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Designing a SCAR molecular marker for monitoring *Trichoderma* cf. *harzianum* in experimental communities*#

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Abstract: Several species of the fungal genus *Trichoderma* establish biological interactions with various micro- and macro-organisms. Some of these interactions are relevant in ecological terms and in biotechnological applications, such as biocontrol, where *Trichoderma* could be considered as an invasive species that colonizes a recipient community. The success of this invasion depends on multiple factors, which can be assayed using experimental communities as study models. Therefore, the aim of this work is to develop a species-specific sequence-characterized amplified region (SCAR) marker to monitor the colonization and growth of *T.* cf. *harzianum* when it invades experimental communities. For this study, 16 randomly amplified polymorphic DNA (RAPD) primers of 10-mer were used to generate polymorphic patterns, one of which generated a band present only in strains of *T.* cf. *harzianum*. This band was cloned, sequenced, and five primers of 20–23 mer were designed. Primer pairs 2F2/2R2 and 2F2/2R3 successfully and specifically amplified fragments of 278 and 448 bp from the *T.* cf. *harzianum* BpT10a strain DNA, respectively. Both primer pairs were also tested against the DNA from 14 strains of *T.* cf. *harzianum* and several strains of different fungal genera as specificity controls. Only the DNA from the strains of *T.* cf. *harzianum* was successfully amplified. Moreover, primer pair 2F2/2R2 was assessed by quantitative real-time polymerase chain reaction (PCR) using fungal DNA mixtures and DNA extracted from fungal experimental communities as templates. *T.* cf. *harzianum* was detectable even when as few as 100 copies of the SCAR marker were available or even when its population represented only 0.1% of the whole community.

Key words: *Trichoderma* cf. *harzianum*, Sequence-characterized amplified region (SCAR), Molecular marker, Experimental fungal communities

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1 Introduction

One of the major challenges in ecology is to understand which factors control the abundance and diversity of species, how these are influenced by natural or human-induced changes, and what are the consequences for ecosystem processes and properties (van der Putten *et al*., 2007). Experimental communities help answering some of these questions (Jessup *et al*., 2005) and are useful to explain the interactive processes between the microbiota of a complex ecosystem and the different environmental conditions (Moore *et al*., 1996). An area of growing interest is biocontrol, which can be studied in the context of biological invasions, since biocontrol isolates must be able to colonize and grow in a recipient community. In this context, *Trichoderma* species can be considered as invasive and their success as biocontrol agents will depend on abiotic factors such as availability of resources, or on biotic factors such as the properties of the recipient community as well as the ability of the invasive species to invade it (Savazzini *et al*., 2009; Zhang *et al*., 2013). Species of the genus *Trichoderma*

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(Family Hypocreaceae; Phylum Ascomycota) are ubiquitous in soil, where they may represent the majority of the fungal biomass (Druzhinina *et al*., 2011), and play important environmental roles, such as degradation of complex organic compounds and establishment of mutualistic interactions with plants (Kubicek *et al*., 2008). Likewise, *Trichoderma* spp. have been reported to act as biological control agents by their antagonistic properties towards fungal pathogens (Viterbo *et al*., 2001; Benítez *et al*., 2004; Harman, 2006; Komon-Zelazowska *et al*., 2007), being suggested that they are even able to attack, parasitize, and gain nutrition from other fungi, such as phytopathogens (Druzhinina *et al*., 2011).

There are a variety of mechanisms by which *Trichoderma* species establish interactions with other organisms; these can be direct, as the aforementioned mycoparasitism, in which *Trichoderma* species parasitize other fungi like those belonging to the genera *Pythium*, *Phytophthora*, *Botrytis*, *Rhizoctonia*, and *Fusarium*, some of which are well known disease agents of plants (Benítez *et al*., 2004; Harman, 2006). Another direct mechanism corresponds to amensalism, which is related to the production of antibiotics and peptaibols that directly affect other fungi, being able to act on a synergistic manner with mycoparasitism (Grondona *et al*., 1997; Benítez *et al*., 2004; Komon-Zelazowska *et al*., 2007). However, interactions can also be indirect, such as competition for nutrients and space, change of environmental conditions, and stimulation of the growth and/or the immune system of plants by endophytism, among others (Harman *et al*., 2004; Harman, 2006). Due to the relevance of *Trichoderma* species in terrestrial ecosystems and their ability to successfully colonize different habitats (Schuster and Schmoll, 2010; Kredics *et al*., 2014), a proper identification of *Trichoderma* strains is crucial for understanding how this fungus colonizes a recipient microbial community. Various procedures have been proposed and designed for monitoring *Trichoderma* strains, both in experimental and environmental communities, which range from their cultivation in selective media (Knudsen *et al*., 1996; Williams *et al*., 2003; Vargas-Gil *et al*., 2009) to the genetic modification of such organisms (Green and Jensen, 1995). Another way of performing this monitoring is through the identification of the strain(s) or species by specific molecular markers (Hermosa *et al*., 2000; Druzhinina and Kubicek, 2005; Kubicek *et al*., 2008). In many cases, sequence-characterized amplified region (SCAR) markers have been developed by converting dominant randomly amplified polymorphic DNA (RAPD) fingerprints into specific molecular markers (Parasnis *et al*., 2000; Koveza *et al*., 2001; Arnedo-Andrés *et al*., 2002; Bautista *et al*., 2003; Rubio *et al*., 2005; Feng *et al*., 2011). The main aim of the present study was to develop a SCAR molecular marker to specifically detect some *Trichoderma* cf. *harzianum* strains for monitoring the colonization and growth of *T.* cf. *harzianum* when it invades experimental communities. This work is a part of a research project on biological invasions in which experimental fungal communities are used as study models to assess the biotic and abiotic factors determining the success of *T.* cf. *harzianum* as an invasive species.

2 Materials and methods

2.1 Fungal samples and culture conditions

Fourteen strains of filamentous fungi belonging to the genus *Trichoderma* were provided by Castillo (2009), labeled as BpT1a, BpT2, BpT5, BpT6, BpT7, BpT9, BpT10, BpT10a, BpTA, BpTC, BpTF, BpTH, BpTP, and BpTR (Table 1). From each sample, monosporic cultures were prepared from serial dilutions of an aqueous conidial suspension (NaCl 0.7 g/ml) derived from 7-d-old cultures, which were spread on potato dextrose agar (PDA; Difco™) medium. The plates were incubated at 25 °C until single colonies were observed (2–3 d), which were then re-inoculated on PDA medium to ensure the purity of the culture (Gams and Bissett, 1998). In addition, 20 DNA samples were provided by Biopacific Co., Ltd. (Chile), 8 of which corresponded to *T.* cf. *harzianum* (1, 2, 3, 4, 6, 7, 8, and 9), 6 to *Penicillium* spp. (Atc4, Atc5, 9a, 9ar1, C1R, and C3F), 3 to *Aspergillus* spp. (HI, HC1, and 4a), and the other 3 to *Davidiella* sp. (HM), *Paecilomyces* sp. (9ar), and *Monascus* sp. (Atc8) (Table 1). In addition, the fungal species *Aspergillus niger* (MR-1), *Purpureocillium* sp. (MR-6), *Paecilomyces variotti* (MR-9), *Beauveria bassiana* (MR-21), *Talaromyces flavus* (MR-25), and *Cladosporium cladosporoides* (MR-12) were obtained from the fungal collection of the laboratory of Microbial Ecology, Faculty of Sciences, University of Chile (Table 1).

Table 1 Fungal strains and DNA samples used in this study

Strain	Species	Origin	ITS accession No.	Most related sequence accession No.*	Identity (%)
BpT1a	*Trichoderma novaezelandiae*	Neltume, Chile	KC812353	AY154925	99 (100)
BpT2	*Trichoderma* sp.	Neltume, Chile	KC812352	KC007277	99 (100)
BpT5	*Trichoderma atroviride*	Neltume, Chile	KC812350	KC171717	99 (96)
BpT6	*Trichoderma* cf. *harzianum*	Laja River, Chile	KC812342	JX088246	100 (100)
BpT7	*Trichoderma* cf. *harzianum*	Laja River, Chile	KC812343	JX088246	100 (100)
BpT9	*Trichoderma atroviride*	Laja River, Chile	KC812349	KC171717	99 (96)
BpT10	*Trichoderma atroviride*	Port Fuy, Chile	KC812348	KC171717	99 (96)
BpT10a	*Trichoderma* cf. *harzianum*	Port Fuy, Chile	KC812341	JN716380	100 (100)
BpTA	*Trichoderma* cf. *harzianum*	Neltume, Chile	KC812340	JX416574	99 (89)
BpTC	*T. atroviride*	Port Fuy, Chile	KC812346	KC171717	99 (96)
BpTF	*Trichoderma* cf. *harzianum*	Laja River, Chile	KC812345	JX088246	100 (100)
BpTH	*Trichoderma* cf. *harzianum*	Port Fuy, Chile	KC812344	JX088246	100 (100)
BpTP	*Trichoderma citrinoviride*	Port Consuelo, Chile	KC812351	JX173874	100 (100)
BpTR	*Trichoderma atroviride*	Laja River, Chile	KC812347	KC171717	99 (96)
1	*Trichoderma* cf. *harzianum*	Biopacific Co., Ltd., Chile	KC812364	JX416561	100 (89)
2	*Trichoderma* cf. *harzianum*	Biopacific Co., Ltd., Chile	KC812365	JX088246	100 (100)
3	*Trichoderma* cf. *harzianum*	Biopacific Co., Ltd., Chile	KC812366	JX416561	100 (89)
4	*Trichoderma* cf. *harzianum*	Biopacific Co., Ltd., Chile	KC812367	JN716380	100 (100)
6	*Trichoderma* cf. *harzianum*	Biopacific Co., Ltd., Chile	KC812368	AY605728	99 (94)
7	*Trichoderma* cf. *harzianum*	Biopacific Co., Ltd., Chile	KC812369	JX416561	100 (89)
8	*Trichoderma* cf. *harzianum*	Biopacific Co., Ltd., Chile	KC812370	JX644593	99 (98)
9	*Trichoderma* cf. *harzianum*	Biopacific Co., Ltd., Chile	KC812371	JX416574	99 (89)
Atc4	*Penicillium* sp.	Biopacific Co., Ltd., Chile	KC812357	JX869567	100 (100)
Atc5	*Penicillium* sp.	Biopacific Co., Ltd., Chile	KC812358	JX406503	100 (100)
9a	*Penicillium* sp.	Biopacific Co., Ltd., Chile	KC812359	FJ025212	100 (100)
9ar1	*Penicillium* sp.	Biopacific Co., Ltd., Chile	KC812360	JN585940	100 (100)
C1R	*Penicillium* sp.	Biopacific Co., Ltd., Chile	NA	NA	NA
C3F	*Penicillium* sp.	Biopacific Co., Ltd., Chile	NA	NA	NA
HC1	*Aspergillus* sp.	Biopacific Co., Ltd., Chile	KC812355	JQ316527	99 (100)
HI	*Aspergillus* sp.	Biopacific Co., Ltd., Chile	KC812356	HM061131	100 (100)
4a	*Aspergillus* sp.	Biopacific Co., Ltd., Chile	KC812354	FR670319	100 (100)
HM	*Davidiella* sp.	Biopacific Co., Ltd., Chile	KC812361	JF911765	100 (100)
9ar	*Paecilomyces* sp.	Biopacific Co., Ltd., Chile	KC812362	GU980015	100 (100)
Atc8	*Monascus* sp.	Biopacific Co., Ltd., Chile	KC812363	JN942665	100 (100)
MR-1**	*Aspergillus niger*	El Romeral, Chile	NA	NA	NA
MR-9**	*Paecilomyces variotti*	El Romeral, Chile	NA	NA	NA
MR-21**	*Beauveria bassiana*	El Romeral, Chile	NA	NA	NA
MR-6**	*Purpureocillium* sp.	El Romeral, Chile	NA	NA	NA
MR-25**	*Talaromyces flavus*	El Romeral, Chile	NA	NA	NA
MR-12**	*Cladosporium cladosporoides*	El Romeral, Chile	NA	NA	NA

* Most related sequence retrieved by BLAST using as query the ITS1-5.8S rDNA-ITS2 region of the samples. ** Strains from the Fungal Collection of the Microbial Ecology Laboratory, Faculty of Sciences, University of Chile. NA: not available

2.2 DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

Genomic DNA (gDNA) was extracted from fresh monosporic mycelia using the PowerSoil™ DNA extraction kit (MoBio Laboratories Inc.) according to the manufacturer's instructions. The quality and integrity of the extracted DNA were visualized in 0.8% (8 g/L) agarose gels in 1× TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA (pH 8.0)) stained with GelRed™ (Biotium). All DNA samples were stored at −20 °C until analysis. A fragment corresponding to the 5.8S ribosomal RNA (rRNA) gene and the two ribosomal internal transcribed spacers (ITS1 and ITS2) of the ribosomal region were amplified by PCR using primers ITS1 (5'-TCCGTA GGTGAACCTGCGG-3') and ITS4 (5'-TCCTCC GCTTATTGATATGC-3') (White *et al*., 1990).

The amplifications were carried out in a total volume of 25 μl containing 1 μl of gDNA (20±5) ng, with primers at a concentration of 400 nmol/L each, and the reagents provided in the GoTaq® Green Master Mix (Promega, USA; GoTaq® DNA polymerase in 1× Green GoTaq® reaction buffer (pH 8.5), 200 μmol/L of each dNTP, and 1.5 mmol/L $MgCl_2$). The thermal cycles consisted of 3 min of initial denaturation at 94 °C, 37 cycles of denaturation at 94 °C for 30 s, then 30 s annealing at 55 °C and 3 min extension at 72 °C, and a final extension of 20 min at 72 °C on a MaxyGene thermocycler (Axygen). PCR products were evaluated on 1.2% (12 g/L) agarose gels, with the conditions above mentioned using the 100 bp (Invitrogen) as the molecular weight marker. Amplicons were sequenced (Macrogen Inc., Seoul, Korea) using a Genetic Analyzer 3730XL (Applied Biosystems). The identity of each sequence was assessed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990). In addition, all sequences belonging to the genus *Trichoderma* were identified by *Trich*OKEY 2.0 (Druzhinina *et al*., 2005).

2.3 RAPD

To carry out RAPD analyses, 16 arbitrary 10-mer primers (Operon, USA) were tested against purified gDNA from different *Trichoderma* species, including some *T.* cf. *harzianum* strains, and non-*Trichoderma* DNA samples from the genera *Aspergillus*, *Penicillium*, and *Monascus* (HI, Atc4, and Atc8 strains, respectively). The PCR reactions were performed as mentioned above. The thermal cycling was as described by Lehmann *et al*. (1992), consisting of an initial denaturation of 5 min at 94 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C, and a final extension of 10 min at 72 °C, using a MaxyGene thermocycler (Axygen). PCR products were analyzed by electrophoresis in 1.5% (15 g/L) agarose gels using the above mentioned conditions.

2.4 Cloning and sequencing of an RAPD amplicon

The band of interest identified in the RAPD analysis (Fig. 1) was gel-excised and purified using the Wizard SV Gel and PCR Clean-Up system kit (Promega), ligated to the pTZ-57R/T vector (Fermentas) and then transformed into *Escherichia coli* using the InsTA clone PCR cloning kit (Fermentas). The transformants were grown in Luria-Bertani (LB) medium and selected by their resistance to ampicillin (50 μg/ml) and their inability to hydrolyze X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in the presence of isopropylthio-β-galactoside (IPTG). The presence of the expected size insert was confirmed by colony-PCR using primers M13F (5'-GTA AAACGACGGCCAG-3') and M13R (5'-CAGGAA ACAGCTATGACC-3').

The amplified product was sequenced (Macrogen Inc., Seoul, Korea) in both directions using a Genetic Analyzer 3730XL (Applied Biosystems). The sequencing result was edited and subsequently evaluated using BLAST-n (Altschul *et al*., 1990) to determine whether this sequence had some identity with any of the known sequences present in the GenBank database of the National Center for Biotechnology Information (NCBI) (Benson *et al*., 2013). Finally, a local BLAST of this sequence with the genome of *T. harzianum* CBS 226.95 available at the Doe Joint Genome Institute web page (http://genome.jgi.doe.gov/) was performed to determine the number of copies of the SCAR marker.

2.5 Design of SCAR primers

The SCAR primers were designed using the NCBI primer-BLAST tool, which uses the Primer3 tool (Rozen and Skaletsky, 1999). Five primers were designed: two forward primers (2F1 and 2F2) and

three reverse primers (2R1, 2R2, and 2R3) (Table 2). To evaluate these primers and to define the PCR annealing temperature, DNA from *T.* cf. *harzianum* BpTF was used as the template. The conditions of the reagents were the same as in the above mentioned amplification protocols, except that the concentration of each primer was 200 nmol/L. The thermal cycling was as follows: initial denaturation of 3 min at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing, where the temperature ranged between 40 and 64 °C, 1 min extension at 72 °C, and a final extension of 7 min at 72 °C. Once the thermal cycling conditions were assessed, the primers were evaluated using 22 DNA samples from *Trichoderma* belonging to five different species in addition to DNA from species of the genera *Penicillium*, *Aspergillus*, *Davidiella*, *Paecilomyces*, and *Monascus*.

2.6 Real-time quantitative PCR (qPCR) assays

Primers 2F2 and 2R2 were additionally tested in real-time qPCR assays. Reactions were carried out in an Mx3000P real-time PCR system (Stratagene) using the GoTaq® Real-Time qPCR Master Mix (Promega). All reactions were performed in a total volume of 20 μl, with 0.3 μmol/L of each primer and 1 μl of template DNA at the concentrations required by each assay. The thermal cycling was as follows: 2 min of denaturation at 95 °C, 40 cycles consisting of 15 s denaturation at 95 °C, then 1 min annealing at 64 °C and 1 min extension at 72 °C.

To quantify the number of SCAR copies specifically detected by the primers evaluated by real-time qPCR, three standard curves in quadruplicate were performed using 10-fold serial dilutions of

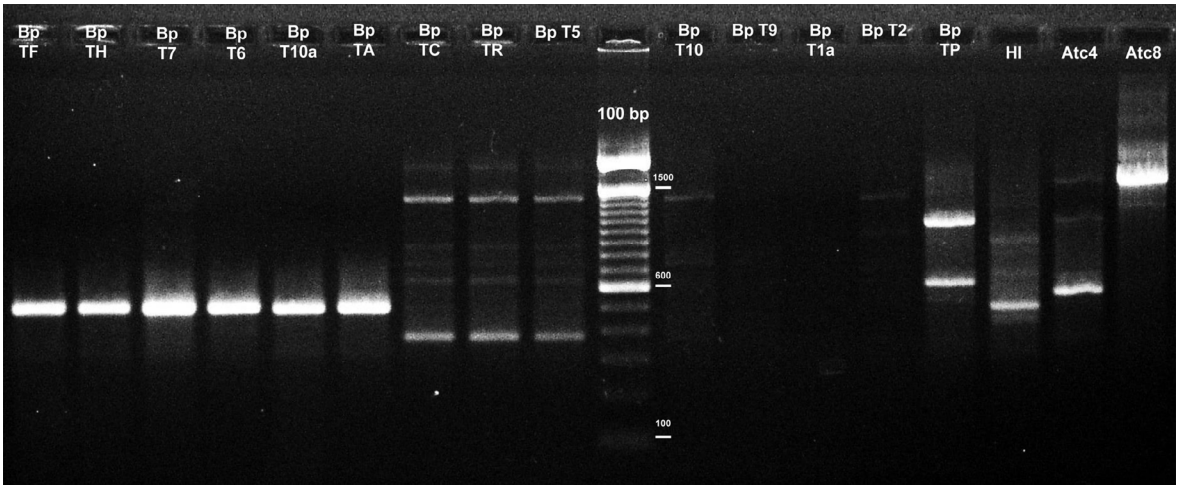


Fig. 1 RAPD fingerprintings of fungal species

Agarose gel showing the RAPD banding profile amplified from fungal gDNA using the OPX02 primer. Lanes labeled as BpTF, BpTH, BpT7, BpT6, BpT10a, and BpTA correspond to isolates of *T.* cf. *harzianum*; BpTC, BpTR, BpT5, and BpT10, BpT9 of *T. atroviride*; BpT1a of *T. novaezelandiae*; BpT2 of *Trichoderma* sp.; BpTP of *T. citrinoviride*; HI of *Aspergillus* sp.; Atc4 of *Penicillium* sp.; and, Atc8 of *Monascus* sp. (Table 1). The 100-bp ladder was used as a molecular weight marker

Table 2 Characteristics of the designed SCAR primers

SCAR primer	Sequence (5'→3')	Position in the RAPD fragment (bp)	GC (%)	T_m (°C)
2F1	TCCGCCACCATCTAGACCAATCG	2–23	56.5	58.28
2F2	TGGCTCGTCGTAGTTCGGAGAAG	51–73	56.5	58.26
2R1	CCACCGCGGGATTCATGCACT	474–494	65.0	58.68
2R2	CCAGATCGGCCACCGAAGAAAC	307–328	59.1	57.92
2R3	GCCACCCACCGCGGGATTCA	480–499	70.0	61.78

GC: guanine-cytosine; T_m: melting temperature

different DNA templates (1–10^{-5} ng): (1) gDNA of *T.* cf. *harzianum* BpT10a, (2) a linearized plasmid (pTZ57R/T) containing the SCAR marker, and (3) an equimolar mix of fungal gDNA from several species (*P. canescens* [MR-13], *A. niger* [MR-1], *C. cladosporoides* [MR-12], *P. variotii* [MR-9], and *T. flavus* [MR-25]). The SCAR copy numbers were determined using the proportion between the nucleotide mass and the nucleotide number according to the ratio 1.096×10^{-21} g/bp; besides, the sizes of the *T. harzianum* CBS 226.95 genome (40.98 Mbp) and the plasmid with the SCAR sequence (3164 bp) were also considered. The threshold cycle (C_t) of each 10-fold serial dilution reaction was plotted versus the number of SCAR copies expressed as log. Using a linear regression analysis, the slope and the determination coefficients (r^2) of the three assays were calculated. In addition, the percentage of efficiency was obtained for each of the three standard curves according to: efficiency=$(10^{-1/\text{slope}}-1)\times100\%$.

The sensibility of the SCAR primers to detect *T.* cf. *harzianum* by qPCR was assessed by using 10-fold serial dilutions of the aforementioned DNA templates. In the latter case, in addition to the equimolar gDNA mix from the five fungal species, six additional mixes were made, where *T.* cf. *harzianum* gDNA was present in decreasing percentages (10%, 5%, 2%, 1%, 0.2%, and 0.1%). The six assays were replicated four times under the same reaction conditions as above mentioned.

Finally, the SCAR primers were evaluated in two experimental fungal communities inoculated with *T.* cf. *harzianum*, where its colonization and growth were monitored by qPCR. The experimental communities were set up in 20 ml flasks using 1 g of rock wool as a support matrix soaked with Vogel medium (supplemented with yeast extract 0.5%, dextrose 0.5%, and chloramphenicol 30 μg/ml) to reach 60% of the water-holding capacity (WHC). Experimental communities were assembled in duplicates and consisted of the following: Community 1 was composed of *T. citrinoviride* (BpTP), *T. novaezelandiae* (BpT1a), *T. atroviride* (Bpt10), *B. bassiana* (MR-21), and *Purpureocillium* sp. (MR-6); and Community 2 was composed of *P. canescens* (MR-13), *A. niger* (MR-1), *C. cladosporoides* (MR-12), *P. variotii* (MR-9), and *T. flavus* (MR-25).

Each community was inoculated with 2×10^4 spores of each fungal isolate and incubated at 25 °C; after 3 d they were inoculated with 2×10^4 spores of *T.* cf. *harzianum* BpT10a. Fungal experimental communities without *T.* cf. *harzianum* BpT10a were built in duplicate as controls. Destructive samplings were carried out 0, 3, 7, and 10 d after inoculation with *T.* cf. *harzianum* BpT10a and the gDNA was extracted with the PowerSoil™ DNA extraction kit (MoBio Laboratories Inc.). gDNA was quantified using the Take3™ Epoch MicroVolume Plate reader (Biotek) and 1 ng was used as the template for the qPCR reactions, which were performed under the same conditions above mentioned. The fungal gDNA-mix standard curve was used to calculate the copy number of the SCAR marker in each community.

3 Results

3.1 Molecular identification of fungal strains

All fungal strains and DNA samples were identified by amplification and sequencing of the molecular marker ITS1-5.8S rDNA-ITS2 and the subsequent search, by means of BLAST-n, in databases from NBCI and the barcode tool for *Trichoderma* (*TrichO*KEY2.0) (Druzhinina *et al*., 2005) when possible. The sequences of the isolates were related with nine different genera (*Trichoderma*, *Penicillium*, *Aspergillus*, *Davidiella*, *Paecilomyces*, *Monascus*, *Beauveria*, *Talaromyces*, and *Cladosporium*) confirming all the previous identifications (Table 1).

3.2 SCAR marker design

The SCAR marker for *T.* cf. *harzianum* was designed through an RAPD assay. The amplicons obtained ranged between 200 and 2350 bp, resulting in highly polymorphic patterns for some of the used primers. However, for the design of the SCAR marker, only those amplicons with high intensity and reproducibility were considered. Using the primer OPX-02, a band of approximately 500 bp was detected, which was common to all the *T.* cf. *harzianum* strains (Figs. 1 and S1). This band of interest was gel-excised and purified from the *T.* cf. *harzianum* BpTF isolate; then it was cloned into the pTZ57R/T vector and transformed into *E. coli*. Five transformants were selected and the insert was recovered using the M13 primers, obtaining a fragment of about 650 bp. The

amplicons were sequenced in both directions using the primers M13F and M13R. The sequences were edited (vector sequences were removed) leaving only the fragment of interest, which resulted in a sequence of 503 nucleotides (Fig. S2). A BLAST-n analysis of this sequence showed no identity with any other sequences in GenBank. Furthermore, the local BLAST analysis performed against the *T. harzianum* CBS 226.95 genome showed that it was present in a single copy (Table S1). Based on the DNA sequence of the cloned 503-bp fragments (FASTA S1), SCAR primers were designed, including the primer sequences initially used for the RAPD analyses. Next, internal primer pairs were designed for reaching better amplification results. The primer positions are shown in Fig. S2.

By means of an *in silico* evaluation of the primers, their sequences, GC (guanine-cytosine) contents, T_m (melting temperature) values, and positions in the RAPD sequenced fragment were determined (Table 2).

3.3 SCAR marker evaluation

SCAR primers were evaluated using a gradient of annealing temperatures, with the gDNA from *T.* cf. *harzianum* BpTF as the template. The SCAR primers were evaluated using different combinations of them. Two primer combinations, 2F2/2R2 and 2F2/2R3, generated an intense band of the expected size in the whole tested temperature range (40–64 °C). To avoid non-specific amplification, the higher annealing temperature was chosen to carry out subsequent assays. The other primer combinations did not yield any amplification products and no non-specific bands were obtained. PCR reactions were conducted using gDNA from all the *Trichoderma* isolates included in Table 1, along with the gDNA corresponding to species of the genera *Penicillium* (Atc4, Atc5, 9a, 9ar1, C1R, and C3F), *Aspergillus* (HI, HC1, and 4a), *Davidiella* (HM), *Paecilomyces* (9ar), *Monascus* (Atc8), and a mixture of species from a compost sample (C3R). Primer pair 2F2/2R2 amplified only the 278-bp expected product from the 14 *T.* cf. *harzianum* DNA samples (Fig. 2), and no amplifications were obtained from the other non-*Trichoderma* species. Similar results were observed with the primer pair 2F2/2R3, which generated the 448-bp expected amplified product in all the reactions where *T.* cf. *harzianum* DNA was used as the template (Fig. S3). Therefore, both primers were suitable for amplifying the SCAR marker by conventional PCR; however, in the case of the qPCR primer pair, 2F2/2R2 was preferred because it yielded the shortest fragment.

Using the database available at http://genome.jgi.doe.gov/, the SCAR sequence was aligned with 366 fungal genomes, and no hit was observed with non-*Trichoderma* fungi. In the *Trichoderma* group, six genomes were available, and no alignment was detected with *T. asperellum*, *T. atroviride*, or *T. longibrachiatum*. In the case of *T. harzianum* CBS 226.95, as expected, the alignment reported a unique match of 220 bp, indicating that the SCAR marker is present in one copy in this genome. On the other hand, from the two available *T. reesei* genomes, only a 28-bp match with the SCAR sequence was detected, but this was not coincident with the areas where the primers were expected to anneal. Similarly, in the case of *T. virens* genome, a 99-bp match was found, but it did not include the annealing region for the 2F2 primer (Table S1). Additionally, the SCAR marker from the tested *T.* cf. *harzianum* strains was sequenced to evaluate its variability (Fig. S4). The full-length of the fragments matched, showing only 10 polymorphic sites, the most variable strain being the one of *T.* cf. *harzianum* BpT10a (FASTA S2). Moreover, the SCAR marker variability was compared with that of other markers, such as calmodulin (*cal1*) and translation elongation factor (*tef1*) genes, each one exhibiting a similar tree topology (Figs. S4 and S5).

3.4 Real-time qPCR

Primer pair 2F2/2R2 was used for the real-time qPCR assays. Only in the qPCR reactions where *T.* cf. *harzianum* DNA was used as template, was a signal (C_t) detected and a single melting peak observed (data not shown), which indicates that the primers were specific for the SCAR marker of *T.* cf. *harzianum*. These results were further checked using electrophoresis in agarose gels, in which only the expected size fragment was observed. In the three standard curves performed using 10-fold serial dilutions of different DNA templates: (1) gDNA of *T.* cf. *harzianum* BpT10a, (2) a linearized plasmid containing the SCAR marker, and (3) an equimolar mix of fungal gDNA from several species, qPCR reactions with similar levels of efficiency were reached (Fig. 3). The efficiency of each curve and r^2 values of regression

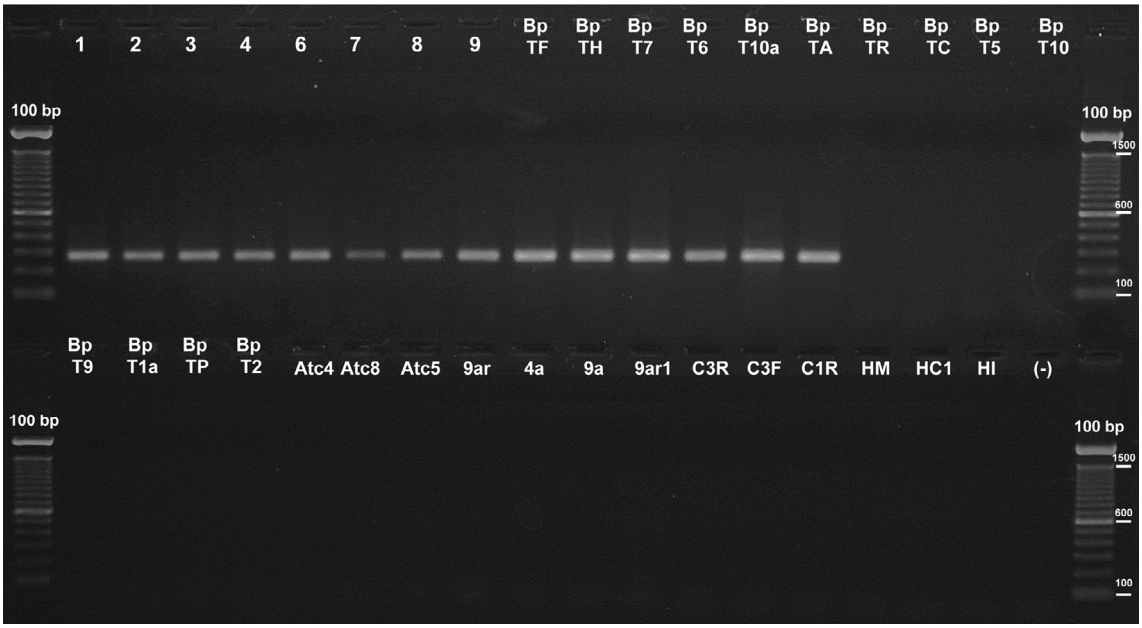
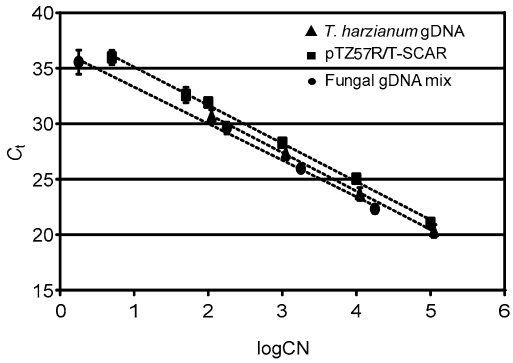


Fig. 2 Evaluation of the SCAR marker primers by PCR

The 278-bp fragments amplified from different *T. harzianum* strains using the SCAR 2F2 and 2R2 primers are shown in the agarose gel together with the absence of amplification from other fungal species. Lanes labeled as 1, 2, 3, 4, 6, 7, 8, 9, BpTF, BpTH, BpT7, BpT6, BpT10a, and BpTA correspond to gDNA from *T.* cf. *harzianum* isolates; BpTR, BpTC, BpT5, BpT10, and BpT9 from *T. atroviride*; BpT1a from *T. novaezelandiae*; BpTP from *T. citrinoviride*; BpT2 from *Trichoderma* sp.; Atc4, Atc5, 9a, 9ar1, C1R, and C3F from *Penicillium* spp.; HI, HC1, and 4a from *Aspergillus* spp.; HM from *Davidiella* sp.; 9ar from *Paecilomyces* sp.; Atc8 from *Monascus* sp. (see Table 1 for details); and C3R from a compost sample. (−) is a negative control. As a molecular weight marker the 100-bp ladder was used



Template	Linear regression	r^2	Efficiency (%)
T. harzianum gDNA	C_t=−3.4667logCN+37.795	0.9996	94
pTZ57R/T-SCAR	C_t=−3.4417logCN+38.544	0.9983	95
Fungal gDNA mix	C_t=−3.2991logCN+36.607	0.9967	101

Fig. 3 Evaluation of SCAR marker primers by real-time quantitative PCR (qPCR)

The SCAR primers were evaluated by qPCR in three different contexts: *T.* cf. *harzianum* gDNA, a plasmid with the SCAR fragment, and a mix of fungal gDNA. For the three assays, the linear regression, r^2, and the efficiency of the reaction were calculated. C_t: threshold cycle; CN: copy number

curves are shown in Fig. 3. In all the three standard curves, the linear regressions showed that the target SCAR fragment was detected even when the template included less than 100 copies of the target genome.

The sensibility of the SCAR marker for monitoring *T.* cf. *harzianum* BpT10a in a fungal DNA mix was evaluated, and the results showed that it was possible to detect *T.* cf. *harzianum* even at the lowest dilution tested, which represented 0.1% of the total gDNA used for the qPCR reactions.

Finally, the SCAR marker sensibility was also assayed in the two experimental fungal communities. In both communities, *T.* cf. *harzianum* BpT10a was detected, but at 7 and 10 d after inoculation, respectively. The SCAR copy numbers in Community 2 were 100 times higher than those in Community 1 (Fig. 4). In this community context, when the DNA was extracted 2 h after the inoculation of spores from *T.* cf. *harzianum* BpT10a (0 d), the SCAR primers were only able to detect 1% of the expected SCAR copy numbers assuming that each spore contains one genome. In Community 1, the copy number showed

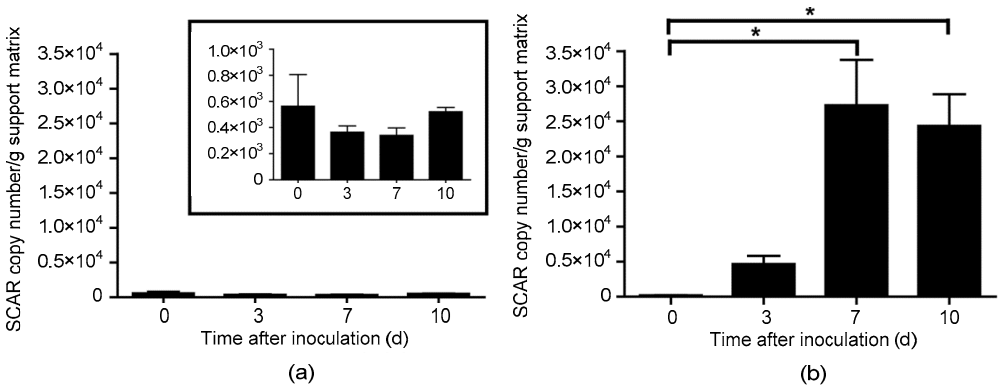


Fig. 4 SCAR marker copy numbers in experimental fungal communities at the different sampling time

T. cf. *harzianum* was monitored by qPCR with the SCAR primers after inoculating it in two experimental fungal communities. (a) Community 1 composed of *T. citrinoviride* (BpTP), *T. novaezelandiae* (BpT1a), *T. atroviride* (Bpt10), *B. bassiana* (MR-21), and *Purpureocillium* sp. (MR-6); (b) Community 2 composed of *P. canescens* (MR-13), *A. niger* (MR-1), *C. cladosporoides* (MR-12), *P. variotii* (MR-9), and *T. flavus* (MR-25). Data are shown mean and standard deviation of duplicate measures. * represents significantly different values according to the Tukey HSD test ($P \leq 0.05$). The inset in the graph of Community 1 is included to show a higher resolution of the scale

no significant differences (P=0.5717) during the whole incubation time, indicating that the fungus is able to stay in this community, but not successfully colonize; in Community 2, the population of *T.* cf. *harzianum* significantly increased after 10 d of incubation (P=0.0189). No signals were detected in the control communities.

4 Discussion

RAPD analysis has been a very useful fingerprinting technique for studying DNA polymorphisms in various organisms, among them fungal strains, although methodological difficulties such as reproducibility and co-dominance are some of the disadvantages associated with this method. To overcome this situation, RAPD assays can be made more robust by converting them into a PCR-based in more specific and reliable markers known as SCAR. These molecular markers facilitate the detection and monitoring process by being able to directly evaluate the presence of species, strains, or isolates in complex environmental samples or experimental microbial assemblages. In this work, an RAPD marker was converted into a SCAR marker to develop a sensitive and reliable assay for monitoring *T.* cf. *harzianum* in experimental fungal communities. Based on the sequence data of the cloned RAPD fragment, specific primer pairs were designed and evaluated to amplify SCAR markers. Two species-specific primer pairs, 2F2/2R2 and 2F2/2R3, amplified a 278-bp and 448-bp DNA fragment, respectively, when tested using the gDNA from various strains of *T.* cf. *harzianum*. The specificity of the SCAR primer pairs was empirically checked using DNA extracted from various fungal species and from fungal experimental communities as templates; *T.* cf. *harzianum* was distinguished from all the other tested *Trichoderma* species by generating the expected amplification product. The alignments of the SCAR marker sequences amplified with the 2F2/2R2 primer set from the tested *T.* cf. *harzianum* strains showed a high homology. Moreover, the SCAR sequence variability of *T.* cf. *harzianum* strains used in this study was similar to those of *cal1* and *tef1* genes according to the neighbor-joining (NJ) tree topologies.

The SCAR sequence was also compared with 366 fungal genomes, and no significant sequence homology was observed with fungi belonging to genera different to *Trichoderma*. In the *Trichoderma* group, six genomes were actually available, and no alignment was found with *T. asperellum*, *T. atroviride*, or *T. longibrachiatum*. In the case of the *T. reesei* and *T. virens* genomes, partial matches were detected, but these were not coincident with the areas where the primers were expected to anneal.

According to the sequence alignment data, this marker is present only once in the *T. harzianum* CBS 226.95 genome, therefore allowing it to assess a quantitative detection, by qPCR, of the number of

genomic units present in a complex sample and to study the dynamics of a population of *T.* cf. *harzianum* in an experimental fungal community.

Although the SCAR marker designed in this study was not able to discriminate among different *T.* cf. *harzianum* strains, it is highly sensitive, with a detection limit of at least 100 genome copies per nanogram of total DNA, being more sensitive than other reported SCAR markers (Rubio *et al*., 2005; Cordier *et al*., 2007). In addition, this SCAR marker is sensible enough to detect *T.* cf. *harzianum* representing 0.1% in 1 ng of gDNA mix. This SCAR marker was also probed in fungal experimental communities, where the copy numbers determined by qPCR in a community composed of fungal species closely related to *T.* cf. *harzianum* BpT10a (Community 1) was not statistically different during the sampling time. Conversely, in Community 2, which comprised fungal species not as closely related to *T.* cf. *harzianum* BpT10a, its colonization was more successful, increasing the SCAR copy numbers from 7 d after inoculation. This result is in line with some previous studies and suggests that the composition of the recipient community affects the colonization by the alien fungus (Bae and Knudsen, 2001; 2005). Interactions, such as competition and amensalism, and the organic nutrient sources are reported to affect the populations and activities of biocontrol agents in the soil (Hoitink and Boehm, 1999). Moreover, in the experimental communities at 0 d after *T.* cf. *harzianum* BpT10a inoculation, the SCAR marker detected only 1% of the expected SCAR copy numbers, assuming that each spore in the inoculum contains one genome, which confirms that the DNA extraction methods for different sample types (spores, mycelia, support matrix, soil, etc.) could lead to underestimating the actual population of the fungus (Black and Foarde, 2007; Martin-Laurent *et al*., 2001).

SCAR markers derived from an RAPD assay have been used to detect different organisms at isolate or strain levels, such as *Gliocladium catenulatum* (Paavanen-Huhtala *et al*., 2000) and *Fusarium oxysporum* (del Mar Jiménez-Gascó and Jiménez-Díaz, 2003; Pasquali *et al*., 2006; Lievens *et al*., 2008). In addition, SCAR markers derived from an RAPD assay have been also previously reported and used to detect different *Trichoderma* species or strains in different samples with varying detection limits. Rubio *et al.* (2005) reported the specific detection, by means of a SCAR marker, of the *T. harzianum* 2413 strain both in a pure culture and an artificially inoculated sterile soil, but their specificity was not great enough to detect and quantify *T. harzianum* 2413 among a mixture of strains. Cordier *et al*. (2007) using a SCAR marker designed to specifically identify *T. atroviride* T1 strain monitored this strain in soil without cross-detection of other native populations of *Trichoderma*; the quantification of *T. atroviride* T1 by real-time PCR was possible at different fungal densities, even at the lowest concentration of 1×10^3 colony-forming unit (CFU) per gram of soil. Feng *et al*. (2011), using SCAR markers, quantified two *Trichoderma* biocontrol strains in artificially inoculated sterile soils by multiplex qPCR, which were found at approximately 10^7–10^8 copies per gram of soil. SCAR markers have also been designed to detect *Trichoderma* at the genus level based in the ITS region sequence and used to detect and quantify *Trichoderma* spp. in different soils by real-time PCR (Hagn *et al*., 2007).

Other SCAR markers have been developed from sequences of endochitinase genes, for monitoring the biocontrol agent *T. atroviride* SC1 strain in field assays, with the detection limit 6×10^3 haploid genome copies per gram of soil (Savazzini *et al*., 2008), and from intergenic regions using universal primer combinations to distinguish the *T. harzianum* AS12-2 strain, without detection limits evaluated (Naeimi *et al*., 2011).

The design carried out in the present study focused on obtaining a molecular marker useful for a wide range of *T.* cf. *harzianum* strains, then allowing the invasion and colonization processes of this fungal species. These markers may be useful not only for monitoring the effect of *Trichoderma* in experimental communities, but could be also used as a quality control system to confirm the identity of *T.* cf. *harzianum* in the industrially batch production of this fungus to market it as a biocontrol agent. SCAR-based real-time PCR has been successfully used to monitor biological control agents such as *Aureobasidium pullulans* L47 strain (Schena *et al*., 2002), *Plectosphaerella cucumerina* (Atkins *et al*., 2003), *Candida oleophila* (Massart *et al*., 2005), and *Pseudomonas fluorescens* (Pujol *et al*., 2005).

Eventually, these SCAR markers could be used to detect *Trichoderma* in environmental samples;

however, since *T.* cf. *harzianum* is a cosmopolitan and diverse species, very frequent and abundant in soils, it is highly likely to find native strains very phylogenetically close to *T.* cf. *harzianum*, which could also be amplified with the designed primers. For this reason, this SCAR marker might not be useful for assessing the presence of *T.* cf. *harzianum* in environmental samples such as soil. In fact, it is very difficult to rule out cross-reactions with native strains not yet described and to discriminate between *Trichoderma* endogenous populations and those artificially inoculated, as has been reported by other authors in field trials (Feng *et al*., 2011). In any case, this SCAR marker can be used as a highly sensitive diagnostic tool, either by conventional PCR or qPCR, for tagging *T.* cf. *harzianum* populations in experimental fungal communities.

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Compliance with ethics guidelines

Gabriel PÉREZ, Valentina VERDEJO, Clarissa GONDIM-PORTO, Julieta ORLANDO, and Margarita CARÚ declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 Randomly amplified polymorphic DNA (RAPD) fingerprintings of *Trichoderma* cf. *harzianum*

Fig. S2 Randomly amplified polymorphic DNA (RAPD) sequence used to design the SCAR primers

Fig. S3 Evaluation of the sequence-characterized amplified region (SCAR) marker primers by polymerase chain reaction (PCR)

Fig. S4 Sequence-characterized amplified region (SCAR) sequence analyses of six *Trichoderma* cf. *harzianum* isolates

Fig. S5 Relationships between *Trichoderma* cf. *harzianum* isolates using different molecular markers

FASTA S1 Randomly amplified polymorphic DNA (RAPD) sequence based on which the sequence-characterized amplified region (SCAR) primers were designed

FASTA S2 Sequence-characterized amplified region (SCAR) sequences of *Trichoderma* cf. *harzianum* isolates

Table S1 BLAST analysis performed to align sequence-characterized amplified region (SCAR) sequence with different fungal genomes

中文概要：

本文题目：SCAR 分子标记监测菌群中的哈茨木霉

Designing a SCAR molecular marker for monitoring *Trichoderma cf. harzianum* in experimental communities

研究目的：开发具有种属特异性序列特征性扩增区域（SCAR）标记物来监测哈茨木霉在其入侵的试验菌群中的定殖和生长，为哈茨木霉应用于生物防治等生态和生物技术中提供支撑。

创新要点：多种木霉属真菌能与各种微观和宏观的生物有机体建立相互作用。利用这些相互作用，木霉可做为原生种群的入侵物种而用于生物防治。本文通过使用试验菌群为研究模型，利用随机扩增多态性 DNA（RAPD）技术和序列特征性扩增区域（SCAR）标记物来监测菌群中哈茨木霉的生长状态。

研究方法：利用随机扩增多态性 DNA（RAPD）技术，从 16 个 10 聚体引物进行多态性筛选，其中 1 个引物扩增出对应哈茨木霉的条带。对该条带进行克隆测序，并设计 5 个 20–23 聚体引物。成功利用引物对 2F2/2R2 和 2F2/2R3278 分别特异性地扩增出哈茨木霉 BpT10a 菌株 278 bp 和 448 bp 的 DNA 片段。同时，用这两个引物对 14 个哈茨木霉菌株和几种不同的真菌菌株进行特异性对照试验，也只成功扩增出哈茨木霉菌株。此外，使用真菌 DNA 混合物和试验真菌群的 DNA 为模板，采用实时聚合酶链式反应（PCR）对引物对 2F2/2R2 进行评估。当仅使用 100 份 SCAR 标记物或哈茨木霉仅占整个菌群的 0.1%时，仍能检测出哈茨木霉。

重要结论：本研究所建立的 SCAR 分子标记能有效监测菌群中的哈茨木霉的定殖和生长，具有较高特异性、灵敏度和准确度。

关键词组：哈茨木霉；序列特征性扩增区域（SCAR）；分子标记；试验菌群