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Article

SSR Markers for *Trichoderma virens*: Their Evaluation and Application to Identify and Quantify Root-Endophytic Strains

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Abstract: Using biological fertilizers and pesticides based on beneficial soil microbes in order to reduce mineral fertilizers and chemical pesticides in conventional agriculture is still a matter of debate. In this regard, a European research project seeks to elucidate the role of root-endophytic fungi and to develop molecular tools to trace and quantify these fungi in the rhizosphere and root tissue. To do this, the draft genome sequence of the biocontrol fungus *Trichoderma virens* (*T. virens*) was screened for simple sequence repeats (SSRs) and primers were developed for 12 distinct loci. Primers were evaluated using a global collection of ten isolates where an average of 7.42 alleles per locus was detected. Nei's standard genetic distance ranged from 0.18 to 0.27 among the isolates, and the grand mean of haploid diversity in AMOVA analysis was 0.693 ± 0.019. Roots of tomato plants were inoculated with different strains and harvested six weeks later. Subsequent PCR amplification identified root-endophytic strains and co-colonization of roots by different strains. Markers were applied to qPCR to quantify *T. virens* strains in root tissue and to determine their identity using allele-specific melting curve analysis. Thus, the root-endophytic lifestyle of *T. virens* was

confirmed, strains in roots were quantified and simultaneous colonization of roots by different strains was observed.

Keywords: *Hypocrea virens*; simple sequence repeat; microsatellite; molecular marker; fragment analysis; root endophyte; biocontrol; strain identification; qPCR; monitoring

1. Introduction

DNA sequencing of the ITS1, 5.8S and ITS2 region identified the ascomycete *Hypocrea virens* (Chaverri, Samuels and Steward) as the teleomorph of *Trichoderma virens* (Miller, Giddens and Foster) Arx [1–3]. By further analyzing the newly identified *H. virens* isolate and comparing its ITS barcode with four different reference strains of the anamorph *T. virens*, both types were found to be indistinguishable [1]. *T. virens* is well known under its common name *Gliocladium virens* Mill. and is also referred to as *Gliocladium flavofuscum* Mill. or *Trichoderma flavofuscum* Mill. The genus *Hypocrea/Trichoderma* consists of about 200 species described by molecular data [4]. In molecular studies for the reconstruction of phylogenetic relationships based on RNA polymerase II subunit b (rpb2) and translation elongation factor 1 alpha (tef1) [5] as well as on ITS sequences of the rDNA repeat [6], *T. virens* shows a distinct grouping in the resulting phylogenetic trees. Tree positions are supported by high bootstrap values and are localized between sections *Longibrachiatum/Pachybasium* and within section *Pachybasium* B, respectively, with *T. crassum* Bisset being the closest relative in both cases. From these data, it can be concluded that *T. virens* is a well-defined species that can certainly be identified by molecular taxonomy approaches.

Trichoderma spp. have attracted growing interest due to their economic value and are widely used in several branches of the industry. A key aspect is the production of diverse extracellular enzymes. Enzyme preparations and living strains of *Trichoderma* are products that are commonly used in agricultural biotechnology. Living strains are utilized for bioremediation of contaminated agricultural land and *T. asperellum* Samuels H15 has demonstrated that it is able to degrade polycyclic aromatic hydrocarbons (PAHs) in soils [7], *T. viride* Pers. strain NFCCI-2745 has the capability of decomposing phenolic pollutants in soil [8] and the *T. viride* strain FRP3 has the ability to biodegrade glyphosate and to be potentially useful in areas where this herbicide is extensively used [9]. In agricultural crop production and plant protection, living strains, mainly of *T. harzianum* Rifai, *T. virens*, *T. atroviride* Karst, *T. koningii* Oudem, *T. hamatum* Bonord and *T. asperellum*, are placed close to the root system in greenhouse and field crops. Strains of these species have been identified as avirulent plant symbionts that enter root cells and have been characterized as root endophytes that form intracellular structures [10] which are assumed to be responsible for substrate exchange and molecular crosstalk. Penetration of the root system by *Trichoderma* frequently causes elevated resistance levels in their host plants via salicylate and jasmonate induced pathways [11]. Further positive effects of the root connected hyphal network include a delay in drought symptoms, enhanced root growth and nutrient uptake. Therefore they are often used as so-called biofertilizers or biostimulants and are available on the market as formulated spore-inoculum products. In natural ecosystems, *Trichoderma* species have been recognized as common and persistent members of the soil microbiome and rhizosphere microbial communities [12].

Mycoparasitism is a common feature among *Trichoderma* spp. but is only rarely reported in other families of the fungi [13,14]. The antagonistic mode of primarily *T. virens*, *T. harzianum* and *T. asperellum* against other fungi and particularly soil-borne plant pathogenic fungi is exploited by the development of biocontrol agents and biological control strategies in integrated plant protection. *Trichoderma* biocontrol products are well established on the market and available as spore-inocula. Numerous reports exist about inhibition of prominent fungal plant pathogens by *Trichoderma* spp., among these, *Rhizoctonia solani* Kühn, *Fusarium solani* Mart. and *Sclerotinia sclerotiorium* de Bary [15]; *Fusarium culmorum* Sm. and *Fusarium graminearum* Schwabe [16]; and *Alternaria altanata* Kreissl., *Botrytis cinerea* Pers. and *Fusarium* spp. [17]. The mode of action in terms of direct interaction is based on a mycotrophic lifestyle including the production of hydrolytic cell wall degrading enzymes like chitinase and ß-1-3 glucanase [18] as well as biocidal substances [19]. *Trichoderma* spp. actively attack other fungi by coiling around their hyphae releasing the above-mentioned substances and feeding on the parasitized mycelia, which often leads to de-vitalization of the prey fungus. This sometimes also causes negative impact in the commercial production of culinary fungi like *Agaricus bisporus* Lange and *Pleurotus ostreatus* Krumm. There are even isolates that are effective against members of the Oomycota through direct interaction [20] or by triggering defense reactions in their host plants [21].

The estimation of root colonization efficiencies of different *Trichoderma* strains, their ability to induce systemic resistance in host plants and their capability of inhibiting plant pathogenic fungi in the rhizosphere under field conditions requires precise re-identification to track and trace strains experimentally released to the root system. In this context, diagnostic molecular markers are a prerequisite for studying plant-microbe interaction, the persistence of released strains in the rhizosphere and their impact on rhizosphere microbial communities. Despite the fact that three complete genome sequences (*T. reesei* Simmons, *T. virens* and *T. atroviride*) are available on the ISTH webpage (www.isth.info/links.php, International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy) genomic data have not yet been intensively exploited for the development of locus-specific molecular markers. Reports about this marker type are sparse in *Trichoderma* spp. and comprise solely the development of sequence characterized amplified regions (SCAR markers) obtained from sequence data of cloned random amplified polymorphic DNA (RAPD) fragments [22,23]. The vast majority of studies on discriminating the strains of *Trichoderma* species are based on anonymous markers like (universally primed) UP-PCR, RAPDs or restriction fragment length polymorphisms (RFLPs) [24–26]. The anonymous approach also holds true for *Trichoderma* microsatellite studies, where ISSRs (inter simple sequence repeat) and RAMS (random amplified microsatellites) have been used so far [27,28]. To our knowledge, no sequence-tagged microsatellite site (STMS) markers based on PCR amplification of unique SSR loci [29] are currently available for *Trichoderma* spp. Therefore, the invaluable genomic information of *T. virens* was exploited to develop a set of SSR markers that is capable of identifying strains in root tissue, rhizosphere soil and environmental samples. Moreover, markers were tested for their potential use in real-time PCR applications to estimate and quantify root colonization efficiencies of certain strains and combinations of strains. Furthermore, transferability of the markers to other *Trichoderma* species, in particular to *Trichoderma harzianum*, was evaluated.

2. Experimental Section

2.1. Screening of Databases and Primer Design

The *T. virens* genome sequence was accessed via the ISTH website by clicking on the links button and going to the genome portal of the Department of Energy Joint Genome Institute [30]. In total, 93 scaffolds ranging from 4.407 bp (scaffold 7) to 3,456,807 bp (scaffold 6) were available and downloaded, of which scaffold 7 was by far the smallest. The majority of the scaffolds were larger than 20 kb and 43 were larger than 50 kb. Roughly 9 scaffolds were at least 1.7 Mb in length and covered half of the genome. The total genome size was 39 Mb containing 12,427 structurally and functionally annotated genes. Further information is available on the homepage of the U.S. Department of Energy's Joint Genome Institute. All scaffolds (in FASTA format) were loaded into the BioEdit Sequence Editor (Ibis Biosciences: Carlsbad, CA, USA) by using the select/slide mode. This made it possible to search for all scaffolds in one go. Screening the genome for microsatellite motifs was based on published data about existing motifs that were detected in *T. virens* by ISSR and RAMS analysis [31,32]. Microsatellite loci sufficiently flanked by unique sequences were selected for primer design using the Primer3 software [33] with parameter settings that included the GC-clamp option and an allowance for primer annealing at approximately 60 °C. The primer sequences obtained were searched in the genome of *T. virens* to avoid multiple annealing sites. After that, about half of the developed primers (Table 1) displayed clear single-band PCR amplification products on agarose gels, while in some cases stuttering bands became visible on PAA gels when the same primers were used.

2.2. Fungal Strains

In order to evaluate the variability at the selected microsatellite loci, a set of *T. virens* isolates of geographically diverse origin (Table 2) was obtained from the CBS-KWAS (Centraalbureau Schimmelcultures–Koninklijke Nederlandse Academie van Wetenschappen) Fungal Biodiversity Centre (Utrecht, The Netherlands). The cultures were grown according to the supplier's recommendations and the DNA was isolated from liquid cultures as described below. The molecular fingerprints of the CBS isolates obtained were compared to a local strain of *T. virens*, which was isolated from maize roots in August 2012. Briefly, the roots of a healthy *Zea mays* L. plant from a maize field in Central Germany (silty loam loess chernozem, pH 7.2–7.5, 2.5%–3.0% humus, 80 m a.s.l., 51°80′ N, 11°73′ E, 511 mm average annual precipitation, 9.7 °C average annual temperature) were obtained by carefully removing residual soil and selecting fine roots (1–2 mm in diameter). The roots were thoroughly washed with sterile water and cleaned with a soft brush to remove any remaining soil. The cleaned roots were submerged in 3% Na-hypochlorite for 5 min, rinsed with sterile water, cut into pieces (approximately 1.5 cm long) and placed on a Petri dish containing potato dextrose agar (PDA, 1.5%, pH 5.8). Outgrowing mycelia were transferred to fresh plates and grown at room temperature until sporulation. A 20 µL spore suspension was taken, diluted 100-fold and 10–100 µL were plated on fresh PDA. One colony originating from a single spore was selected and the mycelium cultivated further. Agar blocks (approximately 5 mm × 5 mm × 5 mm) were excised from actively growing mycelium and stored at −80 °C in a solution containing 50% glycerol, 25% culture medium and 25% distilled water. A liquid culture was prepared and DNA isolated as described below. The ITS region of the nuclear rDNA was

amplified using the primers ITS1F [34] and ITS4 [35] and the resulting PCR fragment directly sequenced following the Sanger method. An online homology search was carried out in the NCBI GenBank nucleotide database by means of the algorithm BLASTn and results confirmed the identity of the isolate, which was then designated as *Trichoderma virens* strain Maize9B.

In order to test for cross-species amplification the primers derived from the *T. virens* genome were applied to genomic DNA of a commercially available *T. harzianum* isolate, notably strain T22 (Koppert B.V.: Berkel en Rodenrijs, The Netherlands).

2.3. Culture Conditions

Conserved fungal cultures were retrieved from −80 °C stocks by thawing the tubes on ice, removing one agar block, rinsing it with sterile water to wash off residual glycerol and placing it on a Petri dish containing potato dextrose agar (1.5%, pH 5.8). Plates were incubated at 20 °C in a 12/12 h day/night cycle. Outgrowing mycelia from the agar blocks were either left until sporulation to harvest spores for root inoculation or—after 4 to 5 days of growth—small agar blocks were taken from the actively growing front of the mycelium and placed in Erlenmeyer flasks containing 50 mL potato dextrose bouillon (pH 5.8). Cultures were incubated on a rotary shaker (120 rpm) under the same conditions as above and mycelia for DNA isolation were harvested after 2 to 3 days by filtration.

2.4. Plant Material and Root Inoculation

All root inoculation experiments were performed with tomato plants *Solanum lycopersicum* L. cv. MOBIL (Breeding Company: ZKI, Zöldségtermesztési Kutató Intézet, Kecskemét, Hungary). This variety is a robust cultivar frequently used in Hungary for field production of tomatoes.

Well sporulated plates of *T. virens* were covered with a layer of 25 mM $MgSO_4$ solution, left to rest for 20 min and then gently stirred with a glass rod to obtain a dark green spore suspension which was transferred to fresh tubes. Spore concentrations were determined using an Abbe-Zeiss counting cell chamber and spore concentrations adjusted to 5×10^7 spores/mL by either diluting or centrifuging the suspension.

Tomato seeds were germinated in Fruhstorfer Soil Type P (Hawita-Group: Vechta, Germany) and seedlings (10 to 12 cm in height) were transferred to 2.5 L planting pots containing 2 kg (dry weight) of local natural farmland topsoil as described above for the maize field in Central Germany. Before transplanting, the soil was pre-wetted, a planting hole opened in the pots, and 2.5×10^4 spores/g substrate pipetted into the holes (1 mL of the stock solution). Seedlings were carefully removed from the germination soil and transplanted into the prepared planting cavity. In the case of co-inoculation with two different *T. virens* strains half of the amount of each isolate was pipetted into the same planting cavity to reach the same spore concentration as single inoculations (2 × 0.5 mL stock solution from each isolate). Inoculated seedlings were grown under semi-controlled greenhouse conditions. Pots were watered on demand from the trivet, temperatures ranged from 18 to 26 °C with a day/night cycle of 10/14 h at an illumination intensity of 20,000 Lux. Roots were harvested 42 days after inoculation and the DNA was immediately isolated as described below.

Table 1. PCR primer sequences for unique microsatellite loci in *T. virens*. Repeat numbers in primer names refer to the *T. virens* reference genome. "Fragment Sizes" were determined in isolate Maize9B; "Range" refers to the other strains analyzed in this study and "Reference Sequence" to the virtual fragment size in the reference genome. "Scaffold No." marks the super contig from which loci were retrieved and the *T. harzianum* column shows which markers were transferable to *T. harzianum*. Primers for multiplex PCR are in bold.

Primer Name	Primer Sequence (5′-3′)	Locus No.	Tm	Fragment Sizes in bp: Maize9B, (Range), Reference Sequence	NCBI Accession No.	Alleles	Scaffold No.	*T. harzianum*
$TvCTT_{56}$f	CTTGATGACAAGCCAAAAGG		58.4	289 (283–439) 456	KM010303	8	64	−
$TvCTT_{56}$r	GAAGAGAGGACATAGGGTCTGG	L1	59.2					
$TvCAT_{32}$f	GTGTAGCAGCCCAACAGTCC		60.7	409(364–457) 481	KM010304	8	89	−
$TvCAT_{32}$r	CAGGTGTCGTGACAGATTCG	L2	60.3					
$TvCTTT_{29}$f	GGAAGATAGCACGATGAAGTCG		61.1	350 (291–410) 402	KM010305	9	81	−
$TvCTTT_{29}$r	AACCGTGGAAGTAGGTGTCG	L3	60.0					
$TvCTTTT_{27}$f	TCATCCACCCTGCTAACTCG		61.2	420 (378–563) 482	KM010306	9	81	−
$TvCTTTT_{27}$r	CGCTGCGTCATCCTAAACC	L4	61.7					
$TvAAC_{21}$f	CACCATTCCATTATTACGCGACG		60.4	234 (210–269) 268	KM010307	7	2	+
$TvAAC_{21}$r	CTGCACTCCCTCCCAATGC	L5	60.8					
$TvCAG_{13}$f	CCCAGGAAACCCTCAGAACG		60.3	180(161–180) 206	KM010308	7	92	−
$TvCAG_{13}$r	TCTTTGCAGTTTCCAAGTCGG	L6	59.1					
$TvGAAA_{34}$f	GGGGTGCTGAATAGCTAACG		59.7	325 (315–491) 423	KM010310	6	3	+
$TvGAAA_{34}$r	TGCCGTCTTGTCTTATTTTCG	L7	60.3					
$TvTGTC_{18}$f	GTGGTGAGGACTTGCTTGG		59.3	425 (393–469) 483	KM010311	7	2	+
$TvTGTC_{18}$r	TCTGCCTGTCAGTTGTTTGC	L8	60.0					
$TvGAT_{18}$f	GGGATCTGATTTGGCCTACC		60.7	371 (333–423) 387	KM010312	8	3	+
$TvGAT_{18}$r	ACTTCCCCCATCCAATAACG	L9	60.9					
$TvCA_{39}$f	GCATCTGCACCTGATATATTCC		58.6	256 (236–271) 306	KM010313	8	6	−
$TvCA_{39}$r	CCTTGTACGATCTCCAGAACC	L10	58.7					
$TvGTT_{23}$f	GCATCAAAGCGTGCTGTTGG		60.8	216 (206–237) 279	KM010309	4	87	−
$TvGTT_{23}$r	GCAAACACAAGCTGACAATGC	L11	60.6					
$TvAG_{29}$f	TGTGCCCACTGAGATTTCG		60.8	449 (423–462) 470	KM010314	8	93	−
$TvAG_{29}$r	TCAGCATGAGATTACACATACCG	L12	60.0					

Table 2. CBS numbers of the utilized *T. virens* strains with additional information retrieved from the CBS catalogue.

Isolate Origin	CBS Number	Habitat	Year of Isolation
Ivory Coast	CBS 123790	soil	Unknown
Italy	CBS 116947	sandy soil, *Pinus pinea*	1982
Iran	CBS 111249	soil	2001
Guadeloupe	CBS 100946	rain forest soil	1998
Papua New Guinea	CBS 350.96	coastal region soil	1995
The Netherlands	CBS 609.95	compost	1995
Australia (Perth)	CBS 497.84	sandy soil, lettuce	1984
Moldova	CBS 512.66	soil	1966
USA (Maryland)	CBS 430.54	soil	1954
Germany	-	maize roots	2012

2.5. DNA Extraction

2.5.1. Mycelia

Fungal DNA of approximately 100 mg fresh mycelium from liquid culture was extracted by using the peqGOLD Fungal DNA Mini Kit (VWR Peqlab: Erlangen, Germany). Mycelia were filtered, briefly dried between tissue paper and mechanically homogenized in 2 mL tubes containing 700 µL peqGOLD lysis buffer in a FastPrep24 instrument (MP Biomedicals: Heidelberg, Germany) for 3 × 30 s at a speed of 6 m/s with 1.0 mm silica spheres and an additional 0.25 inch ceramic bead. After each homogenization cycle samples were cooled on ice for 1 min. DNA was further extracted following the manufacturer's instructions. The DNA was tested on 1% agarose gels and concentrations were determined using the NanoDrop ND-1000 photometer (VWR Peqlab).

2.5.2. Roots

Tomato roots were carefully removed from the planting pots, thoroughly cleaned with water and a soft brush until no residual soil adhered, briefly dried between paper towels, and cut into small pieces. Approximately 100 mg of fine roots were placed in 2 mL tubes containing 1.0 mm silica spheres and a single 0.25 inch ceramic bead (MP Biomedicals) and 700 µL peqGOLD lysis buffer, homogenized for 30 s at a speed of 6 m/s three times (FastPrep 24 device, MP Biomedicals). After each cycle, samples were cooled on ice for 1 min. DNA was extracted with the peqGOLD Fungal DNA Kit (VWR Peqlab) following the manufacturer's instructions. DNA quality was determined as described above.

2.5.3. Leaves

DNA from tomato leaves was extracted with the Plant Fast DNA Spin Kit (MP Biomedicals: Heidelberg, Germany), following the supplier's instructions. Tissue disruption with the FastPrep 24 device was performed similarly to that of root tissue. Leaf DNA was used to ensure that the *T. virens* SSR primers did not show cross-reactions with tomato DNA, which could cause confusion when examining results from in-root detection of fungal endophytes.

2.6. PCR Conditions

2.6.1. Touchdown PCR

PCR was carried out in 20 µL volumes containing 10 ng of extracted DNA, 0.5 µM of each primer and 2× Phusion High-Fidelity PCR Master Mix (Thermo Scientific: Schwerte, Germany). Microsatellite amplification was performed in a thermal cycler (Labcycler, SensoQuest: Göttingen, Germany) with the following thermal profile: initial denaturation at 96 °C for 3 min, 9 cycles consisting of 95 °C for 20 s, 64 °C for 25 s (−0.5 °C per cycle), 72 °C for 30 s, followed by 31 cycles consisting of 95 °C for 20 s, 60 °C for 25 s, and 72 °C for 30 s. A final elongation step at 72 °C for 5 min completed the protocol.

2.6.2. Multiplex PCR

For multiplex PCR the primer pairs $TvCTTTT_{27}$, $TvGAAA_{34}$ and $TvCAG_{13}$ (0.5 µM each) were used in a single PCR. PCR conditions and thermal profile conformed to touchdown PCR conditions.

2.6.3. In-Root Detection of Fungal DNA

In-root detection of endophytic *T. virens* strains was performed with primer pairs $TvGAAA_{34}$ and $TvCTT_{56}$, respectively. PCR conditions and thermal profile were identical to touchdown PCR, except that 50 ng of total root DNA was used for specific amplification of the fungal DNA fraction.

2.6.4. Real-Time PCR

Real-time PCR was performed using a PikoReal 96 real-time PCR system (Biozym: Hessisch Oldendorf, Germany). PCR reactions consisted of 0.5 µM forward and reverse primers ($TvGAAA_{34}$ and $TvCTT_{56}$, respectively), 2× Phusion High-Fidelity PCR Master Mix, 1× SybrGreen I Nucleic Acid Stain (Lonza: Basel, Switzerland), and a dilution series of DNA with a total volume of 20 µL. The following thermal profile was used: initial denaturation at 96 °C for 3 min, 37 cycles consisting of 95 °C for 20 s, 60 °C for 25 s, and 72 °C for 30 s. After amplification, a melting curve was created by increasing the temperature from 60 °C to 95 °C in 0.2 °C steps. The results were analyzed using the PikoRealTM 2.2 software.

2.7. Electrophoresis

2.7.1. Agarose Gels

PCR products from microsatellite amplification were checked on 3% agarose gels containing 2.5 µL Roti-GelStain/50 mL 1× TAE buffer (Carl Roth: Karlsruhe, Germany). Electrophoresis was performed at 6 V/cm for approximately 45 min. Then the gels were documented under UV light (Genius Bio Imaging System, Syngene: Cambridge, UK).

2.7.2. Polyacrylamide (PAA) Gels

5% Mini-Protean TBE precast PAA gels (Bio-Rad: Munich, Germany) were used to analyze small molecular weight differences caused by variable microsatellite repeats. Electrophoresis was conducted

in a 1× TBE buffer at 10 V/cm for 90–120 min depending on fragment sizes using the Mini-Protean Tetra Cell System (Bio-Rad). Gels were stained with ethidium bromide (5 µL ethidium bromide (10 mg/mL)/100 mL 1× TBE buffer) for 15 min on a rotary shaker. After washing with water, the gels were de-stained with double-distilled water for 15 min on an orbital shaker. Finally, the gels were photographed under UV light (Genius Bio Imaging System, Syngene: Cambridge, UK), the images were imported into the phoretix grabber software version 3.01 (Phoretix International: Newcastle upon Tyne, UK), and fragments sizes were calculated by the software according to molecular weight standards co-separated with the SSR fragments.

2.7.3. Capillary Gel Electrophoresis

The CEQ 8000 capillary sequencer device was used (Beckman Coulter: Pasadena, CA, USA) for fragment analyses of multiplex PCR. Multiplex PCR was performed with Cy5-labeled forward primers and unlabeled reverse primers. PCR products were diluted 100–200 fold and then mixed with a 30 µL sample loading solution (Beckman Coulter) and 0.5–1 µL of 10 fold diluted 20 bp DNA standard ladder (CEQ DNA Size Standard Kit—600, Beckman Coulter). A drop of mineral oil was placed on the sample and the method "Frag-4" was started (capillary temperature 50 °C, sample denaturation at 90 °C for 120 s, injection with 2.0 kV for 30 s, and separation with 4.8 kV for 60 min). Fragment sizes were determined using the CEQ Main Software, Version 9.0.25.

2.8. Re-Sequencing

Extracted DNA of *T. virens* strain Maize9B was amplified using all 12 microsatellite primer pairs (Table 1). PCR products were excised from agarose gels and purified using the Wizard SV Gel and PCR Clean-Up Kit (Promega: Madison, WI, USA). The concentration of the purified fragments was determined using the NanoDrop ND-1000 photometer (VWR Peqlab). Fragments were ligated into the pJET 1.2 blunt cloning vector (CloneJET PCR Cloning Kit, Life Technologies: Paisley, UK) and transformed in competent *E. coli* JM109 cells. One positive clone of each microsatellite amplification product was sequenced in forward and reverse orientation using a 30 pmol primer in 10 mM Tris/HCL following the Sanger method. Sequence alignments were carried out using the Vector NTI Advance 11.5 software (Life Technologies: Carlsbad, CA, USA). Complete microsatellite sequences of 12 distinct loci were submitted to NCBI (GenBank accession numbers: KM010303 to KM010314).

2.9. Statistical Analysis

To establish the relationships and to assess the genetic diversity of the analyzed *T. virens* isolates, a 0/1 matrix was prepared from the absence and presence of bands at distinct molecular weights through microsatellite amplification. The resulting matrix (Supplementary Material 1) was then used to calculate D_{ST}, Nei's standard genetic distance [36]. The molecular variance (AMOVA) was analyzed using GenAlEx 6.5 software [37,38]. Haploid diversity by population and haploid diversity by locus were determined and values for numbers of different alleles (Na), numbers of effective alleles (Ne), Shannon's information index (I), diversity (h) and unbiased diversity (uh) were calculated. For AMOVA, isolates were split into 2 populations (the first five isolates originating from the Eurasian continent and the second

five isolates originating from the Americas, Australia, the Pacific and Africa). Phi_{PT} (modified F_{ST}) was calculated among and within populations by applying 999 permutations to estimate the total molecular variance and molecular variance by locus. The probability (P) of Phi_{PT} was based on standard permutations across the full data set (Supplementary Material 2).

3. Results and Discussion

3.1. Primer Evaluation on a Global Set of Isolates

Next-generation sequencing technologies accelerate and facilitate SSR marker development from whole genome data. This was recently exploited for diverse plant and fungal species [39,40]. As is frequently found, the 39 Mb draft genome sequence of *T. virens* also harbors considerable amounts of repetitive DNA, especially simple sequence repeats suited for microsatellite marker development. Common motifs were found on almost all scaffolds larger than 40 kb, among them long stretches of perfect tandem repeats flanked by unique sequences adequate for primer design. This was confirmed by re-sequencing all selected loci in *T. virens* strain Maize9B, in which the sequenced fragments contained the expected microsatellite motifs (Table 1). All primers generated amplification products in every strain tested and no null alleles were observed. In other cases, null alleles were observed in up to eight of the 24 loci analyzed [41]. This indicates intense genomic variability or points to a species group with vague species boundaries. Further findings detected null alleles as a consequence of host-specific differentiation followed by reproductive isolation [42]. The absence of null alleles in the diverse collection of *T. virens* presented is another indicator for a well-defined species concept in this regard. Furthermore, all isolates displayed single bands as is expected with haploids; there was no evidence of gene duplication events or even dikaryotic phases in the tested specimens. Gene duplication events are rarely detected by microsatellite analysis of haploids, but examples do exist e.g., for *Beauveria bassiana* Vuill. (*Hypocreales*), an entomopathogenic fungus that is also commercialized as a biocontrol agent in the integrated pest management of insects [43]. Fragment analysis (Figure 1) revealed in our survey that especially the di-nucleotide repeats AG/CT_n and CA/TG_n from scaffolds 93 and 6 showed stuttering bands on PAA gels, visible in the image sections as double bands, while fragments from the larger motifs (tri-, tetra- and penta-nucleotide motifs) displayed clear single banding patterns. This problem has been described in SSR fingerprinting and led to the optimization of SSR marker sets for genotyping by avoiding di-nucleotide repeats [44], with the advantage of better separation of alleles and the elimination of uncomfortable stuttering, but with the draw-back that polymorphic markers are excluded.

The numbers of amplified alleles per locus ranged from four and six for L11 and L7, respectively, up to nine alleles for loci L3 and L4 (Table 1). Fragment sizes ranged from 161 bp (L6) to 563 bp at L4. In the latter, the greatest variation at a single locus was also observed with the smallest allele being 378 bp and the largest 563 bp in size. No correlation between motif and information content could be observed. On the one hand, the motifs $(CTTT)_n$ and $(CTTTT)_n$ were the most informative (nine alleles within 10 individuals), while the motifs $(GAAA)_n$ and $(GTT)_n$ were only moderately informative with four and six alleles detected in the sample set. The dinucleotide motifs at loci L10 and L12 were highly informative (eight alleles each), as commonly reported in SSR studies, where di-nucleotide repeats are recognized as being the most common and polymorphic in many plant and fungal species [45].

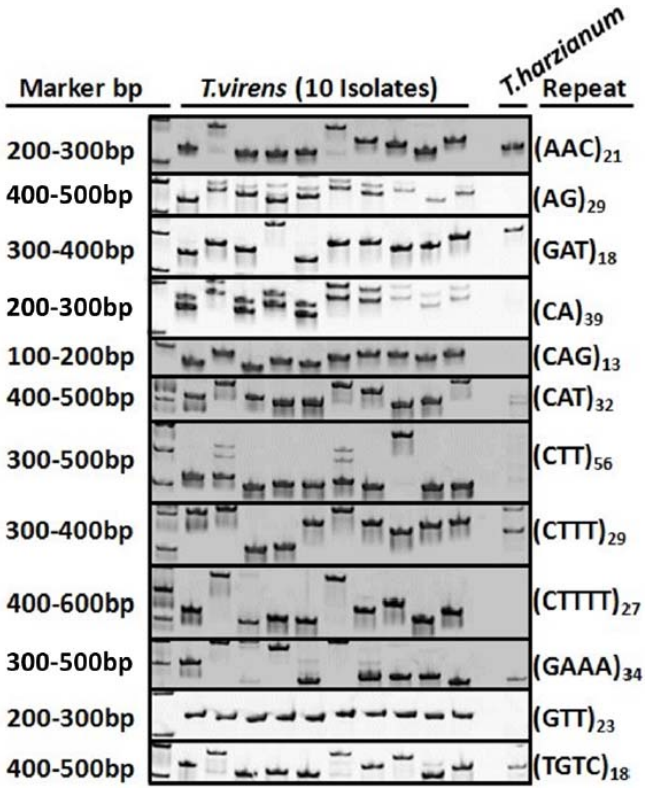


Figure 1. Fragment analysis at 12 microsatellite loci of *T. virens* examined on 5% PAA gels. Sample order from left to right: 100 bp DNA length standard, 10 *T. virens* isolates (Italy, Australia, Iran, Papua New Guinea, Ivory Coast, USA, Germany, The Netherlands, Guadeloupe, and Moldova), blank lane, *T. harzianum* isolate T22.

Data for transferability of the developed primer pairs to *T. harzianum* are shown in Figure 1. Primers developed from loci L5, L7, L8 and L9 (Table 1) also generated fragments in the expected size ranges in *T. harzianum*. Primers from L3 produced a clear double band, while the remaining primers did not generate any signals or display minor diffuse bands not suited for routine use in *T. harzianum*. Since only one single isolate of *T. harzianum* was tested, no conclusion can be drawn about the information content of these primers in this species. Further tests for cross-species amplification of the primer set—mainly to evaluate its specificity for *T. virens*—were conducted with further species of the *Trichoderma* genus (*T. lutea*, *T. koningii*, and *T. reesei*), with specimens from the order *Hypocreales* that include *Trichoderma* (*Neonectria* and *Eucasphaeria*) and further isolates from the Ascomycota (*Penicillium*), *Mucoromycotina* (*Mortierella*) and *Basidiomycota* (*Piriformospora* and *Tricholomata*). Minor bands in the expected size ranges were visible in the tested *Trichoderma*/*Hypocrea* species at loci $(ATT)_{21}$, $(CAG)_{13}$, $(CTT)_{56}$ and $(CTTTT)_{27}$. In the species other than *Trichoderma*, minor bands were detectable at the $(CAG)_{13}$ locus (*Mortierella* and *Neonectria*) and in *P.indica* Varma at locus $(CTT)_{56}$ (Supplementary Material 3). Cross-species amplification of genomic SSRs from non-coding regions has also been reported in fungi [46], but efficiency of transferability is considerably higher with SSRs developed from expressed genomic regions. The consequence is that a growing number of SSRs are isolated by mining expressed sequence tags to obtain so-called EST-SSRs [47]. These display higher synteny and are well suited for transferability across species or even genus borders.

No considerable bands were observed in control PCRs solely containing leaf DNA from tomato *Solanum lycopersicum* L. cv. MOBIL. This indicates that there are no foreseeable difficulties in verifying root colonization of endophytic *T. virens* strains in this tomato variety.

3.2. Data Analysis

Nei's genetic distance was calculated from 89 SSR alleles (average 7.42 alleles per locus) converted into a 0/1 matrix (Supplementary Material 1). Calculations revealed that isolates from the USA and Australia differed in only eight closely related alleles, as estimated from the fragment sizes. This is contrary to expectations due to the wide geographical separation (Maryland, USA and Western Australia) and over 30 years of diverging sampling times (Table 2). Since both isolates were obtained from the same strain collection, a mislabeling cannot be excluded. DNA fingerprinting techniques are frequently used to verify strain and germplasm collections and successfully discover mislabeling or identify duplicates [48]. Pairwise genetic distances within the *T. virens* set ranged from 0.18 to 0.27 (Table 3), with the least genetic distance between the isolates from the USA and Australia (0.18).

AMOVA results indicated low regional differentiation in the sample set. Based on 68 best differentiable alleles (Supplementary Material 2) and by dividing the sample set into two populations (five isolates from the Eurasian continent and five isolates from the rest of the world) analysis revealed that most molecular variance occurred within (96.92%), rather than between, populations (3.08%). This is quite a common observation in ubiquitously existing species and especially in sexually reproducing fungi with airborne ascospores [49,50]. Differentiation between populations in our study was detected at only four of the twelve loci (Table 1; L5, L6, L7 and L10) ranging between 2% and 15%.

Table 3. Pairwise genetic distances matrix from the *T. virens* set of isolates. Range: 0.18–0.27, number of sequences: 12, active sites: 89, number of constant sites: 0, number of non-parsimony informative sites: 31, fit: 97.97, least squares fit: 99.93.

	Italy	**Austr**	**Iran**	**PapNG**	**IvoryC**	**USA**	**Germa**	**Netherl**	**Guade**	**Moldo**
Italy	0.00	0.270	0.247	0.225	0.270	0.270	0.241	0.247	0.225	0.270
Australia	0.270	0.00	0.247	0.270	0.270	0.180	0.247	0.247	0.247	0.247
Iran	0.247	0.247	0.00	0.202	0.202	0.247	0.247	0.270	0.270	0.247
Papua New Guinea	0.225	0.270	0.202	0.00	0.247	0.270	0.270	0.270	0.225	0.270
Ivory Coast	0.270	0.270	0.202	0.247	0.00	0.270	0.247	0.270	0.247	0.270
USA	0.270	0.180	0.247	0.270	0.270	0.00	0.247	0.247	0.270	0.270
Germany	0.247	0.247	0.247	0.270	0.247	0.247	0.00	0.247	0.225	0.247
The Netherlands	0.247	0.247	0.270	0.270	0.270	0.247	0.247	0.00	0.247	0.247
Guadeloupe	0.225	0.247	0.270	0.225	0.247	0.270	0.225	0.247	0.00	0.247
Moldova	0.270	0.225	0.247	0,270	0.270	0.270	0.247	0.247	0.247	0.00

Haploid diversity (h) by locus (Table 4) was calculated and the main indicators for diversity (h and I) were highest and had equal values at L3, L9 and L10 (h = 0.800, I = 1.609) for population 1 (Eurasian continent) and at L2 and L4 (h = 0.800, I = 1.609) for population 2 (rest of the world). The grand mean (total) and standard error (SE) for all loci and populations was h = 0.639 ± 0.019 and I = 1.269 ± 0.055. The means of I and h for haploid diversity by population (Table 5) were 1.323 ± 0.075 and 0.713 ± 0.025 for population one, and 1.214 ± 0.081 and 0.673 ± 0.029 for population two,

respectively. This indicates that the isolates from the Eurasian continent were more diverse than the isolates from population 2. Based on the mean for all loci and populations, loci L3 and L4 were the most informative with h = 0.760 ± 0.04 and I = 1.471 ± 0.139. This is also indicated by the high values for Na and Ne at L3 and L4, where Ne (4.286 ± 0.714) approximated Na (4.5 ± 0.5, Table 4). Haploid diversity values in a range of approximately 0.75 account for high genetic diversity in the *T. virens* collection, although in some cases h-values higher than 0.9 were found, for instance in a large collection of *Aspergillus flavus* Link strains (87 isolates) that were checked at 29 EST-SSR loci with up to 24 alleles per locus and h-values as high as 0.91 [51].

Table 4. Haploid diversity by locus. Number of loci = 12, number of samples = 10 (5/5), number of populations = 2. **Na** = number of different alleles; **Ne** = number of effective alleles (= 1/(Sum pi^2)); **I** = Shannon's information index (= 1 − Sum (pi × Ln (pi))), **h** = diversity (= 1 − Sum pi^2); **uh** = unbiased diversity (= (N/(N − 1)) × h), where pi is the frequency of the ith allele for the population and Sum pi^2 is the sum of the squared population allele frequencies.

		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	Total
	Na	4	3	5	4	4	4	4	4	5	5	2	4	**48**
	Ne	3.571	2.778	5.000	3.571	3.571	3.571	3.571	3.571	5.000	5.000	1.923	3.571	
Pop.1	**I**	1.332	1.055	1.609	1.332	1.332	1.332	1.332	1.332	1.609	1.609	0.673	1.332	
	h	0.720	0.640	0.800	0.720	0.720	0.720	0.720	0.720	0.800	0.800	0.450	0.720	
	uh	0.900	0.800	1.000	0.900	0.900	0.900	0.900	0.900	1.000	1.000	0.600	0.900	
	Na	4	5	4	5	4	2	3	4	3	3	3	4	**44**
	Ne	3.571	5.000	3.571	5.000	3.571	1.923	2.273	3.571	2.273	2.273	2.273	3.571	
Pop.2	**I**	1.332	1.609	1.332	1.609	1.332	0.673	0.950	1.332	1.055	0.950	1.055	1.332	
	h	0.720	0.800	0.720	0.800	0.720	0.480	0.560	0.720	0.640	0.560	0.640	0.720	
	uh	0.900	1.000	0.900	1.000	0.900	0.600	0.700	0.900	0.800	0.700	0.800	0.900	
Mean and SE of Loci for each Population and Grand Mean (Total) and SE of Loci and Populations														
Mean	**Na**	4.000	4.000	4.500	4.500	4.000	3.000	3.500	4.000	4.000	4.000	2.500	4.000	**3.833**
SE		0.000	1.000	0.500	0.500	0.000	1.000	0.500	0.000	1.000	1.000	0.500	0.000	**0.177**
Mean	**Ne**	3.571	3.889	4.286	4.286	3.571	2.747	2.922	3.571	3.889	3.636	2.350	3.571	**3.524**
SE		0.000	1.111	0.714	0.714	0.000	0.824	0.649	0.000	1.111	1.364	0.427	0.000	**0.194**
Mean	**I**	1.332	1.332	1.471	1.471	1.332	1.003	1.141	1.332	1.332	1.280	0.864	1.332	**1.269**
SE		0.000	0.277	0.139	0.139	0.000	0.330	0.191	0.000	0.277	0.330	0.191	0.000	**0.055**
Mean	**h**	0.720	0.720	0.760	0.760	0.720	0.600	0.640	0.720	0.720	0.680	0.560	0.720	**0.693**
SE		0.000	0.080	0.040	0.040	0.000	0.120	0.080	0.000	0.080	0.120	0.080	0.000	**0.019**
Mean	**uh**	0.900	0.900	0.950	0.950	0.900	0.750	0.800	0.900	0.900	0.850	0.700	0.900	**0.867**
SE		0.000	0.100	0.050	0.050	0.000	0.150	0.100	0.000	0.100	0.150	0.100	0.000	**0.024**

For Phi statistics based on locus, data was entered as a haploid distance matrix and the Phi_{PT} (modified fixation index) was calculated in order to analyze molecular variance (Table 6). As mentioned above, fixation of alleles in one of the two populations was only observed at four loci (L5, L6, L7 and L10). Thus, positive values for Phi_{PT} ranging from 0.022 to 0.148 were only observed at these loci, while the remaining loci exhibited negative values, indicating no differentiation between the two populations. Differences between populations can be expected since conditioning, selection and genetic bottlenecks, paired with fitness components like germination and growth rate, drive allele fixation [52]. In contrast,

fluctuation of spores in a global meta-population (e.g., transport of goods in a globalized world) leads to repetitive founder effects that counteract selection, decrease fixation probability and influence time to fixation, where distribution and re-distribution of spores acts as a regional mixing force [53]. This is congruent with the conclusion that most genetic variance in this study was detected within, rather than between, populations. Nevertheless, values for probability of Phi_{PT} ranged from 0.129 to 1.0 and, consequently, were not significant at the $p < 0.05$ level. This is most probably due to the small sample size and the rather artificial division of the sample set into two populations. Significant results are achieved, especially in large sample sets, that were collected following a strict sampling strategy [54], while non-significant results are mainly restricted to studies where only small sample sets were available or short geographic distances were examined [55,56]. Although we are aware of the small sample size, we are convinced that a marker survey on a diverse set of isolates provides initial insights into the feasibility of markers and the possibility of detecting polymorphisms and, thus, genetic diversity within the species *T. virens*.

Table 5. Haploid diversity by population. Number of loci = 12, number of samples = 10 (5/5), number of populations = 2. **Na** = number of different alleles; **Ne** = number of effective alleles; **I** = Shannon's information index; **h** = diversity; **uh** = unbiased diversity.

Pop.		Na	Ne	I	H	uh
Pop. 1	**Mean**	4.000	3.725	1.323	0.713	0.892
	SE	0.246	0.264	0.075	0.025	0.031
Pop. 2	**Mean**	3.667	3.323	1.214	0.673	0.842
	SE	0.256	0.285	0.081	0.029	0.036
Total	**Mean**	3.833	3.524	1.269	0.693	0.867
	SE	0.177	0.194	0.055	0.019	0.024

Table 6. Analysis of molecular variance by locus. Number of samples = 10, number of populations = 2, number of regions = 1, number of permutations = 999. Probability for Phi_{PT} is based on standard permutation across the full data set. Phi_{PT} = AP/(WP + AP) = AP/TOT (AP = estimated variation among populations, WP = estimated variation within populations).

	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	Total
Phi_{PT}	−0.071	−0.023	−0.080	−0.080	0.022	0.148	0.091	−0.125	−0.023	0.115	−0.029	−0.125	−0.012
Phi_{PT} max	0.100	0.100	0.050	0.050	0.100	0.250	0.200	0.100	0.100	0.150	0.300	0.100	0.133
Phi'$_{PT}$	−0.714	−0.227	−1.591	−1.591	0.217	0.591	0.455	−1.250	−0.227	0.764	−0.098	−1.250	−0.088
Probability Phi_{PT}	1.000	0.701	1.000	1.000	0.624	0.129	0.352	1.000	0.719	0.155	0.501	1.000	0.556

3.3. Strain Discrimination Using Multiplex PCR

Several primer combinations were tested for multiplexing, but in most cases poor quality results were obtained due to a loss of fragments or additional bands. The best outcome was achieved by combining primers $TvCAG_{13}$, $TvCTTTT_{27}$ and $TvGAAA_{34}$ (Figure 2). Every allele was clearly detectable, with the minor exception of isolate 3 (Iran), where the upper band (approximately 500 bp) appears with lower intensity, but is still sufficient to detect polymorphisms. A disadvantage of this primer combination was that it cannot discriminate between the isolates from Australia and the USA, since the primers that could

(e.g., the di-nucleotide repeat primers or $TvGTT_{23}$ and $TvCAT_{32}$) were not suited for multiplexing. Multi-locus haplotypes of the remaining *T. virens* strains were explicitly distinguishable by this primer combination, which implies a high probability of this multiplexing approach also being feasible in other diverse sample sets. The minor artifact double bands appearing in some isolates at approximately 300 bp are negligible measuring errors since the major bands are much more prominent and can be differentiated without ambiguity.

Multiplex PCR in fungal research is widely used in identifying different fungal species in a single PCR reaction. Primer design for this purpose is mainly based on ITS, tef1 and rpb2 sequences and up to 14 species could be distinguished by applying this approach [57]. A similar multiplex PCR system has also been developed for the genus *Trichoderma*, which is capable of differentiating four species of the genus in a single PCR reaction [58]. Multiplexing of PCR-amplified SSR loci has only rarely been applied in fungal research and is limited to a study on the maize and sorghum pathogen *Exserohilum turcicum* Luttr. Here, three, four and six SSR primer pairs, respectively, could be multiplexed successfully in three different PCR reactions [59].

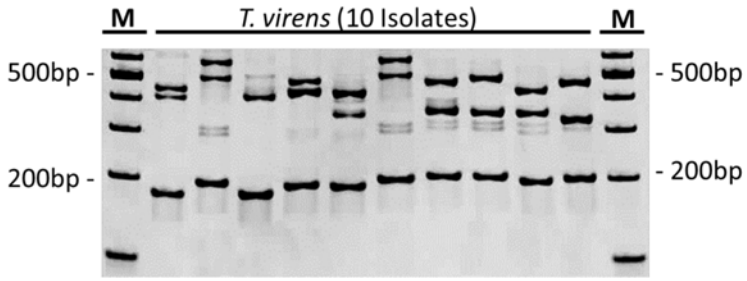
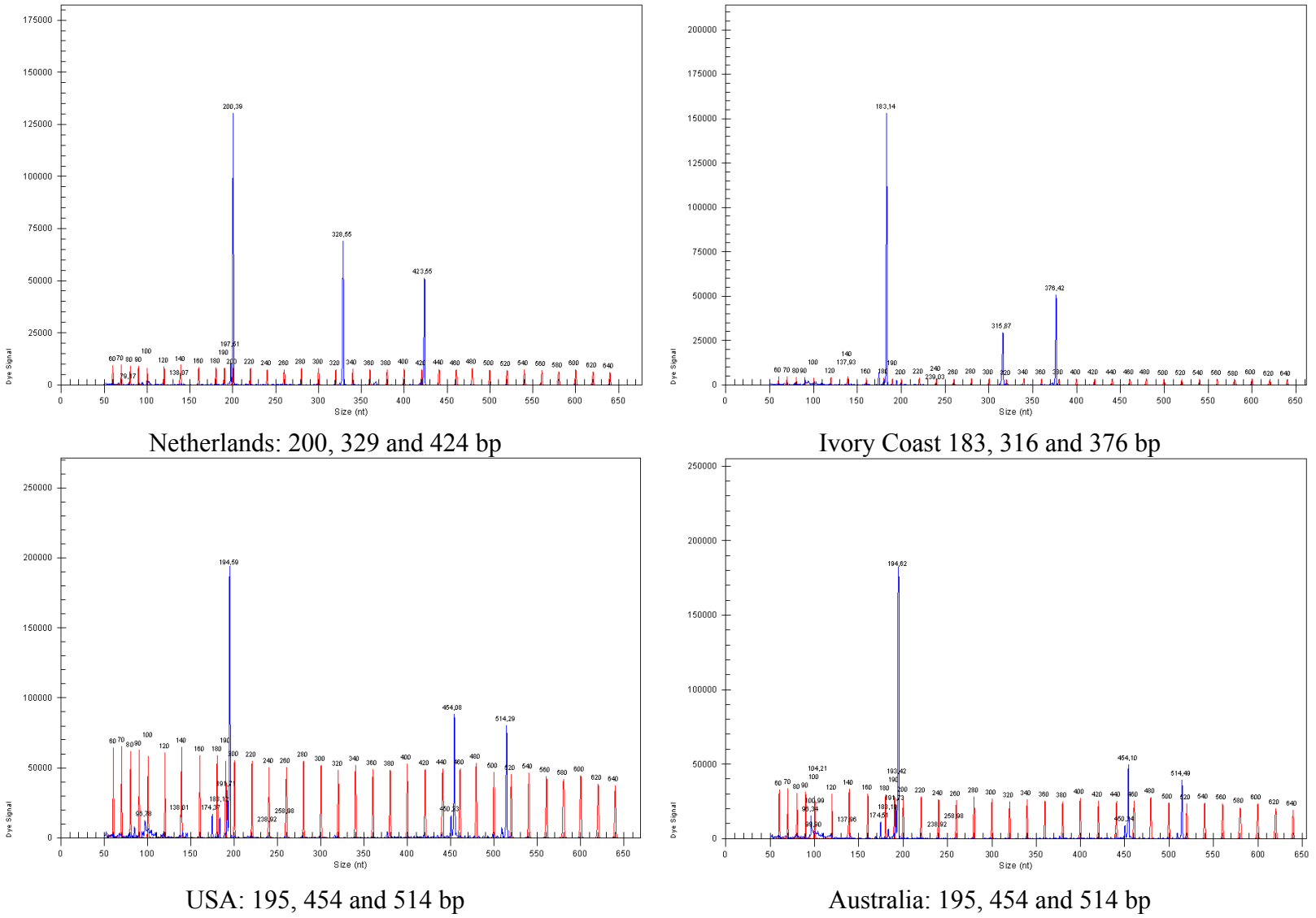


Figure 2. Multiplex PCR with primers $TvCAG_{13}$, $TvCTTTT_{27}$ and $TvGAAA_{34}$ examined on a 5% PAA gel. The order of isolates corresponds to Figure 1. M = 100 bp DNA length standard.

To further investigate minor SSR fragment length polymorphisms that were not detectable by PAA gel electrophoresis, multiplex PCR was also performed with fluorescence labeled primers and fragments were separated by means of capillary gel electrophoresis (Supplementary Material 4), which is a commonly used methodology for fragment analysis, also in the case of high-throughput. Analysis revealed that the fragments generated from the isolates from Australia and the USA (Figure 3) were absolutely identical with these primers and displayed signals at 195, 454 and 514 base pairs. The minor double band at approximately 300 bp did not emit any signals, suggesting that the non-labeled reverse primers were responsible for these artifacts.



Netherlands: 200, 329 and 424 bp

Ivory Coast 183, 316 and 376 bp

USA: 195, 454 and 514 bp

Australia: 195, 454 and 514 bp

Figure 3. Capillary gel electrophoresis from multiplex PCR with Cy5 labeled forward primers. Blue: fluorescence signals from SSR fragments, red: DNA length standard 20 bp ladder. Representative examples are shown for the isolates from The Netherlands, Ivory Coast, USA and Australia.

3.4. Parallel In-Root Detection of Endophytic T. virens *Strains*

In order to test the applicability of the developed markers in tracing the endophytic strains of *T. virens* in root tissue and—even more importantly—in the in-root tracing of different strains according to the molecular weight of their SSR fragments, single tomato plants were grown in pots containing non-sterilized field soil, cultivated in the greenhouse, inoculated with different strains and double-inoculated with two distinct strains as described above. Roots were harvested 42 days after inoculation. Tomato plants showed healthy growth and no evidence of pathogenic effects or additional stress. Strains for inoculation were chosen according to the fragment sizes they generated with certain markers. Suitable combinations were found with the isolates from Germany and Australia and detected with markers $TvCTT_{56}$ and $TvGAAA_{34}$. These two markers displayed a small molecular weight difference in the two isolates at the CTT/AAG_n locus and a larger difference at the $GAAA/TTTC_n$ locus, as seen in Figure 4. As tested in advance, no signal was detectable by using pure tomato DNA. Consequently, from tomato root DNA, no cross-reaction with the *T. virens* primers occurred from the host plant. For inoculated roots the best results were obtained at the CTT/AAG_n locus where single inoculations and double inoculations generated clear bands. This revealed that both strains are able to simultaneously enter root tissue of a single plant and suggests that neither interdependent inhibition among the two fungi [60] nor effective defense reactions, enhanced local resistance possibly mediated by H_2O_2, nor reactive oxygen species occur from the host plant [61].

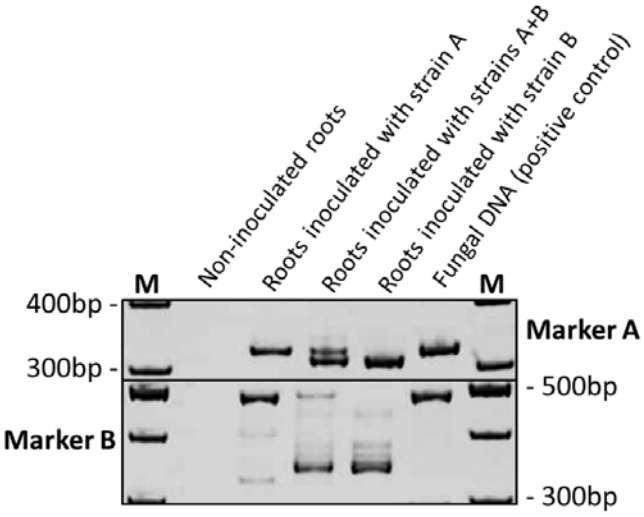


Figure 4. 5% PAA gel with samples from single and double root inoculation. Marker A = $TvCTT_{56}$; marker B = $TvGAAA_{34}$; strain A = Australia; strain B = Germany, M = DNA length standard 100 bp ladder. Dual root colonization by both strains could be confirmed in the middle lanes.

Up-to-date studies on co-colonization of plant roots are restricted to reports about dual colonization by different fungal species or by fungal and bacterial species, e.g., dark septate endophyte (DSE) fungi and ectomycorrhizal fungi in trees [62], DSE fungi and arbuscular mycorrhizal (AM) fungi in *Medicago sativa* L. [63], *Piriformospora indica* and *Trichoderma harzianum* in *Piper nigrum* L. [64], AM and DSE fungi in grasses [65] and the fungus *Acremonium strictum* Gams and bacterium

Acinetobacter sp. in the *Asteraceae* species [66]. To the best of our knowledge, this is the first report on the detection of dual root colonization by different strains of the same fungal species as characterized by their molecular fingerprint.

Detailed analysis of the gel indicates a clear-cut result for marker $(CTT)_{56}$, where no additional bands were observed at all. Marker $(GAAA)_{34}$ produced some extra minor bands in the inoculated root samples with the major bands clearly distinguishable. Single inoculation results appear as expected with both markers and strains, while with double inoculation, bands from the Australian isolate appear to be less intensive than the bands from the German isolate. This was observed in the case of both markers. Taking this as a semi-quantitative result, root colonization efficiency seems to be higher in the German than in the Australian isolate.

3.5. Determination of Strain-Specific Root Colonization Efficiencies by Quantitative PCR

To further investigate the root colonization abilities of the two isolates primer pairs, $TvGAAA_{34}$ and $TvCTT_{56}$ were applied to a qPCR Sybr Green assay with the same root DNA as above. In a test (performed as duplicates) including dilution series with pure fungus DNA from the isolates from Australia and Germany (R^2-values ranged from 0.9963 to 0.9997) differences in the amounts of the fungal DNA fractions could be detected in the root samples (Supplementary Material 5). The utilization of qPCR Sybr Green assays to quantify root endophytes with SSR primers has been demonstrated before using the *Alternaria brassicicola* Schwein./*Alternaria brassicae* Sacc. system [67]. In the present study, single inoculation with the isolate from Germany yielded 0.027 and 0.032 pg fungal DNA per ng root DNA in a repeated determination, while the isolate from Australia yielded only 0.0091 and 0.0092 pg/ng root DNA with single inoculation. This confirmed the semi-quantitative results from conventional PCR and PAA gel electrophoresis (Figure 4). The results from double inoculation yielded 0.012 and 0.014 pg fungal DNA per ng root DNA in the repeat determination, which points to a slightly complementary inhibiting effect since approximately 0.019 pg/ng would have been expected in the case of equal colonization behavior as in single inoculations.

An additional feature of qPCR applying microsatellite primers was determined using a melting curve analysis. According to allele sizes, the melting temperatures of the two PCR fragments differed by 0.88 °C at locus $(GAAA)_{34}$ and by 0.45 °C at locus $(CTT)_{56}$ (Figure 5). Thus, different SSR alleles of typical strains were also differentiable by qPCR and subsequent conventional melting curve analysis. This result opens up new perspectives for qPCR in terms of whether two different isolates or only one isolate entered, for example, the root tissue by determining the melting temperature(s) of the generated fragment(s). In a quantitative conclusion about the ratio of the extent to which different strains colonized the same root tissue, peak heights of melting curves can be taken into account, especially when high resolution melting (HRM) instead of conventional melting is applied. HRM was not possible in this study, but will be further examined in future experiments.

HRM analysis is broadly applied in single nucleotide polymorphism (SNP) research [68], where melting temperature differences can be detected on the basis of one differing nucleotide in low weight PCR fragments. It seems worthwhile to also evaluate the sensitivity of HRM in SSR analysis. The utilization of specialized HRM laboratory kits, based on more sensitive fluorescent dyes that only emit signals when intercalated into the double helix and/or small unlabeled oligonucleotide probes, may be a

future option to optimize HRM analysis in microsatellite marker detection. An avenue for the quantification of dual or multiple colonization events in analyzed tissues was provided by Capper *et al.*, 2015 [69], who introduced quantitative high resolution melting (qHRM). This new method enabled them to determine SNP allele frequencies in pooled samples along with two or more individuals of known genotype as reference material. Similarly, this approach should also be suitable for quantifying or at least revealing the proportion of different SSR alleles in a background of host DNA.

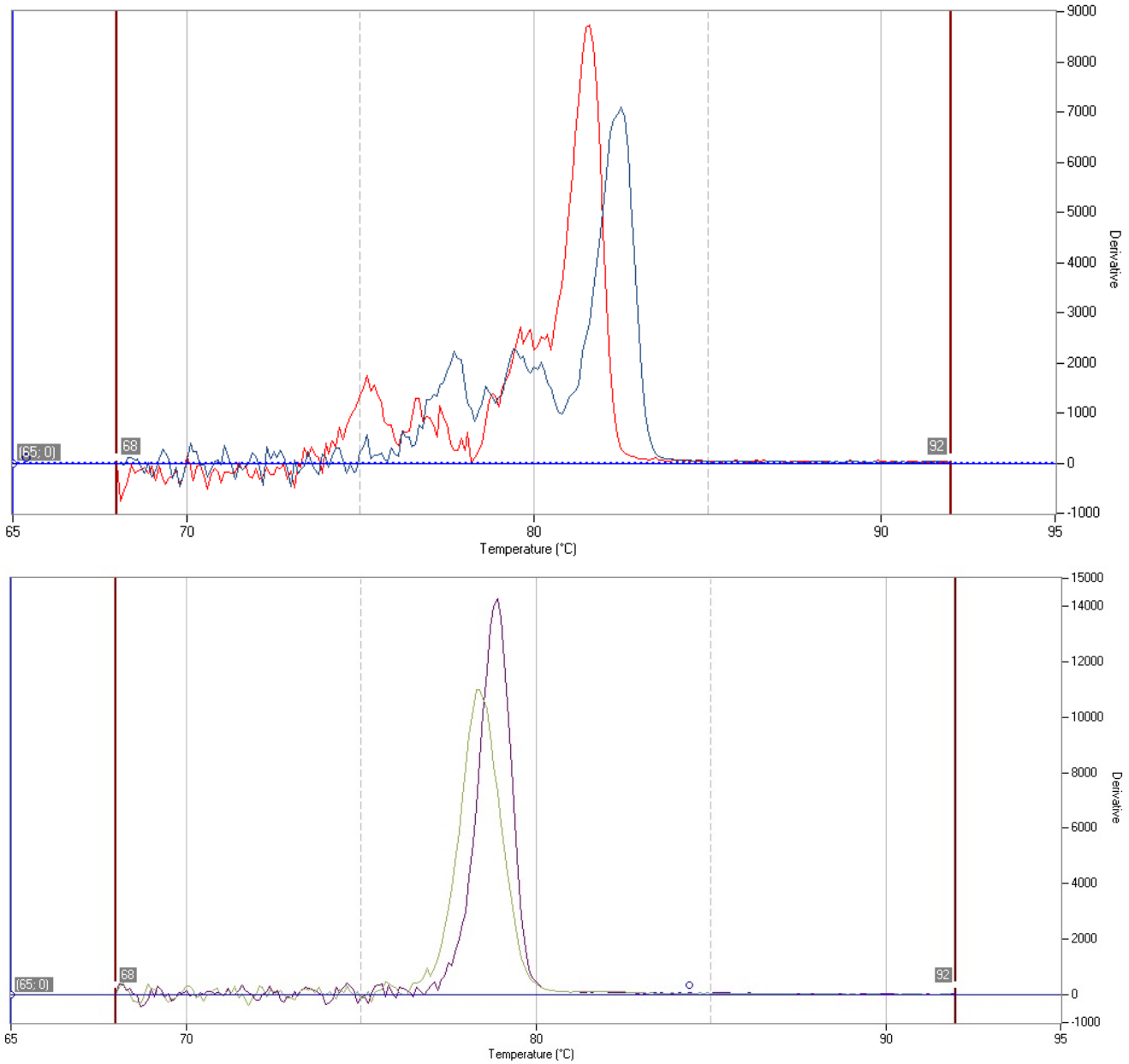


Figure 5. Allele-specific melting curve analysis. The different allele sizes at marker $(GAAA)_{34}$ (**upper panel**) in isolates from Germany (325 bp) and Australia (491 bp) resulted in different melting temperatures of the fragments (differences 0.88 °C and 166 bp). Similarly, this was also observed at the $(CTT)_{56}$ locus, where the melting temperature difference was 0.45 °C and the fragment size difference was only 12 base pairs ((**lower panel**) Australia 301 bp and Germany 289 bp).

4. Conclusions

From the results of this study, it can be concluded that *T. virens* is a well-defined species that harbors moderate to high genetic diversity and exhibits a root-endophytic lifestyle with no negative consequences for the host plant tomato. Single strains of *T. virens* may enter the root system and different strains are able to contemporarily enter root tissue of a single tomato plant as demonstrated in a pot experiment with non-sterilized field soil. Co-colonization events can be monitored with the developed SSR markers and conventional PCR—including multiplex PCR—followed by agarose, polyacrylamide or capillary gel electrophoresis. Markers are also suitable for use in quantitative PCR and are competent to accurately quantify the portion of fungal *T. virens* DNA in total root DNA and, by these means, discriminate between varying root colonization efficiencies of different strains. The developed SSR markers provide a new molecular diagnostic tool to trace and quantify different strains in the environment and also monitor their dispersal in host plants. Furthermore, we demonstrated that dual or multiple root colonization events caused by different *T. virens* strains can also be analyzed by qPCR and subsequent melting curve analysis. The qPCR part of the experiment reveals the total amount of *T. virens* DNA in the roots (as shown in this study), while allele-specific melting curve analysis has the potential of being able to unveil the presence of different alleles originating from different fungal isolates in the roots. In our experiments, conventional melting curve analysis was not sensitive enough to differentiate between fungal allele sizes in a tomato context, but was able to discriminate between allele sizes in pure fungus DNA. A future perspective would be the application of qHRM to quantify different alleles and to reveal the proportion of different alleles present in root tissue. Thus, it appears possible to circumvent laborious combinations of conventional and quantitative PCR and to draw the same amount of information from a single qPCR/qHRM experiment.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/1424-2818/7/4/360/s1.

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Author Contributions

The manuscript was written by Joerg Geistlinger, the experiments were performed by Jessica Zwanzig, the statistical analysis was conducted by Sophie Heckendorff, and the experimental concept was developed by Ingo Schellenberg.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Chaverri, P.; Samuels, G.J.; Stewart, E.L. *Hypocrea. virens* sp. nov., the teleomorph of *Trichoderma. virens*. *Mycologia* **2001**, *95*, 1113–1124.
2. Miller, J.H.; Giddens, J.E.; Foster, A.A. A survey of the fungi of forest and cultivated soils in Georgia. *Mycologia* **1957**, *49*, 779–808.
3. Von Arx, J.A. Plant pathogenic fungi. *Nova Hedwig. Beih.* **1987**, *87*, 1–288.
4. Atanasova, L.; Druzhinina, I.S.; Jaklitsch, W.M. Two hundred *Trichoderma* species recognized based on molecular phylogeny. In *Trichoderma: Biology and Applications*, 2nd ed.; Mukherjee, P.K., Sigh, U.S., Horwitz, B.A., Schmoll, M., Mukherjee, M., Eds.; CABI of Nosworthy Way: Wallingford, UK, 2013; pp. 10–26.
5. Jaklitsch, W.M. European species of *Hypocrea.* Part 1. The green-spored species. *Stud. Mycol.* **2009**, *63*, 1–91.
6. Druzhinina, I.S.; Kopchinskiy, A.G.; Komon, M.; Bissett, J.; Szakacs, G.; Kubicek, C.P. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet. Biol.* **2005**, *42*, 813–828.
7. Zafra, G.; Moreno-Montano, A.; Absalon, A.E.; Cortes-Espinosa, D.V. Degradation of polycyclic aromatic hydrocarbons in soil by a tolerant strain of *Trichoderma asperellum*. *Environ. Sci. Pollut. Res. Int*. **2015**, *22*, 1034–1042.
8. Divya, L.M.; Prasanth, G.K.; Sadasivan, C. Potential of the salt-tolerant laccase-producing strain *Trichoderma viride* Pers. NFCCI-2745 from an estuary in the bioremediation of phenol-polluted environments. *J. Basic Microbiol.* **2014**, *54*, 542–547.
9. Arfarita, N.; Imai, T.; Kanno, A.; Yarimizu, T.; Xiaofeng, S.; Jie, W.; Higuchi, T.; Akada, R. The potential use of *Trichoderma viride* strain FRP3 in biodegradation of the herbicide glyphosate. *Biotechnol. Biotechnol. Equip.* **2013**, *27*, 3518–3521.
10. Hoyos-Carvajal, L.; Orduz, S.; Bisset, J. Growth stimulation in bean (*Phaseolus vulgaris* L.) by *Trichoderma*. *Biol. Control* **2009**, *51*, 409–416.
11. Harman, G.E.; Howell, C.R.; Viterbo, A.; Chet, I.; Lorito, M. *Trichoderma* Species—opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* **2004**, *2*, 43–56.
12. Blaszczyk, L.; Popiel, D.; Chelkowski, J.; Koczyk, G.; Samuels, G.J.; Sobieralski, K.; Siwulski, M. Species diversity of *Trichoderma* in Poland. *J. Appl. Genet*. **2011**, *52*, 233–243.
13. Gauthier, N.W.; Maruthachalam, K.; Subbarao, K.V.; Brown, M.; Xiao, Y.; Robertson, C.L.; Schneider, R.W. Mycoparasitism of *Phakopsora pachyrhizi*, the soybean rust pathogen, by *Simplicillium lanosoniveum*. *Biol. Control* **2014**, *76*, 87–94.
14. Zeng, L.-M.; Zhang, J.; Han, Y.-C.; Wu, M.; Jiang, D.-H.; Chen, W.; Li, G.-Q. Degradation of oxalic acid by the mycoparasite *Coniothyrium minitans* plays an important role in interacting with *Sclerotinia sclerotiorum*. *Environ. Microbiol.* **2014**, *16*, 2591–2610.
15. Qualhato, T.F.; Lopes, F.A.C.; Steindorff, A.S.; Brandao, R.S.; Jesuino, R.S.A.; Ulhoa, C.J. Mycoparasitism study of *Trichoderma* species against three phytopathogenic fungi: Evaluation of antagonism and hydrolytic enzyme production. *Biotechnol. Lett.* **2013**, *35*, 1461–1468.

16. Matarese, F.; Sarrocco, S.; Gruber, S.; Seidl-Seiboth, V.; Vannacci, G. Biocontrol of *Fusarium* head blight: Interactions between *Trichoderma* and mycotoxigenic *Fusarium*. *Microbiology* **2012**, *158*, 98–106.
17. Mulaw, T.B.; Druzhinina, I.; Kubicek, C.P.; Atanasova, L. Novel endophytic *Trichoderma* spp. isolated from healthy *Coffea arabica* roots are capable of controlling coffee tracheomycosis. *Diversity* **2013**, *5*, 750–766.
18. Yang, P. Pathogenic fungi induce the expression of *Trichoderma asperellum* cell wall degrading enzymes in the process of mycoparasitism. *Adv. Mater. Res.* **2014**, *937*, 282–285.
19. Shi, M.; Chen, L.; Wang, X.-W.; Zhang, T.; Zhao, P.-B.; Song, X.-Y.; Sun, C.-Y.; Chen, X.-L.; Zhou, B.-C.; Zhang, Y.-Z. Antimicrobial peptaibols from *Trichoderma pseudokoningii* induce programmed cell death in plant fungal pathogens. *Microbiology* **2012**, *158*, 166–175.
20. Mbarga, J.B.; Begoude, B.A.D.; Ambang, Z.; Meboma, M.; Kuate, J.; Schiffers, B.; Ewbank, W.; Dedieu, L.; Ten Hoopen, G.M. A new oil-based formulation of *Trichoderma asperellum* for the biological control of cacao black pot disease caused by *Phytophthora megakarya*. *Biol. Control* **2014**, *77*, 15–22.
21. Perazzolli, M.; Moretto, M.; Fontana, P.; Ferrarini, A.; Velasco, R.; Moser, C.; Delledonne, M.; Pertot, I. Downy mildew resistance induced by *Trichoderma harzianum* T39 in susceptible grapevines partially mimics transcriptional changes of resistant genotypes. *BMC Genomics* **2012**, *13*, 660–679.
22. Feng, X.M.; Johnsson Holmberg, A.-I.; Sundh, I.; Ricard, T.; Melin, P. Specific SCAR markers and a multiplex real-time PCR for quantification of two *Trichoderma* biocontrol strains in environmental samples. *BioControl* **2011**, *56*, 903–913.
23. Naeimi, S.; Kocsube, S.; Antal, Z.; Okhovvat, S.M.; Javan-Nikkhah, M.; Vagvölgyi, C.; Kredics, L. Strain-specific SCAR markers for the detection of *Trichoderma harzianum* AS12–2, a biological control agent against *Rhizoctonia solani*, the causal agent of rice sheath blight. *Acta Biol. Hung.* **2011**, *62*, 73–84.
24. Hernandez, A.; Jimenez, M.; Arcia, A.; Ulacio, D.; Mendez, N. Molecular characterization of 12 isolates of *Trichoderma* spp. using RAPD and rDNA-ITS. *Bioagro* **2013**, *25*, 167–174.
25. Devi, S.S.; Sreenivasulu, Y.; Saritha, S.; Kumar, M.R.; Kumar, K.P.; Sudhakar, P. Molecular diversity of native *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.), a causal agent of *Fusarium* wilt in tomato (*Lycopersicon esculentum* Mill.). *Arch. Phytopathol. Plant Prot.* **2012**, *45*, 686–698.
26. Lubeck, M.; Bulat, S.; Alekhina, I.; Lieckfeldt, E. Delineation of species within the *Trichoderma viride/atroviride/koningii* complex by UP-PCR cross-blot hybridization. *FEMS Biol. Lett.* **2004**, *237*, 255–260.
27. Shahid, M.; Srivastava, M.; Kumar, V.; Singh, A.; Pandey, S. Genetic determination of potential *Trichoderma.* species using ISSR (microsatellite) marker in Uttar Pradesh, India. *J. Microb. Biochem. Technol.* **2014**, *6*, 174–178.
28. Siddiquee, S.; Tan, S.G.; Yusuf, U.K.; Fatihah, N.H.N.; Hasan, M.M. Characterization of Malaysian *Trichoderma* isolates using random amplified microsatellites. *Mol. Biol. Rep.* **2012**, *39*, 715–722.

29. Geistlinger, J.; Weising, K.; Winter, P.; Kahl, G. Locus-specific microsatellite markers for the fungal chickpea pathogen *Didymella rabiei* (anamorph *Ascochyta rabiei*). *Mol. Ecol.* **2000**, *9*, 1939–1941.
30. Nordberg, H.; Cantor, M.; Dusheyko, S.; Hua, S.; Poliakov, A.; Shabalov, I.; Smirnova, T.; Grigoriev, I.V.; Dubchak, I. The genome portal of the Department of Energy Joint Genome Institute: 2014 updates. *Nucleic Acids Res.* **2014**, *42*, 26–31.
31. Bahkali, A.H.; Abd-Elsalam, K.A.; Guo, J.-R.; Khiyami, M.A.; Verreet, J.-A. Characterization of novel di-, tri-, and tetranucleotide microsatellite primers suitable for genotyping various plant pathogenic fungi with special emphasis on fusaria and *Mycospherella graminicola*. *Int. J. Mol. Sci.* **2012**, *13*, 2951–2964.
32. Consolo, V.F.; Monaco, C.I.; Cordo, C.A.; Salerno, G.L. Characterization of novel *Trichoderma* spp. isolates as a search for effective biocontrollers of fungal diseases of economically important crops in Argentina. *World J. Microbiol. Biotechnol.* **2012**, *28*, 1389–1398.
33. Rozen, S.; Skaletsky, H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **2000**, *132*, 365–386.
34. Gardes, M.; Bruns, T.D. ITS primers with enhanced specificity for basidiomycetes—Application to the identification of mycorrhizae and rusts. *Mol. Ecol.* **1993**, *2*, 113–118.
35. White, T.M.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*, 1st ed.; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: San Diego, CA, USA. 1990; pp. 315–321.
36. Nei, M. Genetic distance between populations. *Am. Nat.* **1972**, *106*, 283–291.
37. Peakall, R.; Smouse, P,E. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* **2006**, *6*, 288–295.
38. Peakall, R.; Smouse, P.E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—An update. *Bioinformatics* **2012**, *28*, 2537–2539.
39. Simpson, M.C.; Wilken, M.; Coetzee, M.P.A.; Wingfield, M.J.; Wingfield, B.D. Analysis of microsatellite markers in the genome of the plant pathogen *Ceratocystis fimbriata*. *Fungal Biol.* **2013**, *117*, 545–555.
40. Gilmore, G.S.; Bassil, N.V.; Barney, D.L.; Knaus, B.J.; Hummer, K.E. Short-read DNA sequencing yields microsatellite markers for *Rheum*. *J. Am. Soc. Hortic. Sci.* **2014**, *139*, 22–29.
41. Anderson, S.J.; Stone, C.L.; Posada-Buitrago, M.L.; Boore, J.L.; Neelam, B.A.; Stephens, R.M.; Luster, D.G.; Frederick, R.D.; Pedley, K.F. Development of simple sequence repeat markers for the soybean rust fungus, *Phakopsora pachyrhizi*. *Mol. Ecol. Resour.* **2008**, *8*, 1310–1312.
42. Bucheli, E.; Gautschi, B.; Shykoff, J.A. Host-specific differentiation in the anther smut fungus *Microbotryum violaceum* as revealed by microsatellites. *J. Evolut. Biol.* **2000**, *13*, 188–198.
43. Reineke, A.; Bischoff-Schaefer, M.; Rondot, Y.; Galidevara, S.; Hirsch, J.; Devi, K.U. Microsatellite markers to monitor a commercialized isolate of the entomopathogenic fungus *Beauveria bassiana* in different environments: Technical validation and first applications. *Biol. Control* **2014**, *70*, 1–8.

44. Cipriani, G.; Marrazzo, M.T.; di Gaspero, G.; Pfeiffer, A.; Morgante, M.; Testolin, R. A set of microsatellite markers with long core repeat optimized for grape (*Vitis* spp.) genotyping. *BMC Plant Biol.* **2008**, *8*, 127, doi:10.1186/1471-2229-8-127.
45. Schoebel, C.N.; Jung, E.; Prospero, S. Development of new polymorphic microsatellite markers for three closely related *Phytophthora* species using 454-pyrosequencing and their potential applications. *Phytopathology* **2013**, *103*, 1020–1027.
46. Prospero, S.; Jung, E.; Tsykun, T.; Rigling, D. Eight microsatellite markers for *Armillaria cepistipes* and their transferability to other *Armillaria* species. *Eur. J. Plant Pathol.* **2010**, *127*, 165–170.
47. Dillon, N.L.; Innes, D.J.; Bally, I.S.E.; Wright, C.L.; Devitt, L.C.; Dietzgen, R.G. Expressed sequence tag-simple sequence repeat (EST-SSR) marker resources for diversity analysis of mango (*Magnifera indica* L.). *Diversity* **2014**, *6*, 72–87.
48. Sehic, J.; Garkava-Gustavsson, L.; Fernandez-Fernandez, F.; Nyboom, H. Genetic diversity in a collection of European pears (*Pyrus communis*) cultivars determined with SSR markers chosen by ECPGR. *Sci. Hortic.* **2012**, *145*, 39–45.
49. Bouajila, A.; Zoghlami, N.; Murad, S.; Baum, M.; Ghorbel, A.; Nazari, K. Genetic differentiation in *Pyrenophora teres* f. *teres* populations from Syria and Tunisia as assessed by AFLP markers. *Lett. Appl. Microbiol.* **2013**, *56*, 389–400.
50. Urbanelli, S.; della Rosa, V.; Fanelli, C.; Fabbri, A.A.; Reverberi, M. Genetic diversity and population structure of the Italian fungi belonging to the taxa *Pleurotus eryngii* and *P. ferulae*. *Heredity* **2013**, *90*, 253–259.
51. Wang, X.; Wadl, P.A.; Wood-Jones, A.; Windham, G.; Trigiano, R.N.; Scruggs, M.; Pilgrim, C.; Baird, R. Characterization of expressed sequence tag-derived simple sequence repeat markers for *Aspergillus flavus*: Emphasis on variability of isolates from the southern United States. *Mycopathologia* **2012**, *174*, 371–382.
52. Zhao, L.; Lascoux, M.; Overall, A.D.J.; Waxman, D. The characteristic trajectory of a fixing allele: A consequence of fictitious selection that arises from conditioning. *Genetics* **2013**, *195*, 993–1006.
53. Aguilée, R.; Claessen, D.; Lambert, A. Allele fixation in a dynamic meta-population: Founder effects *vs.* refuge effects. *Theor. Popul. Biol.* **2009**, *76*, 105–117.
54. Boose, D.; Harrison, S.; Clement, S.; Meyer, S. Population genetic structure of the seed pathogen *Pyrenophora semeniperda* on *Bromus tectorum* in western North America. *Mycologia* **2011**, *103*, 85–93.
55. Saldamondo, C.I.; Velez-Arango, A.M. Host plant association and genetic differentiation of corn and rice strains of *Spodoptera. frugiperda* in Colombia. *Neotropical. Entomology* **2010**, *39*, 921–929.
56. Dong, Z.; Lui, Z.; Lui, D. Genetic characterization of the scyphozoan jellyfish *Aurelia* spp. in Chinese coastal waters using mitochondrial markers. *Biochem. Syst. Ecol.* **2015**, *60*, 15–23.
57. Gordon, M.; van Norman, K. Molecular monitoring of protected fungi: Mycelium persistence in soil after timber harvest. *Fungal Ecol.* **2014**, *9*, 34–42.
58. Prabhakaran, N.; Promeeladevi, T.; Sathiyabama, M.; Kamil, D. Multiplex PCR for detection and differentiation of diverse *Trichoderma* species. *Ann. Microbiol*. **2015**, *65*, 1591–1595.

59. Haasbrock, M.P.; Craven, M.; Barnes, I.; Crampton, B.G. Microsatellite and mating type primers for the maize and sorghum pathogen *Exserohilum turcicum*. *Aust. Plant Pathol.* **2014**, *43*, 577–581.
60. Ohsumi, K.; Watanabe, M.; Fujie, A. AS2077715 is a selective inhibitor of fungal mitochondrial cytochrome BC 1 complex. *J. Antibiot.* **2014**, *67*, 713–716.
61. Su, Z.-Z.; Mao, L.-J.; Li, N.; Feng, X.-X.; Yuan, Z.-L.; Wang, L.-W.; Lin, F.-C.; Zhang, C.-L. Evidence for biotrophic lifestyle and biocontrol potential of dark septate endophyte *Harpophora oryzae* to rice blast disease. *PLoS ONE* **2013**, doi:10.1371/journal p one 0061332.
62. Reininger, V.; Sieber, T.N. Mitigation of antagonistic effects on plant growth due to root co-colonization by dark septate endophytes and ectomycorrhiza. *Environ. Microbiol. Rep.* **2013**, *5*, 892–898.
63. Saravesi, K.; Ruotsalainen, A.L.; Cahill, J.F. Contrasting impacts of defoliation on root colonization by arbuscular mycorrhizal fungi and dark septate endophyte fungi of *Medicago sativa*. *Mycorrhiza* **2014**, *24*, 239–245.
64. Anith, K.N.; Faseela, K.M.; Archana, P.A.; Prathapan, K.D. Compatibility of *Piriformospora indica* and *Trichoderma harzianum* as dual inoculants in black pepper (*Piper nigrum* L.). *Symbiosis* **2011**, *55*, 11–17.
65. Sathiyadash, K.; Muthukumar, T.; Uma, E. Arbuscular mycorrhizal and dark septate endophyte fungal associations in South Indian grasses. *Symbiosis* **2010**, *52*, 21–32.
66. Wang, X.-M.; Yang, B.; Wang, H.-W.; Yang, T.; Ren, C.-G.; Zheng, H.-L.; Dai, C.-C. Consequences of antagonistic interactions between endophytic fungus and bacterium on plant growth and defense responses in *Atractylodes lancea*. *J. Basic Microbiol.* **2015**, *55*, 659–670.
67. Singh, R.; Kumar, S.; Kashyap, P.L.; Srivastava, A.K.; Mishra, S.; Sharma, A.K. Identification and characterization of microsatellite from *Alternaria brassicicola* to access cross-species transferability and utility as a diagnostic marker. *Mol. Biotechnol.* **2014**, *56*, 1049–1059.
68. Dong, Y.; Shi, S.; Bao, Y.; Yao, H.; Jing, Y.; Lin, Z. Development of 90 EST-SNP markers in blood clam (*Tegillarca granosa*) using high resolution melting (HRM). *Conserv. Genet. Resour*. **2015**, *7*, 309–314.
69. Capper, R.L.; Jin, Y.K.; Lundgren, P.B.; Peplow, L.M.; Matz, M.V.; van Oppen, M.J.H. Quantitative high resolution melting: Two methods to determine SNP allele frequencies from pooled samples. *BMC Genet.* **2015**, *16*, 62–75.