Mycorrhiza (2003) 13:159–165
DOI 10.1007/s00572-002-0212-7

ORIGINAL PAPER

Roland Schubert · Stefan Raidl · Rita Funk · Günther Bahnweg · Gerhard Müller-Starck · Reinhard Agerer

Quantitative detection of agar-cultivated and rhizotron-grown *Piloderma croceum* Erikss. & Hjortst. by ITS1-based fluorescent PCR

Received: 26 March 2002 / Accepted: 19 November 2002 / Published online: 15 January 2003

Abstract A real-time quantitative TaqMan-PCR was established for the absolute quantification of extramatrical hyphal biomass of the ectomycorrhizal fungus *Piloderma croceum* in pure cultures as well as in rhizotron samples with non-sterile peat substrate. After cloning and sequencing of internal transcribed spacer (ITS) sequences ITS1/ITS2 and the 5.8S rRNA gene from several fungi, including *Tomentellopsis submollis*, *Paxillus involutus*, and *Cortinarius obtusus*, species-specific primers and a dual-labelled fluorogenic probe were designed for *Piloderma croceum*. The dynamic range of the TaqMan assay spans seven orders of magnitude, producing an online-detectable fluorescence signal during the cycling run that is directly related to the starting number of ITS copies present. To test the confidence of the PCR-based quantification results, the hyphal length of *Piloderma croceum* was counted under the microscope to determine the recovery from two defined but different amounts of agar-cultivated mycelia. Inspection of the registered Ct values (defined as that cycle number at which a statistically significant increase in the reporter fluorescence can first be detected) in a 10-fold dilution series of template DNA represents a suitable and stringent quality control standard for exclusion of false PCR-based quantification results. The fast real-time PCR approach enables high throughput of samples, making this method well suited for quantitative analysis of ectomycorrhizal fungi in communities of natural and artificial ecosystems, so long as applicable DNA extraction protocols exist for different types of soil.

Keywords Ectomycorrhiza · Biomass quantification · Fluorescent polymerase chain reaction · Internal transcribed spacer sequences · TaqMan technology

R. Schubert · G. Müller-Starck
Department of Plant Sciences, Section of Forest Genetics, Weihenstephan Center of Life and Food Sciences, Technical University of Munich, Am Hochanger 13, 85354 Freising, Germany

S. Raidl (✉) · R. Funk · R. Agerer
Department of Biology I and GeoBio-CenterLMU, Biodiversity Research: Systematic Mycology, Ludwig-Maximilians-University Munich, Menzinger Strasse 67, 80638 Munich, Germany
e-mail: s.raidl@botanik.biologie.uni-muenchen.de
Tel.: +49-89-17861236
Fax: +49-89-172638

G. Bahnweg
GSF-National Research Centre for Environment and Health, Institute of Biochemical Plant Pathology, Ingolstädter Landstrasse 1, 85764 Neuherberg, Germany

R. Schubert
Department of Mathematics and Natural Sciences, Biotechnology Group, University of Applied Sciences Zittau/Görlitz, Külzufer 2, 02763 Zittau, Germany

Introduction

Ectomycorrhizal root-colonising fungi commonly form a more or less compact hyphal mantle around the root tips of their host plants (Agerer 1987–1998; Smith and Read 1997). In most species of ectomycorrhizal fungi, contact with the substrate as well as nutrient uptake is performed by the emanating extramatrical mycelium, which consists of single hyphae or strands of bundled hyphae, the so-called rhizomorphs (Allen 1991; Read 1992). Depending on the amount and distribution as well as the organisation of the extramatrical mycelia, several exploration types can be distinguished that strongly differ in structure, extension, and biomass (Raidl 1997; Agerer 2001).

To estimate mycelial biomass of ectomycorrhizal fungi, lengths or weights of hyphae or mycelia can be measured directly (Fogel and Hunt 1978; Bååth and Söderström 1979; Kunzweiler and Kottke 1986; Ineichen et al. 1995). Moreover, numerous chemical and physiological methods are available to quantify fungal mycelia (summarised by Weaver et al. 1999). However, in the investigation of the dynamics of ectomycorrhizal fungal

communities, species-specific quantification of mycelia represents a technological bottleneck, especially when large numbers of rhizosphere samples have to be analysed. The available methods fail when no distinct morphological characters or chemical constituents exist to sort different ectomycorrhizal species or when such structures are not produced at times and places chosen to analyse a particular fungus.

By focusing on the enzymatic amplification of the internal transcribed spacer (ITS) regions ITS1 and ITS2 of the ribosomal gene cluster, polymerase chain reaction (PCR) has significantly improved the DNA-based detection and identification of ectomycorrhizal symbioses (reviewed by Buscot et al. 2000; Horton and Bruns 2001). Based on two oligonucleotide primers, PCR enables the in vitro synthesis of a diagnostic DNA marker fragment specific for the micro-organism studied. Furthermore, the PCR product obtained can be quantified by image analysis subsequent to its electrophoretic detection (e.g. Schubert et al. 1999). Real-time PCR has been developed to replace end-point measurements for accurate and reliable product quantification and avoid post-PCR sample handling (Heid et al. 1996).

To study possible competition in response to nutrient amendment and elevated CO_2 between four selected symbiotic fungi associated with roots of Norway spruce in nature (Matyssek et al. 2002; for detailed information see http://www.forst.tu-muenchen.de/EXT/SFB607/sfb607.htm), we applied TaqMan PCR technology (Heid et al. 1996; Schild 1996). This report concerns the cloning of ITS sequences from the fungi *Piloderma croceum* Erikss. & Hjortst., synonymous to *Piloderma fallax* (Libert) Stalpers, *Tomentellopsis submollis* (Svrcek) Hjortstam, *Paxillus involutus* (Batsch) Fr., and *Cortinarius obtusus* (Fr.) Fr. Following the design of species-specific primers, real-time PCR technology was applied to quantify rhizotron-grown mycelium of *Piloderma croceum*. The findings were validated by measuring the total hyphal length by direct microscopy, an established and reliable tool for hyphal biomass quantification.

Materials and methods

Fungal isolates and culture conditions

Fruitbodies of *Cortinarius obtusus* (SR 929) and *Paxillus involutus* (SR 788), as well as ectomycorrhiza of *Piloderma croceum* (SR 430) and *Tomentellopsis submollis* (SR 806), were collected in Bavarian forests and identified according to Agerer (1987–1998) and Agerer and Rambold (1998). Cultures of *Piloderma croceum* were maintained for 6 weeks at room temperature on square Petri dishes (12×12×1 cm; Merck, Germany) with modified 0.5x Melin-Norkrans (MMN) agar medium (Marx 1969), supplemented with 1% (w/v) tetracycline. Each Petri dish was inoculated with nine small agar plugs and a sterile nylon grid (mesh 80 µm; Draht Center, Stuttgart, Germany) was placed on the agar surface to allow the harvesting of large amounts of young surface mycelium contaminated with only limited amounts of medium.

Ectomycorrhizal inoculation procedures

Surface-sterilised seeds of *Picea abies* (L.) Karst. were grown in a 10:1 peat perlite:agriperl perlite substrate (Dämmstoff GmbH, Darmstadt, Germany) for 2 months. Seedlings were then inserted into specially designed rhizotrons (Raidl 1997) filled with previously sterilised peat (Kölle, Munich, Germany) and inoculated by incorporating MMN-agar plugs containing *Piloderma croceum* into the root zone. Mycorrhiza formed within 3–5 weeks under the conditions given by Peterson and Chakravarty (1991) and Raidl (1997). After successful mycorrhiza formation, rhizotrons were cultivated for a further 5 months according to Brand and Agerer (1988) and Raidl (1997). For the measurement of the rhizotron-grown hyphal length of *Piloderma croceum* by direct microscopy, four separate control rhizotrons containing non-inoculated spruce seedlings were processed in the same way as the four mycorrhizal rhizotrons to differentiate between hyphae of *Piloderma croceum* and hyphae of saprotrophic fungi.

DNA isolation methods

For PCR amplification of ITS regions, genomic DNA was obtained from *Cortinarius obtusus*, *Tomentellopsis submollis*, *Piloderma croceum,* and *Paxillus involutus* according to the techniques described by Agerer et al. (1996). A DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was applied to extract genomic DNA from agar-cultivated *Piloderma croceum* mycelium. To carry out PCR product sequencing and calibration of TaqMan PCR, DNA of recombinant pT7 Blue T plasmid derivatives was extracted from liquid *Escherichia coli* cultures using the Qiagen Plasmid Mini Kit and Qiagen-tip 20 columns. DNA samples were eluted from the spin columns with 400 µl pre-warmed (65°C) AE buffer (0.04 M Tris-acetate; 0.001 M EDTA).

Genomic DNA of rhizotron-grown *Piloderma croceum* was prepared from the rhizosphere employing the protocol reported by Bahnweg et al. (1998). An aliquot of 100 mg fresh weight, consisting of extramatrical mycelium and colonised peat substrate, was extracted per sample. In pursuit of a high DNA yield, preparation step no. 5 was slightly modified by the presence of 2 µl SeeDNA co-precipitant (Amersham Pharmacia Biotech, Freiburg, Germany) and the pellet was dissolved in 100 µl TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA).

DNA samples were spectrophotometrically quantified (A_{260}) with DNA, the quality of which was further proven by agarose gel electrophoresis.

Cloning and sequencing of ITS regions

Using a total reaction volume of 25 µl and approximately 40 ng template DNA, fungal ITS amplification was performed with the universal primer pair ITS1/ITS4 (White et al. 1990) under the conditions described by Agerer et al. (1996). PCR products were separated by horizontal gel electrophoresis, recovered from the agarose using the QIAquick Gel Extraction Kit (Qiagen), and purified by membrane filtration (Nanosep 100 microconcentrator, Pall Filtron Corporation, Northborough, Mass., USA). Purified PCR products were cloned into plasmid pT7 Blue T vector (Novagen, Madison, Wis., USA) and sequenced on both strands employing the Cy5-AutoRead Sequencing Kit, the Cy5-dATP labelling mix and the Alfexpress instrument (Amersham Pharmacia Biotech). To compute maximum matching of pair-wise calculated sequence identities and perform a multiple alignment among the four fungal sequences cloned, nucleotide sequences were subjected to the DNASIS program (version 2.5, Hitachi Software Engineering Europe S.A., Olivet Cedex, France) and were analysed using the Needleman-Wunsch (1970) and Higgins-Sharp (1988) algorithms, respectively.

Microscopic determination of the total hyphal length of Piloderma croceum

Based on the agar film technique (Frankland et al. 1978; Bååth and Söderström 1979), agar-cultivated mycelium was ground in liquid nitrogen, dissolved in 400 µl distilled water and mixed with a definite volume of pre-warmed (50°C) 1.5% (w/v) agar solution (Merck, Darmstadt, Germany). Per sample, six aliquots of the obtained agar film solution were transferred into a pre-warmed (50°C) hemocytometer (Neubauer improved, Merck, Germany). Length of hyphal elements was evaluated with a light microscope (Axioskop 50, Zeiss, Germany) by counting 10 squares per slide. To count rhizotron-grown hyphae, extramatrical mycelium was collected from inoculated seedling roots and mixed with the colonised peat substrate from the rhizotron. Harvested material was ground in liquid nitrogen, dissolved in distilled water and centrifuged (1 min at 1500 *g*). The pellet was resuspended in equal volumes of 90% (w/v) lactic acid (Merck, Germany) and distilled water and three aliquots were transferred into the hemocytometer for microscopic examination (see above). The substrate moisture content was determined by oven drying (2 days, 80°C) a sub-sample so that the moist sample could be diluted on a dry weight basis. The hyphal biomass of saprotropic fungi was estimated by assessing four non-mycorrhizal control rhizotrons using exactly the same microscopic procedure as described above.

Piloderma croceum-specific TaqMan PCR

Based on the sequence data of the ITS1 region of *Piloderma croceum*, a forward primer P1 (5'GAACCTATTGTAAGGGCCCGTAA3'), reverse primer P2 (5'CAACGCTTTTT-AAGGCGAGGACA3') and phosphorylated probe F (5'CCTTTACGTCTTATCACCAACCCATCp3') were designed, yielding PCR-products 103 bp in length. Probe F was labelled at the 5' end with the fluorescent reporter dye FAM, whereas the 3' end was modified with the quencher dye TAMRA. These chemical modifications as well as the phosphorylation of the fluorogenic probe (indicated by p at the 3' end) and oligonucleotide syntheses were provided by Perkin Elmer Applied Biosystems (Weiterstadt, Germany). The universal amplicon design guidelines reported by Schild (1996) in combination with the secondary structure calculation tool, operating in the DNASIS program mentioned above, were used for the selection of primer sequences and fluorogenic probe, avoiding sequences that produce internal secondary structures and focusing on gene probes meeting optimal requirements for TaqMan PCR technology. PCR was performed in 50-µl reactions (MicroAmp optical 96-well plates, PE Biosystems), containing 10 µl of varying concentrations of template DNA, 180 nmol forward primer P1, 240 nmol reverse primer P2, 120 nmol fluorogenic probe F, 25 µl Universal Master Mix (PE Biosystems), and 12 µl Fluka water (Sigma-Aldrich, Taufkirchen, Germany). The thermocycler conditions were: 2 min at 50°C, followed by 10 min at 95°C and 35 cycles of 95°C (15 s) and 60°C (1 min). The automated ABI Prism 7700 sequence detector recorded fluorescent light emission by generating overlapping spectra in the wavelength range from 500 to 660 nm. Data were analysed with the ABI Prism sequence detection software version 1.6 (PE Biosystems). Controls were performed for each amplification run by using 10 µl Fluka water instead of 10 µl template DNA. The absence of fluorescence light emission indicated that the chemical stock solutions were not contaminated with DNA. For the plasmid standards and *Piloderma croceum* samples tested, a 10-fold dilution series of DNA was made with Fluka water. Each DNA sample was thermal cycled in duplicate, confirming identical Ct values for both replicates.

```
1) TCGAAAAGCA ATCCGGGGGG GAGGCGACCG AGCGAAGTTT GGTAGATCGT        50
2) *T****--T* *A**T---*A TG**TTGTT* --------C* ***T--CTC*        35
3) AT****--TG T*TGTC*A*A AG**TTGTA* --------C* **CC--**CA        38
4) *T**G*---- -*TATA**C* AG**TTGTA* --------C* **CC--**TC        35

1) AGGGATTGTC GCTGGCCTTT GGAAACGAAG GCATGTGCAC GTTCCGAGTT       100
2) *****GCA*- *TGCA*AC** *TC*T*TTTA TATCTC-*** -C*GT*CACC        82
3) G***CA***- **ACA**C*G ATC*CATCCA C*TCCCA*** -C*GT*CACC        83
4) G***CA***- **AC***C-G AGCCCTT**T C**CACA*** -C*GT**ACC        82

1) CTCCTTAGTC CTCCTTTGCC TTTCCTTTGG AAAACCCTTT CTTCACACCC       150
2) T*TTG***-- ---A*C**GA *AG*T**CT* **TG**TAGC A**TGGGTTT       127
3) GC*TG***-- ---***G*GA *GA--*CAC* G*G-***--- --*G*TGGG*       120
4) TATTG**A-- ------G*G* CCG--*AAAA **GG**T--- --*T**GT*T       117

1) GTGCACCCAT TGTAGGTCTC CGCGAGGGGA TCTATGTCTT CACATAAACA       200
2) *AAG*TTG*C *-*TTC*G** TTTCTTTATT *TC*G****A TGTT*---*T       173
3) AACG*ATGCC C-*C---G** TAT**ATATT *TC*----CA ***G----*T       158
4) TAT****A*C C--C---A** ---*TAT*TC *-C*----*A G*AT----GT       150

1) CTACGTATGT CTAGGAATGT ATCTAAAAGC GTCGGACGGC TTGGCTTCGT       250
2) *C*TA**CCC *A*T*T**** TATAG**T*T AATAA****- --*C**CT**       220
3) *A*A*****A *AGAATG*A* ***AT*TT** ***T**TAA- --AC----**       201
4) AA*TA*********** *----CTC*C C*TA****** **-T**TAA- --AC----T*       188

1) GCCCGGTCGG CGACGGTAAA GAACCATAAT A                           281
2) ***TA----- ------**** T-----*T** *                          235
3) *---A----- ------C*** T--------- *                          209
4) ---------- ---------* T--------- *                          191
```

Fig. 1 Multiple nucleotide sequence alignment for the ITS1 region of *Paxillus involutus* (line 1) in comparison with *Cortinarius obtusus* (line 2), *Tomentellopsis submollis* (line 3), and *Piloderma croceum* (line 4) as performed by the algorithm-based analysis given in Materials and methods. Sequences are numbered (1–281) from the start of the ITS1 region and bases identical to that of line 1 are indicated by *. Gaps (-) were introduced by the DNASIS software in order to adjust alignment. The *underlined bold areas* indicate the target sequences for the oligonucleotide primer pair P1/P2, whereas the *underlined area* represents the target sequence for fluorogenic probe F- capable of amplifying *Piloderma croceum*-DNA by TaqMan PCR technology

Results

Probe selection for the amplification of *Piloderma croceum* by TaqMan PCR technology

The ITS1/ITS4-amplified regions ITS1 and ITS2 and the 5.8S rRNA gene were cloned and sequenced from the following fungal isolates (EMBL accession numbers given in parentheses): *Cortinarius obtusus* (AJ438981), *Piloderma croceum* (AJ438982), *Tomentellopsis submollis* (AJ438983), and *Paxillus involutus* (AJ438984). Pairwise calculated sequence identities ranged from 50 to 64%, indicating distant relationships between all four species. To meet requirements for optimal PCR technology, a forward primer P1, reverse primer P2 and dual-labelled fluorogenic probe F were designed. By targeting species-differentiating DNA motifs of the ITS region (underlined sequences in Fig. 1), a PCR product exclusively from DNA extracts of *Piloderma croceum* was expected. Inspection of public electronic databases with P1, P2 and F by the Blast similarity search program (http://www.NCBI.nlm.nih.gov/) detected no significant matches in any other organism except the closely related species *Piloderma byssinum* (Karst.) Jül. (Larsen et al. 1997).

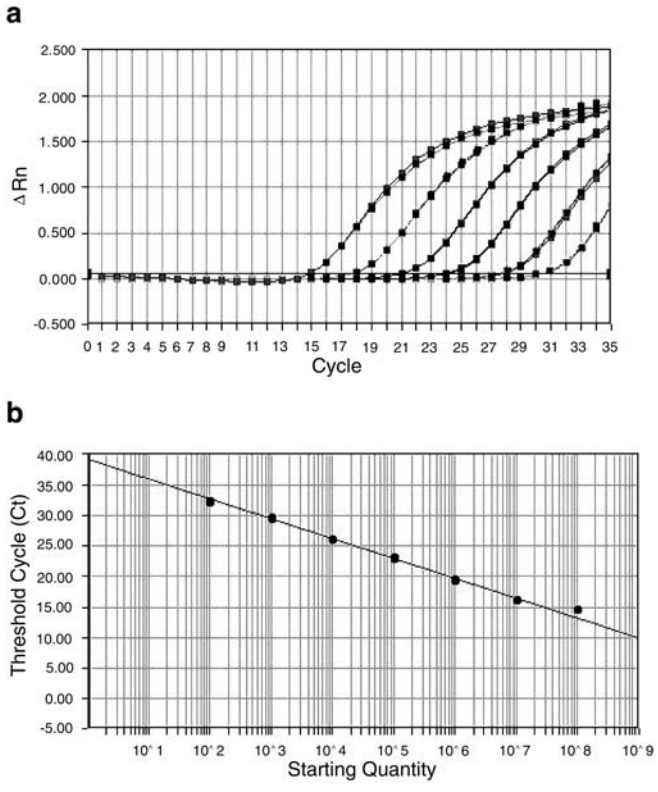

Fig. 2 **a** Real-time amplification plot of recombinant plasmid DNA standards containing genomic sequences from *Piloderma croceum.* Different numbers of starting copies (marked by symbol ■ with individual concentrations not shown here) were analysed and the fluorescence light emission (Δ*Rn*) is plotted versus the number of PCR cycles performed. **b** Calibration curve for the absolute quantification of *Piloderma croceum* DNA. The threshold number of PCR cycles (Ct value), ranging from 0 to 35 cycles, was plotted against the log of the starting number of target copies present per µl extract of plasmid DNA. For gene probes and PCR conditions see Materials and methods. Both plots were produced by the ABI Prism sequence detection software

Calibration of TaqMan PCR for the absolute quantification of *Piloderma croceum*

A recombinant 3.5-kb pT7 Blue T plasmid containing 630 bp ITS1, 5.8SrDNA, and ITS2 sequences from *Piloderma croceum* was used to calibrate real-time PCR. Starting with an initial concentration of 176 µg DNA/ml, which represents 1×10^{12} ITS copies/µl, serial dilutions of the template DNA made with distilled water over 10 orders of magnitude were employed for TaqMan PCR with primers P1, P2 and the fluorogenic probe F. The ABI Prism instrument recorded the fluorescence light emission for all plasmid DNA concentrations tested (Fig. 2a) that accumulated with the number of cycles and depended on the initial number of target copies. By determining the point in time at which the reporter fluorescence exceeds the threshold, Ct values were calculated and plotted against the log of the initial copy number of template DNA (Fig. 2b). The data indicate that starting amounts of the recombinant plasmid ranging from at least 10^8 ITS copies/µl to 10^2 ITS copies/µl permit PCR product amplification within the linear dynamic range of the fluorescence detection assay. The generated plasmid DNA standard curve (r = 0.998), therefore, allows quantification of *Piloderma croceum* DNA in unknown samples over a wide range of at least seven orders of magnitude. No significant fluorescent light emission was detected when recombinant pT7 Blue T plasmids containing ITS1, 5.8SrDNA and ITS2 sequences from *Tomentellopsis submollis* and *Cortinarius obtusus* were tested with oligonucleotides P1, P2 and F. Downstream of PCR cycle no. 30, however, fluorescence signals were detected when very high copy numbers of a recombinant pT7 Blue T plasmid (equivalent to 10^8 ITS copies/µl) containing cloned ITS1, 5.8SrDNA and ITS2 sequences from *Paxillus involutus*, were used (data not shown).

Quantification of agar-cultivated *Piloderma croceum* by TaqMan PCR compared with microscopic determination of fungal biomass

We calculated the input number of DNA molecules as a biomass indicator by amplifying *Piloderma croceum* samples that were isolated from sterile agar-cultivated mycelia (Table 1). After serially diluting DNA extracts, real-time PCR was conducted with oligonucleotides P1, P2 and F. The Ct values obtained allowed quantification of template DNA when applied to the plasmid DNA

Table 1 Quantification of biomass from agar-cultivated *Piloderma croceum* mycelium by direct microscopy of hyphae (mean of six replicates, plus standard deviations are shown as ±) and TaqMan-based amplification of DNA extracts (mean of two replicates). The ratio is the data from 30 mg of mycelium (fresh weight) divided by the data from 10 mg of mycelium

Petri dish	Mycelium (mg)	Hyphal length (m)	Initial ITS copies	Hyphal length / ITS copies (m)
1	10	123.49±25.91	5.01×10^8	2.47×10^{-7}
	30	393.05±75.62	1.64×10^9	2.39×10^{-7}
	ratio	3.18	3.28	
2	10	112.65±17.63	4.08×10^8	2.76×10^{-7}
	30	322.54±19.01	1.05×10^9	3.07×10^{-7}
	ratio	2.86	2.59	
3	10	149.78±7.44	9.64×10^8	1.55×10^{-7}
	30	485.44±79.38	4.92×10^9	9.87×10^{-8}
	ratio	3.24	5.10	

Table 2 Quantification of biomass from rhizotron-grown *Piloderma croceum* mycelium by direct microscopy of hyphae (mean of three replicates) and TaqMan-based amplification of DNA extracts (mean of two replicates)

Rhizotron	Hyphal length (m per g dry soil)	Initial ITS copies (per g dry soil)	Hyphal length / ITS copies (m)
1	4.27	1.82×10^{7}	2.35×10^{-7}
2	5.84	2.33×10^{7}	2.51×10^{-7}
3	4.30	4.67×10^{7}	9.21×10^{-8}
4	3.60	3.73×10^{7}	9.65×10^{-8}

standard curve depicted in Fig. 2b. To validate the results obtained by the TaqMan PCR technology, we performed a comparison with data produced by direct microscopy. As can be seen in Table 1, 0.01 g and 0.03 g mycelium grown on three different Petri dishes was tested. In general, conventional microscopy indicated a ratio of 3 for all three Petri dishes, comparing the fungal biomass quantification from 0.03 g mycelium with the data from 0.01 g mycelium. On average, quantification by fluorescent TaqMan PCR technology also tended towards a ratio of 3 when the data from 0.03 g and 0.01 g mycelium were compared. There was, however, a higher deviation associated with real-time PCR among the three agar cultures examined, that indicated an exceptionally high biomass determined for Petri dish no. 3 as against a low biomass for Petri dish no. 2, in agreement with the microscopic assay.

Quantifying rhizotron-grown mycelium of *Piloderma croceum*

After calibrating and validating TaqMan PCR, this technology was utilised to quantify rhizotron-grown biomass of *Piloderma croceum*. Following the successful production of ectomycorrhizal seedlings, samples were collected from each rhizotron and processed to isolate high-molecular-weight genomic DNA. Since the DNeasy Plant Mini Kit (Qiagen) failed to recover inhibitor-free DNA suitable for PCR, the preparation method reported by Bahnweg et al. (1998) was employed to prevent the possible co-precipitation of PCR-inhibiting compounds from the rhizotron samples tested. DNA samples were serially diluted and the number of *Piloderma croceum*-specific DNA target copies was estimated by real-time PCR using the calibration curve depicted in Fig. 2b. Comparable amounts of extracted template DNA, representing the same order of magnitude, were found for all four rhizotrons investigated (Table 2). Furthermore, rhizotron-grown hyphal length of *Piloderma croceum* was estimated by direct microscopy. Peat samples obtained from the non-mycorrhizal rhizotrons served as controls to avoid the rhizotron-grown saprotrophic biomass. In this way, we found that rhizotron-grown saprotrophic fungi represented an average of 0.989 m total hyphal length per g of oven-dried soil substrate. This background value was subtracted when counting the hyphal length of rhizotron-grown hyphae of *Piloderma croceum* by microscopy. Notably, dividing the observed total hyphal length of *Piloderma croceum* by the calculated starting copy number of extracted template DNA resulted in a nearly identical ratio for rhizotrons 1 and 2 and for rhizotrons 3 and 4 (Table 2).

Discussion

Real-time PCR, frequently utilised in clinical diagnosis of human pathogens (e.g. Manzin et al. 1995; Mensink et al. 1998; Desjardin et al. 1998) but less frequently in plant and mycological research (Böhm et al. 1999), was employed here to quantify the mycelial biomass of the ectomycorrhizal fungus *Piloderma croceum* on the basis of pure agar cultures and non-sterile rhizotron chambers. Following the design of genome-specific PCR probes, this technology has great potential for performing high-throughput biomass quantification of ectomycorrhizal fungal communities from natural as well as artificial ecosystems, or of species living in intimate contact and usually difficult to analyse (as reported for several species of *Chroogomphus* and *Gomphidius* growing within mantle and rhizomorphs of *Rhizopogon* or *Suillus* species; Agerer 1990, 1991). Therefore, differing morphological characters or physiological features are no longer required to distinguish between species. ITS sequences are promising targets for the design of species-specific gene probes because they are easy to clone (White et al. 1990) and they show sequence variability between species. This makes for extra sensitivity of the PCR assay and robustness due to the repetitive genome organisation and reduced within-species diversity.

Conventional microscopic determination of the hyphal length of *Piloderma croceum* was utilised here as a reliable tool to validate the PCR-based results of biomass quantification. Analysing the results from two given amounts of agar-cultivated mycelia, the microscopic observation technique gave the most reliable results with all three Petri dishes examined. In contrast, fluorescent PCR technology provided unreliable data from two of the three Petri dishes tested, with conspicuous relative differences, especially for dish no. 3 resulting in a 1.5- to 3-fold lower ratio of hyphal length to number of ITS copies.

As fungal cultures start ageing at the centre, resulting in hyphae of different vitality, a lower DNA content in the more senescent part of the mycelium could be a reason for the differences found. However, the cultures used were exactly the same age and had been grown under identical conditions resulting in a regular distribution of old and young hyphae in all the samples. On the other hand, a

non-exponential increment of PCR products may have occurred in dishes 2 and 3. As expected in theory (Schild 1996), Ct values should increase exactly in steps of 3.321 (the decadic logarithm of 10) in the ideal case of a non-inhibited amplification, when a 10-fold dilution series of the template DNA is compared by TaqMan PCR. Taking into account the dilution experiment performed with template DNA of Petri dish no. 1, the detected Ct values fit perfectly with the Ct values predicted (see above). This experiment, therefore gives a ratio of 3 when the PCR-based quantification results from 0.03 g and 0.01 g of mycelium extracted are compared. For Petri dishes 2 and 3, the 10-fold dilution series of template DNA showed Ct values ranging between 3.31 and 3.53 (data not shown). In general, this indicates an imprecise exponential increment of PCR products. Assuming similar DNA recovery rates from small and large amounts of cultivated mycelia, as certainly occurred in the case of Petri dish no.1, PCR-inhibiting substances (e.g. carbohydrates and secondary metabolites; Tan et al. 1998) could be responsible for the non-exponential amplification of template DNA. These substances, co-purified with template DNA, were possibly introduced prior to DNA isolation by harvesting mycelia with small amounts of agar contamination or were produced by the cultivated fungi themselves. Careful inspection of the computer-registered Ct values within a 10-fold dilution series of template DNA thus represents a suitable and stringent quality control standard to recognize artefacts produced by fluorescent PCR technology in association with poor quality of extracts.

The extraction of inhibitor-free template DNA also played a key role in the employment of the TaqMan assay to quantify rhizotron-grown mycelium of *Piloderma croceum.* After replacing Qiagen's standard preparation protocol by the laborious method developed by Bahnweg et al. (1998), we successfully isolated high-molecular-weight genomic DNA from rhizosphere samples of colonised spruce seedlings and peat substrate. We subsequently amplified the 103-bp diagnostic marker fragment by fluorescent PCR technology. We did not include an external control for similar recovery rates of *Piloderma croceum* DNA from all samples extracted and this appears unnecessary in the light of the comparable biomass yields reported here for four different mycorrhizal rhizotrons. Moreover, no abnormal Ct values were obtained among a 10-fold dilution series of template DNA (data not shown). In contrast to the ectomycorrhiza revealed in a large number of seedlings 5 months after inoculation, the biomass measured for the rhizotron-grown fungus using direct microscopy was small relative to the maximum possible growing amount extrapolated by Read (1992) for *Paxillus involutus.* On average, a total hyphal length of 4.5 m per g oven-dried soil was measured in our rhizotron experiments in the case of *Piloderma croceum*, whereas a maximum of 200 m hyphal length per g oven-dry soil was extrapolated for *Paxillus involutus* (Read 1992). This reflects vastly different types of ectomycorrhiza with regard to structure, extension, and biomass (according to Agerer 2001). The results obtained from the TaqMan-based quantification of rhizotron-grown mycelia lie within the range of the results obtained from the sterile cultures, i.e. approximately 0.9–3 x 10^7 m of hyphal length per ITS copy.

Based on the procedure of Bahnweg and co-workers stated above, we failed to obtain DNA of *Piloderma croceum* from a native sandy forest soil (horizon A_h-B_v; mean pH 4.35) previously colonised by this species. However, additional protocols exist for recovering DNA from recalcitrant soil sources (e.g. Cullen and Hirsch 1998; Sanseverino et al. 2002) but these are not expected to be a universal tool for analysing all environmental settings due to the very wide diversity in texture and soil chemical content and their effects on DNA extraction and subsequent PCR assays (reviewed by Nazar et al. 1997; Kowalchuk 1999). The distribution and possible application of real-time PCR technology within the field of ectomycorrhizal research, as recently established for detection and quantification of soil bacteria in pure cultures as well as environmental samples (Bach et al. 2002), will strongly depend on the availability of fungal-DNA extraction protocols for different types of soil.

Acknowledgement The study was supported by the Deutsche Forschungsgemeinschaft (SFB 607, A1/A9/B7).

References

Agerer R (ed) (1987–1998) Colour atlas of ectomycorrhizae. 1st–12th edn. Einhorn, Schwäbisch Gmünd, Germany

Agerer R (1990) Studies on ectomycorrhizae. XXIV. Ectomycorrhizae of *Chroogomphus helveticus* and *C. rutilus* (Gomphidiaceae, Basidiomycetes) and their relationship to those of *Suillus* and *Rhizopogon*. Nova Hedwigia 50:1–63

Agerer R (1991) Studies on ectomycorrhizae. XXXIV. Mycorrhizae of *Gomphidius glutinosus* and of *G. roseus* with some remarks on Gomphidiaceae (Basidiomycetes). Nova Hedwigia 53:127–170

Agerer R (2001) Exploration types of ectomycorrhizal mycelial systems. A proposal to classify mycorrhizal mycelial systems with respect to their ecologically important contact area with the substrate. Mycorrhiza 11:107–114

Agerer R, Rambold G (1998) DEEMY, a DELTA-based information system for characterization and determination of ectomycorrhizae. Version 1.1. Institute for Systematic Botany, Section Mycology, University of Munich

Agerer R, Müller WR, Bahnweg G (1996) Ectomycorrhizae of *Rhizopogon subcaerulescens* on *Tsuga heterophylla*. Nova Hedwigia 63:397–416

Allen MF (1991) The ecology of mycorrhiza. Cambridge University Press, Cambridge

Bach HJ, Tomanova J, Schloter M, Munch JC (2002) Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification. J Microbiol Methods 49:235–245

Bahnweg G, Schulze S, Möller EM, Rosenbrock H, Langebartels C, 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

Bååth E, Söderström B (1979) Fungal biomass and fungal immobilization of plant nutrients in Swedish coniferous forest soils. Rev Ecol Biol Sol 16:477–489

Böhm J, Hahn A, Schubert R, Bahnweg G, Adler N, Nechwatal J, Oehlmann R, Oßwald W (1999) Real-time quantitative PCR: DNA determination in isolated spores of the mycorrhizal fungus *Glomus mosseae* and monitoring of *Phytophthora infestans* and *Phytophthora citricola* in their respective host plants. J Phytopathol 147:409–416

Brand F, Agerer R (1988) Studien an Ektomykorrhizen. XIII. Drei häufige Ektomykorrhizen der Buche (*Fagus sylvatica* L.). Charakterisierung und unsterile Kultivierung von Buchenektomykorrhizen. Sydowia 40:1–37

Buscot F, Munch JC, Charcosset JY, Gardes M, Nehls U, Hampp R (2000) Recent advances in exploring physiology and biodiversity of ectomycorrhizas highlight the functioning of these symbioses in ecosystems. FEMS Microbiol Rev 24:601–614

Cullen DW, Hirsch PR (1998) Simple and rapid method for direct extraction of microbial DNA from soil for PCR. Soil Biol Biochem 30:983–993

Desjardin LE, Chen Y, Perkins MD, Teixeira L, Cave MD, Eisenach KD (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

Fogel R, Hunt G (1978) Fungal and arboreal biomass in a western Oregon Douglas-fir ecosystem: distribution patterns and turnover. Can J For Res 9:245–256

Frankland JC, Lindley DK, Swift MJ (1978) A comparison of two methods for the estimation of mycelial biomass in leaf litter. Soil Biol Biochem 10:323–333

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6:986–994

Higgins DG, Sharp PM (1988) CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. Gene 73:237–244

Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Mol Ecol 10:1855–1871

Ineichen K, Wiemken V, Wiemken A (1995) Shoots, roots and ectomycorrhiza formation of pine seedlings at elevated atmospheric carbon dioxide. Plant Cell Environ 18:703–707

Kowalchuk GA (1999) New perspectives towards analysing fungal communities in terrestrial environments. Curr Opin Biotechnol 10:247–51

Kunzweiler K, Kottke I (1986) Quantifizierung von Myzel im Waldboden. In: Einsele G (ed) Das landschaftsökologische Forschungsprojekt Naturpark Schönbuch. VCH, Weinheim, Germany, pp 429–441

Larsen MJ, Smith JE, McKay D (1997) On *Piloderma bicolor* and the closely related *P. byssinum, Piloderma croceum* and *P. fallax*. Mycotaxon 63:1–8

Manzin A, Solforosi L, Bianchi D, Gabrielli A, Giostra F, Bruno S, Clementi M (1995) Viral load in samples from hepatitis C virus (HCV)-infected patients with various clinical conditions. Res Virol 146:279–284

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

Matyssek R, Schnyder H, Elstner EF, Munch JC, Pretzsch H, Sandermann H (2002) Growth and parasite defence in plants: the balance between resource sequestration and retention: in lieu of a guest editorial. Plant Biol 4:133–136

Mensink E, van de Locht A, Schattenberg A, Linders E, Schaap N, Geurts van Kessel A, De Witte T (1998) Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR. Br J Haematol 102:768–774

Nazar RN, Robb EJ, Hu X, Volossiouk T, Lee SW (1997) Development of PCR-based diagnostics for soil borne plant pathogens. J Plant Biol 40:176–81

Needleman SB, Wunsch CD (1970) Description of the method used in PCOMPARE and SCANISM. J Mol Biol 48:443–453

Peterson RL, Chakravarty P (1991) Techniques in synthesizing ectomycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Techniques for the study of mycorrhiza. Methods Microbiol 23:75–106

Raidl S (1997) Studien zur Ontogenie an Rhizomorphen von Ektomykorrhizen. Bibl Mycol 169:1–184

Read DJ (1992) The mycorrhizal mycelium. In: Allen MF (ed) Mycorrhizal functioning. An integrative plant–fungal process. Chapman & Hall, New York, pp 102–133

Sanseverino J, Layton AC, Sayler GS (2002) Detection of polychlorinated biphenyl-degrading organisms in soil. In: Aquino de Muro M, Rapley R (eds) Gene probes: principles and protocols. Methods in Molecular Biology, vol 179. Humana, Totowa, N. J., pp 193–209

Schild TA (1996) Einführung in die Real-Time TaqMan™ PCR-Technologie. Applied Biosystems GmbH, Weiterstadt

Schubert R, Bahnweg G, Nechwatal J, Jung T, Cooke DEL, Duncan JM, Müller-Starck G, Langebartels C, Sandermann H, Oßwald W (1999) Detection and quantification of *Phytophthora* species which are associated with root-rot diseases in European deciduous forests by species-specific polymerase chain reaction. Eur J For Pathol 29:169–188

Smith SE, Read DJ (eds) (1997) Mycorrhizal symbiosis. Academic, San Diego

Tan S-L, Dossett M, Katze MG (1998) Extraction of genomic DNA suitable for PCR analysis from dried plant rhizomes/roots. BioTechniques 25:796–801

Weaver RW, Angle S, Bottomley P, Bezdicek D, Smith S, Tabatabai A, Wollum A (eds) (1999) Methods of soil analysis. Part II. Microbiological and biochemical properties. Soil Science Society of America, Madison, Wis

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Snisky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, New York, pp 315–321