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Development of a molecular approach to describe the composition of *Trichoderma* communities

Remo Meincke [a,b,1], Nicole Weinert [b], Viviane Radl [c], Michael Schloter [c,*], Kornelia Smalla [b], Gabriele Berg [a]

[a] Environmental Biotechnology, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria
[b] Julius Kühn-Institut, Federal Research Centre for Cultivated Plants (JKI), Institute for Epidemiology and Pathogen Diagnostics, D-38104 Braunschweig, Messeweg 11-12, Germany
[c] Department for Terrestrial Ecogenetics, Helmholtz Zentrum München, Ingolstädter Landstrasse1, D-85758 Oberschleissheim, Germany

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ABSTRACT

Trichoderma and its teleomorphic stage *Hypocrea* play a key role for ecosystem functioning in terrestrial habitats. However, little is known about the ecology of the fungus. In this study we developed a novel *Trichoderma*-specific primer pair for diversity analysis. Based on a broad range master alignment, specific *Trichoderma* primers (ITS*Tr*F/ITS*Tr*R) were designed that comprise an approximate 650 bp fragment of the internal transcribed spacer region from all taxonomic clades of the genus *Trichoderma*. This amplicon is suitable for identification with *Tricho*Key and *Tricho*BLAST. Moreover, this primer system was successfully applied to study the *Trichoderma* communities in the rhizosphere of different potato genotypes grown at two field sites in Germany. Cloning and sequencing confirmed the specificity of the primer and revealed a site-dependent *Trichoderma* composition. Based on the new primer system a semi-nested approach was used to generate amplicons suitable for denaturing gradient gel electrophoresis (DGGE) analysis and applied to analyse *Trichoderma* communities in the rhizosphere of potatoes. High field heterogeneity of *Trichoderma* communities was revealed by both DGGE. Furthermore, qPCR showed significantly different *Trichoderma* copy numbers between the sites.

1. Introduction

Fungi belonging to the genus *Trichoderma*/*Hypocrea* (*Ascomycetes*) can be isolated from nearly every soil, decaying wood, compost or other organic matter (Harman et al., 2004; Berg et al., 2005). Because of their aggressive lytic capacity, *Trichoderma* strains are involved in the degradation of complex organic compounds in soil (Kubicek et al., 2008). Furthermore, many *Trichoderma* isolates are known for their ability to suppress different fungal plant pathogens, e.g. *Botrytis cinerea*, *Fusarium* spp., *Phytophthora cactorum*, *Pythium* spp., *Rhizoctonia solani*, and *Verticillium dahliae* (Weinling, 1932; Chet, 1987; Grosch et al., 2006), and to promote plant growth (Inbar et al., 1994). Therefore, some strains have been used as active ingredients of biofungicides (Monte, 2001; Hermosa et al., 2004). Different mechanisms, including competition for nutrients, antibiosis, production of cell wall lytic enzymes, induction of systemic resistance, and mycoparasitism have been described as the basis for the antagonistic activity (Harman et al., 2004). In addition, the important role of auxin signalling for plant growth promotion was shown for *Trichoderma* spp. in *Arabidopsis* (Contreras-Cornejo et al., 2009). In contrast to the reported beneficial interactions with plants, several *Trichoderma* species are also known as pathogens for diverse eukaryotic hosts, e.g. *Trichoderma longibrachiatum*, an opportunistic pathogen of humans (De Miguel et al., 2005) or *Trichoderma aggressivum*, the causal agent of "green mould disease" in fungi like *Agaricus* and *Pleurotus* (Hatvani et al., 2007).

Due to the importance of *Trichoderma* in terrestrial ecosystems, in the last decade several studies on the ecology and biogeography of *Trichoderma* were published, which were based on the cultivation of selected isolates (Zhang et al., 2005; Migheli et al., 2009; Sadfi-Zouaoui et al., 2009). However, to describe the overall diversity of *Trichoderma* in soil molecular tools are needed to avoid the bias, which is caused generally by cultivation-based approaches. Therefore, Hagn et al. (2007) developed primers targeting a 540 bp fragment comprising the internal transcribed spacer region 1 (ITS 1), 5.8S rRNA gene and internal the transcribed spacer region 2 (ITS 2) of all taxonomic clades of the genus *Trichoderma*. Although this sequence was useful to analyse *Trichoderma* abundance in ecosystems (Zachow et al., 2009), it was not suitable to unambiguously identify *Trichoderma* at the species level using *Trichoderma* identification systems like *Tricho*Key 2.0 (Druzhinina et al., 2005) and *Tricho*BLAST (Kopchinskiy et al., 2005).

Therefore, the objective of our study was to develop a novel primer system that would allow the sequence-based identification of the amplicon with both *Tricho*Key 2.0 and *Tricho*BLAST. In order to evaluate the newly developed primers, amplicons obtained from total community DNA from the rhizospheres of potatoes grown at two field

* Corresponding author. Tel.: +49 0 89/3187 4065; fax: +49 0 89/3187 2800.
E-mail address: schloter@helmholtz-muenchen.de (M. Schloter).
[1] (*in memoriam*).

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sites were cloned and sequenced. In addition, this primer system was adopted for analysis of the *Trichoderma* community composition by denaturing gradient gel electrophoresis (DGGE).

2. Materials and methods

2.1. Field design and sampling of potato rhizospheres

In this study the potato cultivars 'Baltica', 'Désirée', 'Ditta' and 'Selma' were used. Furthermore two genetically modified (GM), zeaxanthin-accumulating, potato lines (SR47, SR48) which are based on the parental line 'Baltica' were included in this study. The construction of the two GM lines SR47/00#18 (co-suppression) and SR48/00#17 (anti-sense) was described in detail by Römer et al. (2002). The accumulation of zeaxanthin in the tuber reached up to 40 μg/g dry weight (dw) (SR47/00#18) and 17 μg/g dw (SR48/00#17) compared to 0.2 μg/g dw of the wildtype.

The field design has been described by Weinert et al. (2009) in detail. The two sites used are located in Southern Germany (Bavaria). The soil of the site Roggenstein was characterised by 26.1% sand, 44.0% silt, 28.1% clay, Corg: 1.1%, Nt: 0.1% and pH 6.6. The soil of the site Oberviehhausen contained 54.6% sand, 31.3% silt, 14.1% clay, Corg: 1.9%, Nt: 0.2%, and had a pH of 6.5. Plant protection management at both sites was performed according to agricultural practice.

The experimental setup was designed as a randomised field trial: each plot had a size of 9×3 m, resulting in 4 rows with a total of 40 potatoes. The cultivars and GM lines were grown in six replicated plots, of which four plots were sampled. Sampling of rhizosphere soil was performed from individual plants during the flowering period. Samples were shock frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Extraction of DNA from fungal isolates and from soil samples

Extraction of DNA from fungal isolates (plated on Sabouraud-Dextrose-Agar (Difco, Detroit, USA) for 7 to 14 days at room temperature) was performed using a modified protocol published by John J. Weiland on the FGSC-site (http://www.fgsc.net/fgn44/weiland.html). In brief: tubes were filled with 200 μg glass beads and wetted with Phenol/Chloroform/Isoamylalcohol (24:24:1, ROTH, approximately 200 μl) before adding the fresh mycelia harvested from agar plates; cell lysis was performed with the FastPrep system (Qbiogene, BIO101® Systems, Carlsbad, USA) for 20 s and 5.5 ms^{-1}.

DNA extraction was done using 0.5 g of the microbial pellet obtained from 10 g of root material with adhering soil as recently described by Weinert et al. (2009) by means of the FastDNA® Spin for Soil Kit (Qbiogene, BIO101® Systems, Carlsbad, USA). The DNA yield was checked by agarose gel electrophoresis (0.8%, 1× TBE) using 5 μl of the DNA suspension. To remove PCR inhibiting substances, the rhizosphere community DNA was purified using the GeneClean® Spin Kit (Qbiogene, BIO101® Systems, Carlsbad, USA).

2.3. In silico primer development

A total of 577 *Trichoderma*-ITS-sequences were obtained from different databases (NCBI and ISTH) and screened for appropriate size (>400 bp). For the screening of possible consensus regions, the sequences were aligned and integrated in the ARB database (Ludwig et al., 2004). The *Trichoderma* assignment was confirmed by BLAST and the probe match tool of the ARB package was used for primer design. The forward and reverse primers were selected based on a medium GC-content, a melting temperature of >50 °C/<60 °C, and an amplicon size of >300 bp that allows their identification by means of *Tricho*Key 2.0 or the ITS2 Database: forward primer ITS1*Tr*F (5′-ACTCCCAAACCCAATGTGAA-3′, Tm: 53.9 °C) and reverse primer ITS4*Tr*R (5′-TGTGCAAACTACTGCGCA-3′, Tm: 54.6 °C).

2.4. PCR conditions

For PCR amplification of genomic DNA from isolates, the following conditions were used: 5 U of *Taq* polymerase (Stoffel fragment, Applied Biosystems, CA, USA), 10× Stoffel buffer, 0.2 mM dNTPs, 3.75 mM MgCl2, 2.5% DMSO, 0.5 μM of each primer, and about 20 ng template. After an initial step for 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 35 s at 53 °C and 2 min at 72 °C, followed by a final extension step at 72 °C for 10 min were performed. The PCR products were analysed by agarose gel electrophoresis (1%, 1× TBE). For cloning experiments the PCR products were purified with the GeneClean® Spin Kit.

For amplification of soil DNA a semi-nested strategy was chosen. The first PCR reaction was performed using the fungal specific ITS forward primer ITS1F (Gardes and Bruns, 1996) and the *Trichoderma*-specific ITS reverse primer ITS4*Tr*R. For the second reaction the *Trichoderma*-specific primer system ITS*Tr*F/ITS*Tr*R developed in this study was used. To establish a *Trichoderma*-specific DGGE system a GC clamp attached to the 5′end of ITS*Tr*F was employed. The conditions for the first amplification were the same as described above except that the annealing temperature was raised to 54 °C and the number of cycles was reduced from 35 to 30. For the second amplification the amplicons of the first PCR were diluted 1:20. The conditions were the same as described above, except that the number of cycles was 25.

For the quantification of *Trichoderma* the qPCR protocol published by Hagn et al.(2007) was used.

2.5. Cloning of PCR products and sequence alignments

Cloning of PCR amplicons was done with the pGEM-T Easy vector system (Promega, Madison, USA). The ligation reaction was performed with 3 μl of the obtained PCR products. Positive clones were picked and cultivated on fresh agar plates containing 85 μg/ml ampicillin. Clones were checked for correct insert size by PCR with the SP6/T7 primer system using cell lysates as template and subsequent agarose gel electrophoresis (1%, 1× TBE). Clones with the correct insert size were sent to *IIT* Biotech (Bielefeld, Germany) for one shot sequencing.

After removing vector sequences using VecScreen (www.ncbi.nlm.nih.gov) the sequences were identified by BLASTn (Basic Local Alignment and Search Tool) as well as *Tricho*Key 2.0 and *Tricho*BLAST (Druzhinina et al., 2005; Kopchinskiy et al., 2005). To compare a set of sequences for similarities, they were first truncated to the same length using the binding sites for the *Trichoderma*-specific primer as a marker.

2.6. Denaturing gradient gel electrophoresis (DGGE) of Trichoderma-specific gene fragments

DGGE analysis was performed in a Biorad apparatus (DCode, Biorad, Germany) with a double gradient composed of 26–58% denaturants (100% denaturants defined as 7 M urea and 40% formamide) and 6.2–9% acrylamide. Approximately 3 μl aliquots of PCR products were loaded on the gel. The electrophoresis run was performed in 1× Tris-acetate-EDTA buffer at a constant voltage of 220 V for 7 h at 58 °C and gels were silver-stained according to Heuer et al. (2001). Stained gels were air-dried and scanned transmissively (Epson 1680 Pro, Seiko-Epson, Japan).

3. Results

3.1. Primer development

The designed primers were first tested *in silico* against the constructed database of 577 sequences obtained from ISTH and NCBI databases. The forward primer ITS*Tr*F had a perfect match with the ITS1 of 378 *Trichoderma* strains mainly belonging to clade I

(*Harzianum* clade) and clade XII (*Viride* clade). Moreover, primer ITS*Tr*F showed more than 90% matches with the sequences of 139 strains that represented all other clades. Of these, 125 sequences had an additional base after position 12 from the 3′-end of the primer and thus it is not assumed that this mismatch would influence amplification. The reverse primer allowed the binding of approximately 94% (545 from 577) of the target sequences with a perfect match (100%). Another 17 sequences showed only one or two mismatches at the 5′-end of the primer that should not influence amplification.

To test for non-target matches of the primers they were evaluated using the probe match feature in the ARB package, the RDP II database and also BLASTn. For the forward primer possible annealing to non-target sequences were observed for several species but due to the high specificity of the reverse primer ITS*Tr*R amplicons are unlikely to be generated. Secondary structure analyses were performed using NetPrimer (http://home.postech.ac.kr/~heony/NetPrimer/netprimer.html). The forward primer did not form any secondary structures. Primer dimer for the reverse primer cannot be excluded but could less likely be formed in PCR reaction with DMSO added. Furthermore, no cross dimerisation was detected for this primer pair.

Direct amplification from DNA of 42 *Trichoderma* isolates (Grosch et al., 2007; Hagn et al., 2007; Meincke, unpublished) was used to verify the applicability of the primer pair. DNA of *Phytophthora infestans* and *Verticillium dahliae* ELV 25 was used as negative control. All isolates except one resulted in a positive signal of the correct size (approx. 450 bp). No amplicons were obtained with non-target strains included as negative controls. The calculated annealing temperature of 53 °C was verified by temperature gradient PCR (data not shown).

3.2. Cloning and sequencing of Trichoderma amplicons obtained from potato rhizosphere

PCR products were obtained with ITS*Tr*F/ITS*Tr*R from DNA of all rhizosphere samples from flowering potato plants grown at both sites. The amplicons of all four replicates per plant genotype were pooled and cloned. Approximately 15 clones from each potato genotype were picked and analysed. Overall, 174 clones were obtained (91 from Oberviehhausen, 83 from Roggenstein). After sequencing, the isolates were identified by BLASTn, *Tricho*Key 2.0 and *Tricho*BLAST. The results are summarised in Table 1. The data obtained by *Tricho*-BLAST indicated that the majority of the sequences were affiliated to *Trichoderma* with a similarity between 95% and 100%. The sequences were assigned to seven of the 16 *Trichoderma* clades with the highest proportion of all clones belonging to the group of *Trichoderma stromaticum* and *Trichoderma viride* (clade V and clade XII, respectively). One group that was only found in Oberviehhausen soil belonged to clade VI and showed a high sequence similarity to *Trichoderma brevicompactum*. Sequences of members from clade I were found in Oberviehhausen less frequently than in Roggenstein. Surprisingly, sequences from *Trichoderma harzianum* were rarely found. Overall, 20 different *Trichoderma* species were identified. The number of species was higher for Oberviehhausen (17) than for Roggenstein (11) indicating pronounced differences between both sites. However, for most of the clones *Tricho*Key 2.0 allowed only the assignment to the genus *Trichoderma.*

3.3. Establishment of a DGGE system to study Trichoderma population structure

For cultivation-independent analysis of *Trichoderma* communities by DGGE, a semi-nested strategy was chosen to obtain sufficient amounts of GC-clamped PCR products from rhizosphere DNA samples. DGGE conditions were optimised and a good separation of bands was achieved with a gradient ranging from 26–58%.

Trichoderma community DGGE fingerprints of rhizosphere samples of four potato genotypes ('Baltica', 'Selma', two transgenic lines) taken at EC90 are exemplarily shown in Fig. 1. The fingerprints displayed a rather low complexity with up to 15–20 bands per replicate. Although several common bands were found for all potato genotypes, a high heterogeneity was observed not only among potato genotypes but also among replicates.

3.4. Abundance of Trichoderma in the rhizosphere of the analysed potato cultivars

Statistically significant differences were observed between both sites (χ^2-test) comparing rhizosphere samples of the four cultivars 'Baltica', 'Désirée',and 'Ditta' as well as the transgenic lines, with higher copy numbers in samples from Oberviehhausen copy number per gram of soil compared to samples from Roggenstein (Fig. 2). Although in general for the samples from 'Ditta' and 'Baltica' higher copy numbers for *Trichoderma* were measured, the effects were not statistically significant due to the high heterogeneity among the replicate plots of each cultivar.

4. Discussion

Due to the enormous importance of *Trichoderma* in terrestrial ecosystems but also in biotechnology, a correct identification of *Trichoderma* strains is crucial. However, species identification using BLAST search can result in misidentification (Druzhinina et al., 2005). To overcome this problem *Tricho*Key 2.0, a nucleotide bar coding system for *Trichoderma* was developed which can be used with *tef*1- or with ITS-sequences (Druzhinina et al., 2005). However, already Druzhinina et al. (2005) recognised that *Tricho*Key 2.0 might fail to correctly identify new species because of slight differences in the species-specific hallmarks. In view of the limitations of previously published *Trichoderma*-specific primers for identification (Hagn et al., 2007; Bo et al., 2008) we decided to design an improved primer system for cultivation-independent studies of *Trichoderma* communities based on the sequences in *Tricho*Key 2.0 and additional sequences from the NCBI database. We tried to use a maximum of sequence-length in order to exploit the four species-specific hallmarks. The primers targeting binding sites inside the ITS2 region were tested with known sequences *in silico* in order to verify that the sequences were theoretically amplified and possessed the diagnostic sites (hallmarks) required. Furthermore, four out-groups (*Fusarium oxysporum*, *Stachybotrys cylindrospora*, *Bionectra coronata* and *Bionectra rossmanie*) of the *Hypocreales* were included to test for non-target annealing of the primers.

In addition to *in silico* testing the novel primer system was tested with genomic DNA from a collection of target and non-target isolates of different origin. With one exception amplicons of the correct size were obtained from all *Trichoderma* isolates but not from non-target isolates. The *Trichoderma* strain which did not give an amplicon was possibly misidentified. Most importantly, the specificity of the primers was shown by cloning and sequencing of PCR products obtained from rhizosphere DNA of seven potato genotypes grown at two different field sites. In contrast to previously published primers (Hagn et al., 2007), all sequences of clones obtained from both sites were assigned by *Tricho*BLAST to *Trichoderma* or *Hypocrea* species. With one exception these clones were also assigned to *Trichoderma*/*Hypocrea* by BLAST. However, *Tricho*Key did not permit the identification of a considerable number of clones (40/83 from Roggenstein and 45/86 from Oberviehhausen) to the species level.

Sequencing of the cloned amplicons revealed not only a high specificity of the primers but also allowed insights into the *Trichoderma* community structure in the rhizosphere of potato plants grown at two sites. While the number of clones analysed per plant genotype was not high enough to investigate cultivar-dependent differences in the

Table 1

Clone	Length (bp)	Hallmark 1	2	3	4	*Tricho*Key	*Tricho*BLAST	Similarity (%)	BLAST	BLAST acc. no.	Similarity (%)	Genbank acc. no
a. Analysis of Trichoderma clones obtained from Oberviehhausen; hallmarks according to the diagnotic regions within ITS1 and IT2 (Kopchinskiy et al., 2005); "x" indicates presence; "–" indicates absence of the corresponding marker.												
O1-1	441	x	x	x	x	Unidentified species	*T. chlorosporum*	92	*H. atrogelatinosa*	DQ023302	93	GQ255655
O1-2	408	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	96	*T. brevicompactum*	EU338330	99	
O1-4	462	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	96	*T. rossicum*	DQ083024	98	GQ255656
O1-6	437	x	x	x	x	Unidentified species	*T. chlorosporum*	92	*H. atrogelatinosa*	DQ023302	92	GQ255657
O1-7	434	x	x	–	–	–	*T. chlorosporum*	92	*H. nigrovirens*	AY737777	93	GQ255658
O1-10	436	x	x	x	x	Unidentified species	*T. chlorosporum*	92	*H. nigrovirens*	AY737777	93	GQ255659
O1-12	406	x	x	x	x	Unidentified species	*T. viride*	100	*T. viride*	EU263995	100	GQ255660
O1-15	435	–	–	–	–	–	*T. petersenii*	87	*T. atroviride*	AF501328	79	GQ255661
O1-16	415	x	x	x	x	Unidentified species	*H. hunua*	96	*T. brevicompactum*	FJ610290	97	GQ255662
O1-18	437	x	x	x	x	Unidentified species	*H. chlorosporum*	92	*H. nigrovirens*	AY737777	93	GQ255663
O1-19	402	x	x	x	x	Unidentified species	*T. taiwanese*	100	*T. asperellum*	EU598544	97	GQ255664
O1-20	418	x	x	x	x	Unidentified species	*T. strictipilis*	93	*H. strictipilis*	FJ442219	93	GQ255665
O1-21	400	x	x	x	x	Unidentified species	*T. taiwanese*	97	*T. asperellum*	EU598544	97	GQ255666
O1-22	408	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	98	GQ255667
O1-24	420	–	–	–	–	–	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255668
O1-25	434	–	x	–	–	–	*T. virens*	90	*T. brevicompactum*	EU338330	93	GQ255669
O1-52	458	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	96	*T. brevicompactum*	EU338330	99	GQ255670
O1-54	472	x	x	x	x	Unidentified species	*T. ceramicum*	92	*T. rossicum*	EU280089	93	GQ255671
O2-0	462	x	x	x	x	*T. rossicum*	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255672
O2-1	424	x	x	x	x	Unidentified species	*H.strictipilosa*	93	*H. strictipilis*	FJ442219	93	GQ255673
O2-2	424	x	x	x	x	*T. rossicum*	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255674
O2-3	420	x	x	x	x	Unidentified species	*T. stromaticum*	96	*T. rossicum*	DQ083024	98	GQ255675
O2-4	423	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255676
O2-5	424	x	x	x	x	*T. rossicum*	*T. stromaticum*	97	*T. rossicum*	DQ083024	100	GQ255677
O2-6	439	x	x	x	x	Unidentified species	*T. chlorosporum*	92	*H. nigrovirens*	AY737777	93	GQ255678
O2-7	424	x	x	x	x	*T. rossicum*	*T. stromaticum*	97	*T. rossicum*	DQ083024	100	GQ255679
O2-8	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	100	GQ255680
O2-9	419	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	96	*T. brevicompactum*	EU338330	100	GQ255681
O2-10	424	x	x	x	x	*T. rossicum*	*T. stromaticum*	97	*T. rossicum*	DQ083024	100	GQ255682
O2-11	408	x	x	x	x	Unidentified species	*T. taiwanese*	96	*T. asperellum*	EU598544	93	GQ255683
O2-12	416	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	94	*T. brevicompactum*	EU338330	97	GQ255684
O2-13	433	x	x	x	–	Unidentified species	*T. harzianum*	93	*T. harzianum*	AF483587	93	GQ255685
O2-14	420	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	96	*T. brevicompactum*	EU338330	99	GQ255686
O2-15	424	x	x	x	x	*T. rossicum*	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255687
O2-16	416	x	x	x	x	Unidentified species	*T. stromaticum*	96	*T. rossicum*	DQ083024	98	GQ255688
O2-17	430	x	x	x	x	Unidentified species	*T. virens*	95	*T. spirale*	FJ442668	96	GQ255689
O2-18	460	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	96	*T. brevicompactum*	EU338330	98	GQ255690
O2-19	433	x	x	x	x	Unidentified species	*T. virens*	96	*T. rossicum*	DQ083024	97	GQ255691
O2-20	443	x	x	x	x	Unidentified species	*T. ceramicum*	93	*T. sp.*	DQ345867	93	GQ255692
O2-21	394	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	96	*T. brevicompactum*	EU338330	98	GQ255693
O2-22	430	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	96	*T. brevicompactum*	EU338330	99	GQ255694
O2-23b	436	x	x	x	x	Unidentified species	*T. hamatum*	99	*T. hamatum*	AM498484	99	GQ255695
O2-24	455	x	x	x	x	Unidentified species	*T. taiwanese*	97	*T. viride*	DQ315461	97	GQ255696
O2-24-2	437	–	x	–	–	Unidentified species	*H. nigrovirens*	97	*H. nigrovirens*	AY737777	90	GQ255697
O2–25	458	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	95	*T. brevicompactum*	EU338330	98	GQ255698
O2-28	475	x	x	x	x	Unidentified species	*T. asperellum*	100	*T. stromaticum*	AY856834	92	GQ255699
O2-29	459	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	96	*T. brevicompactum*	EU338330	99	GQ255700
O2-30a	479	x	x	x	–	Unidentified species	*T. chlorosporum*	92	*H. atrogelatinosa*	DQ023302	93	GQ255701
O2-31	428	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	96	*T. brevicompactum*	EU338330	99	GQ255702
O2-32	479	x	x	x	–	Unidentified species	*T. polysporum*	100	*T. polysporum*	Z48815	92	GQ255703
O2-33	458	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	96	*T. brevicompactum*	EU338330	99	GQ255704
O2-34	456	x	x	x	x	Unidentified species	*T. taiwanese*	99	*T. viride*	DQ315461	96	GQ255705
O2-35	473	x	x	x	x	Unidentified species	*T. chlorosporum*	92	*H. thailandica*	AY737772	93	GQ255706
O2-36	452	x	x	x	x	Unidentified species	*T. taiwanese*	96	*T. asperellum*	EU264001	97	GQ255707
O2-37	462	x	x	x	x	*T. rossicum*	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255708
O2-38	464	x	x	x	x	Unidentified species	*T. stromaticum*	96	*T. rossicum*	DQ083024	97	GQ255709
O2-39	474	x	x	x	x	Unidentified species	*T. chlorosporum*	92	*H. atrogelatinosa*	DQ023302	93	GQ255710
O2-40	465	x	x	x	x	*T. brevicompactum*	*H. virenscentiflava*	94	*T. brevicompactum*	EF417484	96	GQ255711
O2-41	412	x	x	x	x	Unidentified species	*T. taiwanese*	99	*T. sp.*	EU280140	96	GQ255712
O2-42	399	–	–	–	–	–	*H. nigrovirens*	95	*T. agressivum*	FJ442607	87	GQ255713
O2-43	410	x	x	x	x	Unidentified species	*T. taiwanese*	99	*T. viride*	DQ315461	97	GQ255714
O2-44	405	x	x	x	–	–	*T. taiwanese*	99	*T. viride*	DQ315461	96	GQ255715
O2-45	427	x	x	x	x	*T. cerinum*	*Trihcoderma tomentosum*	99	*T. tomentosum*	EU330958	96	GQ255716
O2-46	425	x	x	x	x	Unidentified species	*H. virenscentiflava*	97	*H. virenscentiflava*	AY737768	97	GQ255717
O2-47	422	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	94	*T. brevicompactum*	EU338330	96	GQ255718
O2-50	421	x	x	x	x	Unidentified species	*T. stromaticum*	96	*T. rossicum*	DQ083024	98	GQ255719
O2-51	434	x	x	x	x	Unidentified species	*T. chlorosporum*	92	*H. nigrovirens*	AY737777	93	GQ255720
O2-53	416	–	x	–	–	Unidentified species	*H. melanomagna*	95	*T. brevicompactum*	EF417484	98	GQ255721
O2-56	465	x	x	x	x	*T. harzianum*	*T. harzianum*	97	*H. lixii*	FN396577	97	GQ255722
O2-57	409	x	x	x	x	*T. brevicompactum*	*H. melanomagna*	96	*T. brevicompactum*	EU338330	100	GQ255723
O2-58	315	–	–	–	–	–	*T. brevicompactum*	95	*T. brevicompactum*	EU338330	99	GQ255724
O2-59	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	100	GQ255725
O2-60	424	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255726

Table 1 (*continued*)

Clone	Length (bp)	Hallmark 1	2	3	4	*Tricho*Key	*Tricho*BLAST	Similarity (%)	BLAST	BLAST acc. no.	Similarity (%)	Genbank acc. no
a. Analysis of Trichoderma clones obtained from Oberviehhausen; hallmarks according to the diagnotic regions within ITS1 and IT2 (Kopchinskiy et al., 2005); "x" indicates presence; "–" indicates absence of the corresponding marker.												
O2-61	416	x	x	x	x	Unidentified species	*H. melanomagna*	96	*T. brevicompactum*	EU338330	99	GQ255727
O2-62	458	x	x	x	x	Unidentified species	*H. melanomagna*	96	*T. brevicompactum*	EF417484	98	GQ255728
O2-63	415	x	x	x	x	Unidentified species	*T. stromaticum*	97	*T. rossicum*	DQ083024	98	GQ255729
O2-64	424	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255730
O2-65	410	x	x	x	x	Unidentified species	*T. harzianum*	92	*H. atrogelatinosa*	DQ023302	91	GQ255731
O2-66	423	x	x	x	x	*T. brevicompactum*	*T. virens*	95	*T. brevicompactum*	EU338330	98	GQ255732
O2-67	418	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255733
O2-68	415	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255734
O2-69	425	x	x	x	x	Unidentified species	*H. virenscentiflava*	97	*T. rossicum*	DQ083024	98	GQ255735
O2-70	415	x	x	x	x	Unidentified species	*T. stromaticum*	96	*T. rossicum*	DQ083024	97	GQ255736
O2-71	413	–	x	–	–	–	*T. virens*	92	*T. brevicompactum*	EU338330	93	GQ255737
O2-72	420	x	x	x	x	*T. harzianum*	*T. harzianum*	99	*H. lixii*	FN396581	100	GQ255738
O2-74	451	–	x	–	–	–	*H. citrina*	96	*T. brevicompactum*	FJ610290	88	GQ255739
b. Analysis of Trichoderma clones obtained from Roggenstein; hallmarks according to the diagnotic regions within ITS1 and IT2 (Kopchinskiy et al., 2005); "x" indicates presence; "–" indicates absence of the corresponding marker.												
R1-1	416	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255740
R1-2	267	–	–	–	–	–	*H. crytallligena*	94	*Stachibotrys cylindrospora*	AF081474	97	GQ255741
R1-3	407	x	x	x	x	Unidentified species	*T. taiwanese*	99	*T. viride*	DQ315461	97	GQ255742
R1-4	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	FN396558	99	GQ255743
R1-5	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	99	GQ255744
R1-6	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	100	GQ255745
R1-8	419	x	x	x	x	*T. velutinum*	*T. stramineum*	97	*T. velutinum*	FN396567	97	GQ255746
R1-9	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	100	GQ255747
R1-10	439	x	x	x	–	–	*T. stromaticum*	99	*T. stromaticum*	AY856834	93	GQ255748
R1-11	414	x	x	x	x	*T. rossicum*	*T. stromticum*	97	*T. rossicum*	DQ083024	100	GQ255749
R1-12	412	x	x	x	x	*T. velutinum*	*T. stramineum*	99	*T. gamsii*	EF488161	100	GQ255750
R1-16	414	x	x	x	x	Unidentified species	*T. stromticum*	98	*T. rossicum*	DQ083024	99	GQ255751
R1-18	428	x	x	x	x	*T. velutinum*	*T. stramineum*	99	*T. velutinum*	EF596953	100	GQ255752
R1-19	409	x	x	x	x	Unidentified species	*T. taiwanese*	99	*T. viride*	DQ315461	97	GQ255753
R1-20	429	x	x	x	x	*T. velutinum*	*T. stramineum*	99	*T. sp.*	AY154940	99	GQ255754
R1-21	425	x	x	x	x	*T. cerinum*	*T. tomentosum*	99	*T. tomentosum*	AY605737	100	GQ255755
R1-22	424	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255756
R1-23	424	x	x	x	x	*T. rossicum*	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255757
R1-24	285	–	–	–	–	–	*H. virescentiflava*	98	*H. nigrovirens*	AY737777	96	GQ255758
R1-25	410	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	100	GQ255759
R1-26	410	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. viride*	DQ315461	97	GQ255760
R1-27	418	x	x	x	x	*T. velutinum*	*T. stramineum*	97	*T. velutinum*	DQ083010	98	GQ255761
R1-28	409	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	99	GQ255762
R1-29	425	x	x	x	x	*T. cerinum*	*T. tomentosum*	98	*T. tomentosum*	AY605737	99	GQ255763
R1-30	424	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255764
R1-31	430	–	x	–	–	–	*T. stromaticum*	96	*T. rossicum*	DQ083024	98	GQ255765
R1-32	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	100	GQ255766
R1-33	426	x	x	x	x	*T. cerinum*	*T. tomentosum*	98	*T. tomentosum*	AY605737	99	GQ255767
R1-34	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	100	GQ255768
R1-35	407	x	x	x	x	Unidentified species	*T. hamatum*	99	*T. hamatum*	EU264000	100	GQ255769
R1-36	362	x	x	x	x	*T. cerinum*	*T. tomentosum*	100	*T. tomentosum*	EF488143	100	GQ255770
R1-37	425	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255771
R1-38	426	x	x	x	x	Unidentified species	*T. stromaticum*	98	*T. rossicum*	EU280089	99	GQ255772
R1-39	450	–	x	–	–	–	*H. virescentiflava*	99	*H. thailandica*	AY737772	89	GQ255773
R1-40	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	99	GQ255774
R1-41	422	x	x	x	x	Unidentified species	*T. stromaticum*	97	*T. stromaticum*	DQ083013	97	GQ255775
R1-42	409	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	100	GQ255776
R1-43	422	x	x	x	x	Unidentified species	*T. aggresivum*	97	*T. tomentosum*	AY605737	98	GQ255777
R1-44	432	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488154	99	GQ255778
R1-45	420	x	x	x	x	*T. velutinum*	*T. stramineum*	97	*T. velutinum*	EF596953	98	GQ255779
R1-46	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	100	GQ255780
R1-47	428	x	x	x	x	Unidentified species	*T. stromaticum*	98	*T. rossicum*	DQ083024	97	GQ255781
R1-48	429	x	x	x	x	Unidentified species	*T. stromaticum*	99	*T. stromaticum*	DQ083013	97	GQ255782
R1-49	428	x	x	x	x	Unidentified species	*T. stromaticum*	99	*T. stromaticum*	DQ083013	97	GQ255783
R1-50	442	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255784
R1-51	425	x	x	x	x	*T. cerinum*	*T. tomentosum*	99	*T. tomentosum*	AY605737	99	GQ255785
R1-52	425	x	x	x	x	*T. cerinum*	*T. tomentosum*	99	*T. tomentosum*	AY605737	99	GQ255786
R1-53	427	x	x	x	x	*T. stramineum*	*T. stramineum*	99	*T. sp.*	AY154940	99	GQ255787
R1-54	429	x	x	x	x	Unidentified species	*T. harzianum*	97	*T. sp.*	EF488139	99	GQ255788
R1-55	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	99	GQ255789
R1-56	411	x	x	x	x	Unidentified species	*T. stromaticum*	98	*H. lixii*	FJ441021	98	GQ255790
R1-57	421	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255791
R1-58	411	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*H. vinosa*	DQ841744	100	GQ255792
R1-59	422	x	x	x	x	Unidentified species	*T. harzianum*	96	*T. harzianum*	AY154948	96	GQ255793
R1-60	433	x	x	x	x	*T. gelatinosus*	*T. thailandicum*	95	*T. sp.*	AY941822	98	GQ255794
R1-61	421	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255795

(*continued on next page*)

Table 1 (*continued*)

Clone	Length (bp)	Hallmark 1	2	3	4	*Tricho*Key	*Tricho*BLAST	Similarity (%)	BLAST	BLAST acc. no.	Similarity (%)	Genbank acc. no
b. Analysis of Trichoderma clones obtained from Roggenstein; hallmarks according to the diagnotic regions within ITS1 and IT2 (Kopchinskiy et al., 2005); "x" indicates presence; "–" indicates absence of the corresponding marker.												
R1-62	406	x	x	x	x	Unidentified species	*T. taiwanese*	99	*T. viride*	DQ315461	96	GQ255796
R1-63	424	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255797
R1-64	422	x	x	x	x	*T. polysporum*	*T. polysporum*	98	*T. polysporum*	Z48815	98	GQ255798
R1-65	416	x	x	x	x	Unidentified species	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255799
R1-67	427	x	x	x	x	Unidentified species	*T. harzianum*	92	*T. sp.*	AF408107	93	GQ255800
R1-68	421	x	x	x	x	*T. polysporum*	*T. polysporum*	100	*T. polysporum*	Z48815	99	GQ255801
R1-69	404	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	100	GQ255802
R1-70	419	x	x	x	x	Unidentified species	*T. stromaticum*	97	*T. stromaticum*	DQ083013	98	GQ255803
R1-71	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	99	GQ255804
R1-72	421	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255805
R1-73	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	99	GQ255806
R1-74	399	x	x	x	x	Unidentified species	*T. koningiopsis*	98	*T. gamsii*	EF488162	98	GQ255807
R1-75	430	x	x	x	–	*T. cerinum*	*T. tomentosum*	99	*T. tomentosum*	EF596940	93	GQ255808
R1-76	425	x	x	x	x	*T. polysporum*	*T. polysporum*	98	*H. pachybasioides*	AM498499	98	GQ255809
R1-77	408	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	EF488161	99	GQ255810
R1-78	421	x	x	x	x	*T. polysporum*	*T. polysporum*	100	*T. polysporum*	Z48815	99	GQ255811
R2-1	424	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255812
R2-2	404	–	–	–	–	–	*T. hamatum*	99	*T. hamatum*	EU264000	99	GQ255813
R2-3	418	x	x	x	x	*T. cerinum*	*T. tomentosum*	98	*T. tomentosum*	EF488143	98	GQ255814
R2-4	412	x	x	x	x	Unidentified species	*T. koningiopsis*	99	*T. gamsii*	FN396558	99	GQ255815
R2-5	439	x	x	x	x	Unidentified species	*T. chlorosporum*	92	*H. nigrovirens*	AY737777	93	GQ255816
R2-6	424	x	x	x	x	*T. rossicum*	*T. stromaticum*	97	*T. rossicum*	DQ083024	99	GQ255817
R2-7	424	x	x	x	x	*T. sp. PPRI 3559*	*T. stromaticum*	98	*T. rossicum*	DQ083024	99	GQ255818
R2-8	425	x	x	x	x	Unidentified species	*T. stromaticum*	96	*T. rossicum*	DQ083024	98	GQ255819

community composition, the *Trichoderma* community structure was clearly related to the site under investigation. In comparison to the site in Roggenstein a higher number of *Trichoderma* species was found in Oberviehhausen based on *Tricho*BLAST. This observation paralleled the significantly higher abundance of *Trichoderma*-ITS copy numbers determined for DNA from the cultivars 'Baltica', 'Désirée' and 'Sibu' by qPCR. In Roggenstein, the *Trichoderma* copy numbers determined were approximately three times lower, and also the number of different species was lower. However, qPCR also revealed an enormous variability among the replicates for the Oberviehhausen samples. This heterogeneity was also shown by DGGE fingerprint analysis. Based on the sequence analysis of the clone library, some species were found only or mainly on one site, for example *Trichoderma brevicompactum* that was found only in Oberviehhausen. An influence of the site on the composition of fungal communities has already been described (Berg et al., 2005; Weinert et al., 2009). These differences are assumed to result from local soil characteristics, climate, and management practices. These factors can influence the proportion of saprophytes or fungi that are able to degrade/survive pesticides as described for *Trichoderma* (Katayama and Matsumura, 1993). Many *Trichoderma* strains are known for their high sensitivity against chemical pesticides (Papavizas, 1995). Heterogeneous distributions of fungi in agricultural lands were found also in other studies (Rodriguez-Molina et al., 2000; Costa et al., 2006). In contrast to the site specific effects, no cultivar-specific effects were observed for bacterial communities (.Berg and Smalla, 2009). In conclusion, the novel primer system was shown to be suitable for identification of *Trichoderma* isolates and in depth cultivation-independent analysis of *Trichoderma* communities in soil. The primer system in combination with the DGGE analysis of PCR amplicons can be useful for future studies in *Trichoderma* ecology, e.g. for biogeographical or biocontrol aspects.

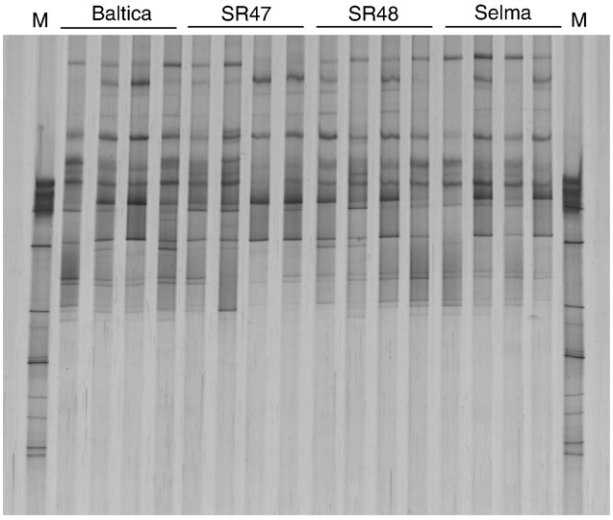

Fig. 1. DGGE fingerprints of the *Trichoderma* community at plant growth stage EC90 generated with four independent repetitions of the commercial cultivars 'Baltica' and 'Selma' and the two GM lines SR47 and SR48 grown at the field trials in Oberviehhausen in 2006. M: Fungal standard comprised of 11 fungal strains (Grosch et al., 2007) with different electrophoretic mobilities.

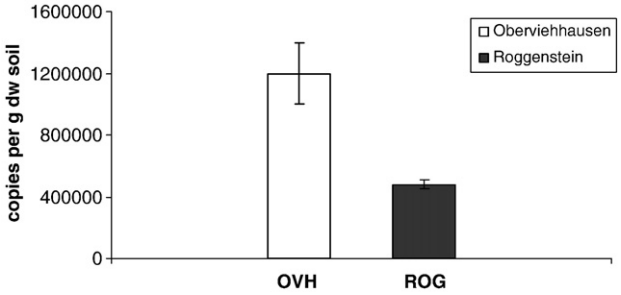

Fig. 2. Abundance of *Trichoderma* in the rhizosphere of flowering potato plants analysed by qPCR at the two sites Roggenstein and Oberviehhausen. Results from the four cultivars 'Baltica', 'Désirée', 'Ditta' and 'Sibu' as well as the transgenic lines SR47 and SR48 were used for calculation. From each cultivar respectively transgenic line replicates from four plots were analysed individually.

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