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Development of a strain-specific SCAR marker for the detection of *Trichoderma atroviride* 11, a biological control agent against soilborne fungal plant pathogens

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Abstract The genus *Trichoderma* includes biocontrol agents (BCAs) effective against soilborne plant pathogenic fungi. Several potentially useful strains for biological control are difficult to distinguish from other strains of *Trichoderma* found in the field. So, there is a need to find ways to monitor these strains when applied to natural pathosystems. We have used random amplified polymorphic DNA (RAPD) markers to estimate genetic variation among sixteen strains of the species *T. asperellum*, *T. atroviride*, *T. harzianum*, *T. inhamatum* and *T. longibrachiatum* previously selected as BCAs, and to obtain fingerprinting patterns. Analysis of these polymorphisms revealed four distinct groups, in agreement with previous studies. Some of the RAPD products generated were used to design specific primers. Diagnostic PCR performed using these primers specifically identify the strain *T. atroviride* 11, showing that DNA markers may be successfully used for identification purposes. This SCAR (sequence-characterised amplified region) marker can clearly distinguish strain 11 from other closely related *Trichoderma* strains.

Key words *Trichoderma* · Biocontrol agents · Molecular markers · SCARs

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Introduction

The filamentous fungi included in the genus *Trichoderma* are among those considered economically important. Several species of this genus are used as biocontrol agents (BCAs) against soilborne plant-pathogenic fungi (Papavizas 1985; Samuels 1996). These species produce extracellular enzymes (Haran et al. 1996) and/or antifungal antibiotics (Ghisalberti and Rowland 1993), or they may be competitors to fungal pathogens (Simon and Sivathamparan 1989), promote plant growth (Inbar et al. 1994), or induce resistance in plants (De Meyer et al. 1998).

Controlled laboratory experiments (Grondona 1994) are usually the initial step towards the identification of strains with potential biocontrol activity. However, the effectiveness of BCAs for the control of pathogens under field conditions needs further study. If the performance of a given BCA in field trials is to be properly understood, two requisites are needed: unequivocal strain identification (that is, the ability to discriminate a specific strain from closely related ones) and rapid means for monitoring its population dynamics. Today, DNA methods are commonly used for identification and phylogenetic classification and different PCR-based strategies have been used to characterise species and strains of *Trichoderma*. Sequence polymorphisms within internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (rDNA) have been used for taxonomic purposes in *T. longibrachiatum* (Kuhls et al. 1997) and in biocontrol isolates of *Trichoderma* spp. (Hermosa et al. 2000). Data produced with randomly amplified polymorphic DNA (RAPD) have been used to differentiate numerous fungi, including *Trichoderma* spp. (Zimand et al. 1994), e.g. subgroups of *Trichoderma* capable of chestnut blight biocontrol (Arisan-Atac et al. 1995), distinguishing strains of *T. harzianum* in mushroom compost (Muthumeenakshi et al. 1994; Ospina-Giraldo et al. 1998; Chen et al. 1999), or mutants distinguishable from their parent *Trichoderma* strains (Schlick et al. 1994). Highly sensitive diagnostic assays, based on the polymerase chain reac-

tion (PCR), have also been implemented successfully for the identification of important plant pathogens (Henson and French 1993; Bounou et al. 1999).

The biotechnological applications of this genus make it necessary to find suitable genetic markers to monitor the population dynamics of patented strains during field trials (Schlick et al. 1994). This has recently been done (Green et al. 1999) by measuring population densities of strains of *Trichoderma* transformed with the β-glucuronidase gene (Thrane et al. 1995). However, the public concern about the use of genetically modified organisms in natural environments makes difficult the introduction of exogenous DNA markers for the detection of BCAs and encourages the use of strains without genetic modifications in agronomic systems. A promising approach to fulfill this requirement is based on the sequence-characterised amplified regions (SCAR) technique, which was first applied to the identification of downy mildew resistance genes in lettuce (Paran and Michelmore 1993) and very recently to a potential biocontrol strain of *Gliocladium catenulatum* (Paavanen-Huhtala et al. 2000). However, little information is available about SCAR markers in strains of *Trichoderma* selected as BCAs.

Sixteen of the *Trichoderma* strains used in this study were previously selected as BCAs against ten isolates of five different soilborne fungal plant pathogens and analysed using morphological, physiological and biochemical criteria (Grondona et al. 1997). Later, a molecular approach was undertaken using their ITS1 sequences and the polymorphisms originated by hybridisation of total genomic DNA with a mitochondrial DNA probe (Hermosa et al. 2000). The ITS1 data gave rise to a phylogenetic analysis, in which the nomenclature applied by Gams and Meyer (1998) to *T. harzianum* s.l. and Lieckfeldt et al. (1999) to *T. asperellum*, was followed. This analysis clearly separated the strains into four groups, according to the species to which they belong.

Two out of these 16 strains, *T. atroviride* 11 and 260 are currently under patent (NBT, Seville, Spain) and have been tested in field trials against the pathogens *Acremonium cucurbitacearum*, *Aphanomyces cochlioides*, *Fusarium oxysporum* f. sp. *radicis licopersici*, *Phoma betae* and *Rhizoctonia solani*. Strain 11 is currently being used in more field trials with more pathogens and different crops. Both strains were closely grouped by the physiological and biochemical analyses, and they showed identical ITS1 and ITS2 sequences (Grondona et al. 1997; Hermosa et al. 2000).

We are interested in providing means to unequivocally identify strains of *Trichoderma* with biocontrol activity and in trying to establish a simple and reliable method to predict biological properties/activities, based upon their phylogenetic relationships from the data obtained with DNA techniques. The aim of this study was to apply the random amplified polymorphic DNA (RAPD) fingerprinting technique to deepen the analysis of the genetic diversity among the 16 strains of *Trichoderma* selected as BCAs and, in particular, to use the RAPD patterns generated to develop a rapid method for discriminating those strains with identical ITS sequences. In this paper we report the identification of a SCAR marker and the development of a PCR-based diagnostic procedure that could be employed to easily distinguish *T. atroviride* 11 from other closely related strains.

Table 1 *Trichoderma* strains used in this work. Strains with an asterisk represent those previously selected as biocontrol agents and used in the random amplified polymorphic DNA analysis in this work. *Ref. no.* is the number by which the strain is referred to in the text and in figure legends. *Culture* numbers are those given by the International Mycological Institute (*IMI*), Egham, UK, or the Spanish Type Culture Collection (*CECT*), Burjasot, Valencia, Spain. *EMBL no.* are the accession numbers of the ITS1, ITS2 and 5.8 S rDNA sequences in the EMBL database

Species	Ref. no.	Geographic origin	Culture	EMBL no.
*T. asperellum**	3	France	IMI 20179	AJ224020
*T. atroviride**	11	France	IMI 352941	AJ224008
*T. inhamatum**	24	Spain	IMI 352940	AJ224006
*T. atroviride**	260	England	IMI 352939	AJ224007
*T. atroviride**	2923	Zimbabwe	IMI 281112	AJ224009
*T. inhamatum**	2924	India	IMI 293168	AJ224010
*T. harzianum**	2925	Colombia	IMI 296235	AJ224011
*T. inhamatum**	2926	England	IMI 298371	AJ224012
*T. inhamatum**	2927	England	IMI 298372	AJ224013
*T. inhamatum**	2928	England	IMI 298373	AJ224014
*T. inhamatum**	2929	England	IMI 298374	AJ224015
*T. inhamatum**	2930	India	IMI 304056	AJ224016
*T. inhamatum**	2931	India	IMI 304057	AJ224017
*T. longibrachiatum**	2932	India	IMI 304058	AJ224018
*T. inhamatum**	2933	Sri Lanka	IMI 300082	AJ224019
*T. asperellum**	ThVA	Spain	IMI 20268	AJ224021
Hypocrea rufa	4	France	IMI 131883	
T. aureoviride	5	Philippines	IMI 288110	
T. hamatum	7	India	IMI 224801	
T. longibrachiatum	12	Sierra Leone	IMI 61758	
T. koningii	14	Colombia	IMI 296239	
T. pseudokoningii	15	Philippines	IMI 288112	
T. reesei QM9414	17	M. Mandels	IMI 2414	
T. saturnisporum	20	Australia	IMI 177881	
T. viride	26	England	IMI 238904	
T. harzianum	2413	USA	CECT 2413	
T. asperellum	T48.1	Spain		
T. harzianum	ThCh	Spain		

Materials and methods

Fungal strains and culture conditions

The strains of *Trichoderma* used in this work are listed in Table 1. In addition to the non-BCA strains listed, another 14 monoconidial isolates of *T. harzianum* Rifai, collected during the 1999 campaign from different strawberry crops in Cartaya, Huelva (Spain), were used to test the SCAR marker (see Results). Cultures were maintained on potato dextrose agar (Difco) at 25 °C.

Genomic DNA extraction

Each strain was cultured in 100 ml of potato dextrose broth (Difco) for 48 h at 25 °C and 120 rpm. The mycelium was then harvested, washed with sterile distilled water, frozen and lyophilised. Total DNA extraction was performed using 40 mg of mycelium by the miniprep procedure of Raeder and Broda (1985).

Standard recombinant DNA procedures

General procedures for plasmid DNA purification, cloning and transformation of *Escherichia coli* DH5α strain were according to Sambrook et al. (1989). pGEM-T Easy vector (Promega) was used for all cloning experiments. DNA fragments for subcloning or labelling were recovered from agarose gels and purified using the Geneclean kit (BIO 101, La Jolla, Calif.). Sequencing-grade plasmid DNA was obtained using the Wizard Plus Minipreps DNA Purification System (Promega), according to the manufacturer's recommendations. Sequencing of both strands of the cloned PCR products was carried out using the forward and reverse universal primers in an ABI 377 Prism Sequencer (Applied Biosystems). Electrophoresis and transfer of DNA onto nylon filters (Hybond N^+, Amersham), were performed as described by Sambrook et al. (1989). Labelling, hybridisation and immunological detection were carried out using the Non-radioactive labelling and immunological detection kit and CDP-Star as the chemiluminescent substrate (Roche Biochemicals), following the manufacturer's recommendations. Hybridisations were carried out at 65 °C, with two final high-stringency washes at 65 °C. Chemiluminescent signals were detected by exposing the filter to X-ray film (Fuji Photofilm) for up to 5 min.

DNA and protein sequence comparisons and alignments were carried out using the BLAST sequence analysis program.

PCR-amplification conditions

Thirty arbitrary decamers (OPC1–OPC20 and OPF1–OPF10; Operon Technologies), were tested for reproducible RAPD pattern generation. Optimal amplification conditions were found to be: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 100 nM primer, 5–40 ng genomic DNA and 1.25 U of *Taq* polymerase (Perkin-Elmer) in a final volume of 50 μl. Amplification reactions were carried out in a Perkin-Elmer thermocycler for 35 cycles of 5 s at 93 °C, 30 s at 36 °C and 1 min at 72 °C. A sample (20 μl) of each PCR reaction was analysed by electrophoresis on 1.2% agarose gels, stained with ethidium bromide and photographed under UV light. All amplification reactions were performed at least twice for each strain, in two separate experiments and using different batches of DNA. Only reproducibly amplified fragments were used for analysis.

Cluster analysis

Comparison of the profiles obtained with the selected primers was done on the basis of the presence versus absence (1/0) of RAPD products of the same size. The analyses were based on the Dice coefficient $[-2a/(2a + b + c)-]$, where a is the number of positive DNA bands scored by the two strains in a pair, and b and c are the number of positive DNA bands present in only one of the strains in a pair (Dice 1945). A dendrogram was derived from the distance matrix by the unweighted pair-group method algorithm (UPGMA) in the computer program package RAPDistance 1.04. The dendrogram that best fitted the similarity matrix based on cophenetic values was chosen.

Diagnostic PCR

The sequences of the 24-bp oligonucleotide primers designed for the amplification of the SCAR markers are: 11-A1: 5′-GGA AGC TTG GCG TTT ATT GTA CAA-3′, 11-A1c: 5′-GGA AGC TTG GGT ATT GAG CTG GGC-3′, 11-A2: 5′-GGA AGC TTG GGA GCT CAG GAG GGG-3′, 11-A2c: 5′-GGA AGC TTG GAT GTA TAG GCA CGT-3′. Each pair of primers was used in PCR reactions as described above, with the following modifications: 5 min at 94 °C, 30 cycles of 1 min 30 s at 93 °C, 2 min at 68 °C (11-A1/11A1c primers) or 55 °C (11-A2/11A2c primers) and 2 min at 72 °C, with a final extension of 5 min at 72 °C. A sample (20 μl) of each PCR reaction was analysed by agarose gel electrophoresis as before.

Results

RAPD analysis of *Trichoderma* strains

Thirty arbitrary decamer primers were used to generate RAPD marker patterns from the 16 strains of *Trichoderma* spp. selected as BCAs. Only three primers, OPC15, OPC16 and OPC20, gave rise to polymorphic fragments useful for RAPD-PCR analysis. The size of the amplified DNA fragments obtained with the three primers ranged over 0.2–4.5 kb and only strong, reproducibly amplified products were scored for presence versus absence (1/0) for all strains studied. Amplification products generated with OPC16 are shown in Fig. 1.

The combined data from all strains were analysed using the Dice coefficient (Dice 1945) to produce a dendrogram (Fig. 2). At a genetic distance of 0.35, the 16 *Trichoderma* strains were separated into four groups. The first group, termed RAPD-1, included the strain *T. asperellum* 3 from France. A second group, RAPD-2, comprised two strains, *T. atroviride* 11 and 260 from France and the United Kingdom, respectively. These two strains showed similar RAPD patterns and the genetic distance between them in the dendrogram is only 0.1. The third group, RAPD-3, included seven non-European strains, corresponding to *T. longibrachiatum* 2932, *T. atroviride* 2923, *T. harzianum* 2925 and four strains of *T. inhamatum*. Finally, the fourth group, RAPD-4, included five strains of *T. inhamatum* collected in Europe (United Kingdom and Spain) and the strain *T. asperellum* ThVA, also from Spain. The dendrogram in Fig. 2 does not show a clear correlation between RAPD groups and species or geographic origin. However, the five European *T. inhamatum* strains (RAPD-4) appear to be different from the four non-European *T. inhamatum* strains (RAPD-3). Interestingly, groups RAPD-1 and RAPD-2 include strains of *T. asperellum* and *T. atroviride* that showed good capabilities for biocontrol of *P. betae* in glasshouse experiments

(Grondona 1994). However, *T. atroviride* strain 2923, which was grouped by the ITS sequence analysis with the other two *T. atroviride* strains (11 and 260), is here located in the RAPD-3 group.

Development of SCAR markers for diagnostic PCR

One of the arbitrary decamers (OPF10) used for the RAPD analysis failed to generate useful polymorphic patterns. However, the PCR amplification products obtained using this primer gave rise to different RAPD patterns for strains *T. atroviride* 11 and *T. atroviride* 260 (Fig. 3). Two DNA fragments 11-A1 (about 1000 bp) and 11-A2 (about 400 bp), amplified from DNA of *T. atroviride* 11, were selected for their ability to differentiate this strain from the closely related strain *T. atroviride* 260.

The two DNA fragments were purified and cloned into the pGEM-T Easy vector, resulting in plasmids pG11-A1 and pG11-A2 respectively. Nucleotide sequencing confirmed that the two RAPD fragments were 996 bp and 361 bp respectively, with the primer sequence (OPF10) at both ends. Although no ORF could be detected in either of the two DNA fragments, both showed a fairly high [G + C] content (43.3% and 48.8%, respectively) and no significant homology to the nucleotide and protein databases.

A Southern blot was carried out in order to confirm that the two DNA fragments indeed corresponded to *T. atroviride* 11 genomic DNA, to detect cross-hybridisations and to check whether they were single- or multiple-copy. Genomic DNA from the sixteen strains was digested with *Eco*RI and probed against the 11-A1 and 11-A2 DNA fragments. The DNA fragment cloned in

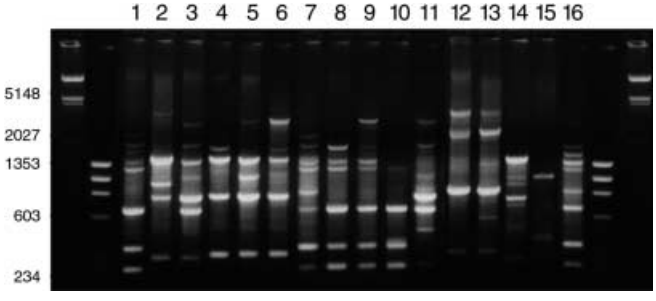

Fig. 1 Agarose gel showing RAPD profiles of 16 strains of *Trichoderma* spp amplified with arbitrary primer OPC16. *Numbers* at the top correspond to strains in Table 1: *lane 1* 24, *lane 2* 2924, *lane 3* 2925, *lane 4* 2926, *lane 5* 2927, *lane 6* 2928, *lane 7* 2929, *lane 8* 2930, *lane 9* 2931, *lane 10* 2932, *lane 11* 2933, *lane 12* 11, *lane 13* 260, *lane 14* 2923, *lane 15* 3, *lane 16* ThVA. The molecular weight standards were *Eco*RI + *Hin*dIII double-digested λ DNA and *Hae*III ϕX174

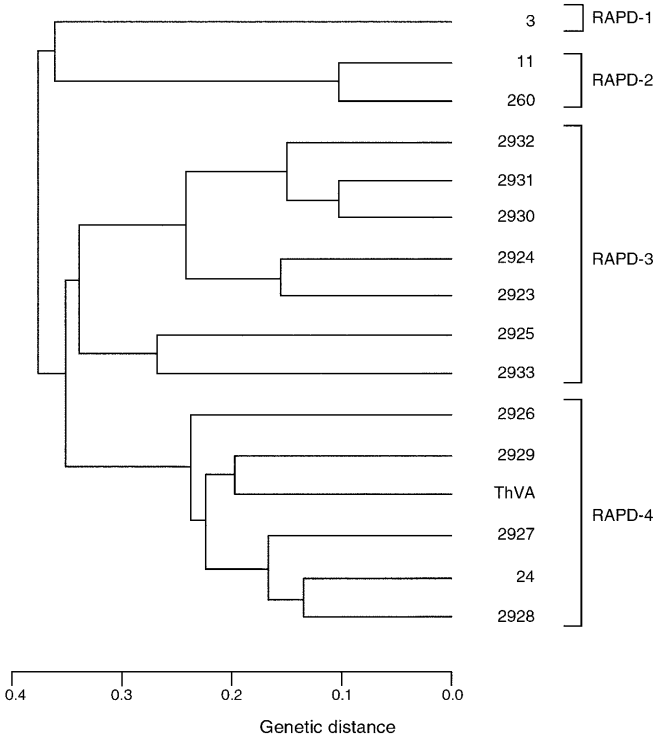

Fig. 2 Unweighted pair-group method algorithm dendrogram of relative genetic distance among 16 strains of *Trichoderma* spp., calculated from a random amplified polymorphic DNA (RAPD)-(0/1)-data matrix including OPC15, OPC16 and OPC20 primers

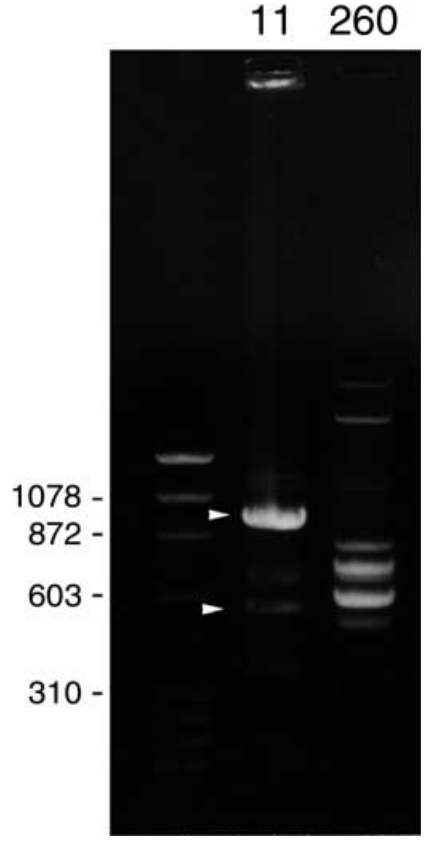

Fig. 3 Agarose gel showing RAPD profiles of *T. atroviride* 11 and 260, amplified with the arbitrary primer OPF10. *White arrows* point to the fragments of approximately 1,000 bp and 400 bp. The molecular weight standard was *Hae*III-digested ϕX174

plasmid pG11-A1 gave rise to a single hybridising band with the strains *T. atroviride* 11, 260 and 2923 and *T. asperellum* 3, but no hybridisation signal was detected with genomic DNA from the other 12 strains (data not shown). The probe derived from pG11-A2 hybridised to *Eco*RI-digested DNA from all 16 strains analysed, but resulted in different banding patterns. The stronger hybridising bands corresponded also to DNA from strains *T. atroviride* 11, 260 and 2923, and *T. asperellum* 3 (Fig. 4).

Specific primers 24 bp in length, coded 11-A1/ 11-A1c and 11-A2/11-A2c, corresponding to both ends of 11-A1 and 11-A2 inserts, respectively, were designed to be used in diagnostic PCR. Primers 11-A1/11-A1c generated the corresponding SCAR fragment only from genomic DNA of *T. atroviride* 11 (Fig. 5). This product was designated as SCAR11-A1. Lowering the annealing temperature by 5 °C (from 68 °C to 63 °C) resulted in multiple fragments of minor intensity that lacked reproducibility.

To check the validity of the SCAR11-A1 marker, genomic DNA from another 26 strains, corresponding to seven *Trichoderma* species (see Table 1 and Materials and methods), was used as a template in amplification experiments, using the primers 11-A1/11-A1c; and in no case was a single DNA fragment amplified.

Primers 11-A2/11A2c amplified a fragment of the expected size (about 360 bp) from DNA of 13 of the 16 strains selected as BCAs. No fragments were amplified from the genomic DNA of the strains 2930, 2932 and 2933. Fragments from the strains 3 and 260 were gel purified and sequenced using 11-A2/11-A2c primers. Both fragments showed an identical sequence to the amplified fragment from the genomic DNA of *T. atroviride* 11. Therefore, the combination of primers 11-A2/ 11-A2c did not allow us to obtain a SCAR marker suitable for distinguishing *T. atroviride* 11 from the other strains.

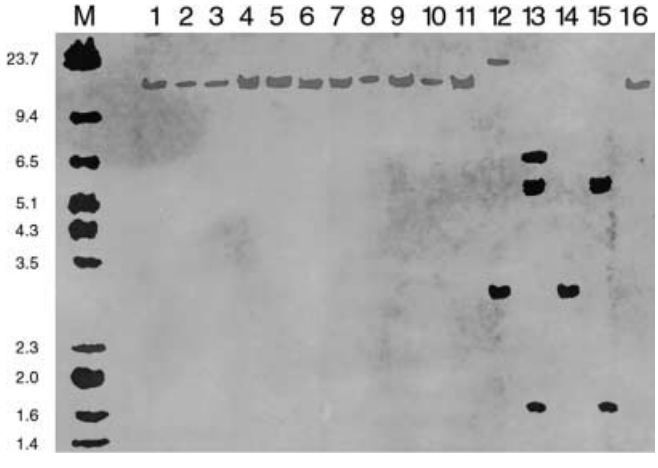

Fig. 4 Southern blot analysis of *Eco*RI-digested genomic DNA of *Trichoderma* strains hybridised with a pG11-A2-derived probe (see text). *Numbers* at the top correspond to the strains in Table 1: *lane 1* 24, *lane 2* 2924, *lane 3* 2925, *lane 4* 2926, *lane 5* 2927, *lane 6* 2928, *lane 7* 2929, *lane 8* 2930, *lane 9* 2931, *lane 10* 2932, *lane 11* 2933, *lane 12* 11, *lane 13* 260, *lane 14* 2923, *lane 15* 3, *lane 16* ThVA. The molecular weight standard was *Eco*RI + *Hin*dIII double-digested λ DNA (*lane M*)

Discussion

Many *Trichoderma* strains have biotechnological potential and are protected under patents, due to their ability to perform as BCAs against soilborne plant pathogens. Despite significant advances in our knowledge of the genus in recent years, the taxonomy of *Trichoderma* is still rather incomplete and the distinction of species in this genus remains problematic (Gams and Meyer 1998). The more extensive and complex the involvement of *Trichoderma* in biocontrol, the more useful and necessary is their accurate classification. However, morphological and nutritional criteria cannot be used as suitable markers to verify the identity of each strain within this genus. Molecular data provide a more objective measure of the genetic variability of individuals than do phenotypic characters. Classification should thus not be based on single characters, but on a combination of sequence analysis of conserved rDNA, analysis of genomic DNA by fingerprinting/RAPD and analysis of mitochondrial DNA. This seems to be the most appropriate way to resolve species complexes for *Trichoderma* (Lieckfeldt et al. 1998).

Sixteen strains of different *Trichoderma* species were selected as potential BCAs against five different soilborne fungal plant pathogens. Their physiological, biochemical and isoenzymatic features were analysed (Grondona et al. 1997), together with their ITS sequences and mitochondrial DNA (Hermosa et al. 2000). While the first approach is usually non-specific at the isolate level for most fungi (Miller 1996), DNA data is considered to be more informative from the phylogenetic point of view. Conserved genome regions such as the ITS sequences of the rRNA genes have been chosen for taxonomic purposes at the species level in almost

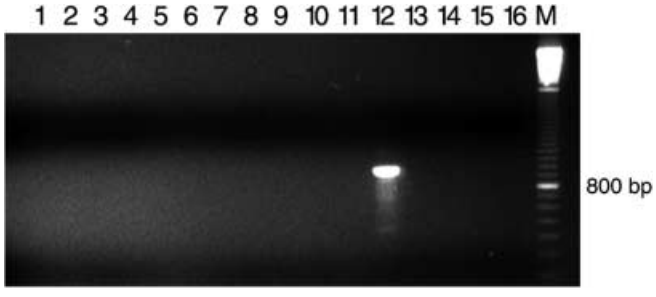

Fig. 5 Agarose gel showing the PCR products amplified from the genomic DNA of 16 *Trichoderma* strains, using the 11-A1/11-A1c primers. *Numbers* at the top correspond to the 16 *Trichoderma* strains: *lane 1* 24, *lane 2* 2924, *lane 3* 2925, *lane 4* 2926, *lane 5* 2927, *lane 6* 2928, *lane 7* 2929, *lane 8* 2930, *lane 9* 2931, *lane 10* 2932, *lane 11* 2933, *lane 12* 11, *lane 13* 260, *lane 14* 2923, *lane 15* 3, *lane 16* ThVA. The molecular weight standard was a 100-pb ladder (Roche)

every fungus analysed (Bruns et al. 1991). The analysis of the ITS sequences of the 16 strains selected as BCAs separated them into four groups, according to the closely-related species they enclose, but several strains were indistinguishable, since they showed identical ITS sequences (Hermosa et al. 2000).

The RAPD technique generates neutral markers that may reflect, at least in theory, the whole genotype of an individual. This procedure has been used to differentiate numerous fungi, including *Trichoderma* species (Zimand et al. 1994; Arisan-Atac et al. 1995; Chen et al. 1999). It has also been used in plants to find DNA markers linked to specific features such as resistance genes in wheat (Robert et al. 1999), or the male sex in hemp (Mandolino et al. 1999). We are interested in devising a method to select (or discard) strains of *Trichoderma* before the exploration of their potential capacity for biocontrol in natural environments. Therefore, we decided to perform a RAPD analysis on strains already selected as BCAs, which might somehow reflect a correlation between DNA data and biological activity. The low level of polymorphism obtained in our results is in accordance with the taxonomic proximity of the five species of *Trichoderma* included in this work (Hermosa et al. 2000). However, additional useful polymorphic markers could be obtained by increasing the number of primers used.

The dendrogram shown in Fig. 2 essentially matches previous results in which the ITS sequences were analysed. These studies showed a closer genetic relationship between the species *T. asperellum*/*T. atroviride* and between *T. inhamatum*/*T. harzianum*, than between both groups of species (Gams and Meyer 1998; Lieckfeldt et al. 1999; Hermosa et al. 2000). In fact, Bissett (1991) suggested that *T. inhamatum* and *T. harzianum* should be synonyms. However, using physiological and biochemical criteria (Grondona et al. 1997) and molecular techniques (Gams and Meyer 1998), these species were shown to be slightly different. Several particular results in the dendrogram obtained with the RAPD analysis must be taken into account. RAPD-1 and RAPD-2 groups included *T. asperellum* and *T. atroviride* strains; and RAPD-3 and RAPD-4 included *T. inhamatum* and *T. harzianum* strains, together with *T. longibrachiatum* 2932, *T. asperellum* ThVA and *T. atroviride* 2923. The location of these three strains within the *T. inhamatum*/*T. harzianum* group needs further attention. The strain *T. longibrachiatum* 2932 has been grouped with strains of *T. inhamatum*, either by physiological and biochemical criteria, mitochondrial DNA polymorphisms or the analysis of ITS sequences (Grondona et al. 1997; Hermosa et al. 2000); and this species probably constitutes a monophyletic group (Arisan-Atac et al. 1995; Kuhls et al. 1997; Lieckfeldt et al. 1998). The RAPD analysis confirms the location of *T. longibrachiatum* 2932 (RAPD-3), within the *T. inhamatum*/*T. harzianum* group. In a similar way, although the ITS sequence analysis alone located the strain *T. asperellum* ThVA close to strains of *T. hamatum*/*T. asperellum*, the RAPD analysis shows that this strain is closer to the *T. inhamatum*/*T. harzianum* group. This result has also been obtained with biochemical analyses, the polymorphisms of mitochondrial DNA (Grondona et al. 1997; Hermosa et al. 2000) and even with the hybridisation of genomic DNA with the 11-A1 and 11-A2 DNA fragments from *T. atroviride* 11 (Fig. 4). Finally, strain *T. atroviride* 2923, which by RAPD analysis is located in the RAPD-3 group, has always been unequivocally co-located with the other two *T. atroviride* strains (11 and 260). This strain was shown to be very similar to *T. atroviride* 11 by the ITS sequence and the mitochondrial DNA patterns. The 11-A1 and 11-A2 DNA fragments from *T. atroviride* 11 also produced similar patterns of hybridisation with the genomic DNA of *T. atroviride* 2923. The only significant difference between the *T. atroviride* strains in RAPD-2 group and *T. atroviride* 2923 is that the strains in the RAPD-2 group showed strong capabilities for biocontrol of *P. betae* in glasshouse experiments (Grondona 1994), while *T. atroviride* 2923 showed low levels of extracellular enzyme activity and low antagonistic activity (Grondona et al. 1997). These results suggest that there might be a correlation between RAPD pattern grouping and biocontrol activity, as has also been suggested for other *Trichoderma* strains capable of chestnut blight biocontrol (Arisan-Atac et al. 1995). Thus, RAPD markers might be used as a rapid method for the screening of strains to search for potential BCAs.

The RAPD analysis here reported also reveals the existence of two genetically distinct groups within *T. inhamatum* (RAPD-3 and RAPD-4), which correlate to their European and non-European origin. The distribution of these strains into two different groups was also observed using physiological and biochemical criteria (Grondona et al. 1997).

RAPD analyses have proven to be useful for detecting genomic polymorphisms (Williams et al. 1990). Although the RAPD technique appears to be the simplest and the most flexible method for assessing the BCA populations, the low annealing temperature and short primer size used for RAPD generation may affect amplification reproducibility. Thus, this method could be inappropriate for diagnosis purposes. *T. atroviride* strains 11 and 260 were selected on the basis of their biocontrol activity against five different pathogens and are currently under patent. They were closely grouped by physiological and biochemical analyses and they showed identical ITS1 and ITS2 sequences (Grondona et al. 1997; Hermosa et al. 2000). To this end, we decided to check the efficiency of SCAR markers and, therefore, we decided to convert two RAPD fragments generated during PCR with OPF10 primer, both of which specifically tagged *T. atroviride* 11, into SCAR markers. Both SCAR markers, SCAR11-A1 and SCAR11-A2, allowed us to distinguish between the *T. atroviride* strains 11 and 260 when used as probes in hybridisation experiments. However, from a practical point of view, hybridisation methods are difficult and time-consuming when analysing large BCA populations.

On the contrary, a PCR-based assay would enable rapid monitoring to be carried out.

The first question to test the validity of the SCAR11-A1 marker, amplified when using the combination of 11-A1/11-A1c primers under suitable PCR conditions, is to demonstrate that it is strictly specific for *T. atroviride* 11. The results of the southern blot showed that the SCAR-A1 marker was single-locus and that it was present only in the *T. asperellum* and *T. atroviride* strains, but no homologous sequences could be detected in the other 12 strains included in the analysis. In addition to the 16 *Trichoderma* strains, another 26 strains belonging to seven different *Trichoderma* species were assayed for amplification of the SCAR11-A1. The primer pair 11-A1/11-A1c did not amplify the SCAR11-A1 when using DNA from any of these strains (B. Rubio, unpublished results). This number of species and strains can be regarded as being representative, since the number of *Trichoderma* species isolated from soil samples is relatively low. A survey of native populations of *Trichoderma* demonstrated that 12 soil samples gave rise to 180 isolates which corresponded to only five species (Roiger et al. 1991). *T. hamatum* (Bonord) Bainier, *T. harzianum* Rifai and *T. koningii* Ouden were the most common species, occurring in most soils.

Another important issue in biological control is the ability to detect and identify a single strain within complex populations. Since BCAs are already being used in field trials, studies of their population dynamics are needed. The traditional method of counting colony-forming units on a *Trichoderma*-selective medium does not distinguish between closely related strains or species and DNA markers have proven useful to this point. Recently, isolate-specific markers for *T. hamatum* 382 were identified, converted into SCAR markers and used successfully to verify the presence of this BCA in nine different soil, compost and potting mix samples (Abbasi et al. 1999). However, the performance of a given marker depends on its stability to enable repeated sampling and monitoring of strains during field trials. Although teleomorphic stages of *Trichoderma* are known, most strains have not been associated with a sexual state and are believed to be mitotic and clonal. Therefore, the mechanisms of asexual variation are of primary importance in the taxonomy of these fungi, their adaptability to changing environmental conditions and their interactions with other organisms, including other strains of *Trichoderma* found in the field. Among those mechanisms of asexual variation, chromosomal polymorphism has been shown to occur slowly in field populations of filamentous fungi and strains remain stable over several years (Zolan 1995).

It has been demonstrated in laboratory experiments that there exists in *Trichoderma* a certain degree of asexual genetic recombination (Harman et al. 1998), but it is not clear whether these interactions do really occur in natural populations (Gómez et al. 1997). Clearly, the nature and extent of exchange of genetic information in wild *Trichoderma* populations requires further investigation. However, it can be considered that the probability with which a SCAR marker can be lost during field trials is relatively low. The development of more strain-specific markers for a single strain would reduce this probability to a minimum.

The SCAR marker here developed can be a rapid and convenient method for monitoring *T. atroviride* 11 when screening large numbers of samples.

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