeisse, R. (2001) Correspondence between genetic diversity and spatial distribution of above- and below-ground populations of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Mol. Ecol. 10, 1121–1131.
[32] Guidot, A., Lumini, E., Debaud, J.C. and Marmeisse, R. (1999) The nuclear ribosomal DNA intergenic spacer as a target sequence to study intraspecific diversity of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* directly on *Pinus* root systems. Appl. Environ. Microbiol. 65, 903–909.
[33] Jargeat, P., Gay, G., Debaud, J.C. and Marmeisse, R. (2000) Transcription of a nitrate reductase gene isolated from the symbiotic basidiomycete fungus *Hebeloma cylindrosporum* does not require induction by nitrate. Mol. Gen. Genet. 263, 948–956.
[34] Aanen, D.K., Kuyper, T.W., Boekhout, T. and Hoekstra, R.F. (2000) Phylogenetic relationships in the genus *Hebeloma* based on ITS1 and 2 sequences, with special emphasis on the *Hebeloma crustuliniforme* complex. Mycologia 92, 269–281.
[35] Van Kan, J.A.L., Van den Ackerveken, G.F.J.M. and De Wit, P.J.G.M. (1991) Cloning and characterization of the cDNA of avirulence *avr* 9 of the fungal pathogen *Cladosporium fulvum*, the causal agent of tomato leaf mold. Mol. Plant-Microbe Interact. 4, 52–59.
[36] Sequerra, J., Marmeisse, R., Valla, G., Normand, P., Capellano, A. and Moiroud, A. (1997) Taxonomic position and intraspecific variability of the nodule forming *Penicillium nodositatum* inferred from RFLP analysis of the ribosomal intergenic spacer and random amplified polymorphic DNA. Mycol. Res. 101, 465–472.
[37] Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J.C., Soulas, G. and Catroux, G. (2001) DNA extraction from soils: old bias for new microbial diversity analysis methods. Appl. Environ. Microbiol. 67, 2354–2359.

[38] Bahnweg, G., Sculze, S., Möller, E.M., Rosenbrock, H., Langebartels, C. and Sandermann, H. (1998) DNA isolation from recalcitrant materials such as tree roots, bark, and forest soil for the detection of fungal pathogens by polymerase chain reaction. Anal. Biochem. 262, 79–82.

[39] Cullen, D.W. and Hirsch, P.R. (1998) Simple and rapid method for direct extraction of microbial DNA from soil for PCR. Soil Biol. Biochem. 30, 983–993.

[40] Kuske, C.R., Banton, K.L., Adorada, D.L., Stark, P.C., Hill, K.K. and Jackson, P.J. (1998) Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. Appl. Environ. Microbiol. 64, 2463–2472.

[41] Miller, D.N. (2001) Evaluation of gel filtration resins for the removal of PCR-inhibitory substances from soils and sediments. J. Microbiol. Methods 44, 49–58.

[42] Cassidy, J.R., Moore, D., Lu, B.C. and Pukkila, P.J. (1984) Unusual organization and lack of recombination in the ribosomal RNA genes of *Coprinus cinereus*. Curr. Genet. 8, 607–613.

[43] Dons, J.J.M. and Wessels, J.G.H. (1980) Sequence organization of the nuclear DNA of *Schizophyllum commune*. Biochim. Biophys. Acta 607, 385–396.

[44] Desjardin, L.E., Chen, Y., Perkins, M.D., Teixeira, L., Cave, M.D. and Eisenach, D. (1998) Comparison of the ABI 7700 System (TaqMan) and competitive PCR for quantification of *IS6110* DNA in sputum during treatment of tuberculosis. J. Clin. Microbiol. 36, 1964–1968.

[45] Manter, D.K., Kelsey, R.G. and Stone, J.K. (2001) Quantification of *Phaeocryptopus gaeumannii* colonization in Douglas-fir needles by ergosterol analysis. For. Pathol. 31, 229–240.

[46] Horton, T.R. and Bruns, T.D. (2001) The molecular revolution in the ectomycorrhizal ecology: peeking into the black-box. Mol. Ecol. 10, 1855–1871.