M. Maymon *et al.* (2004) *Phytoparasitica* 32(4):370-375

NOTE: Identification of *Trichoderma* Biocontrol Isolates to Clades According to ap-PCR and ITS Sequence Analyses

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A collection of *Trichoderma* isolates, with different biocontrol capabilities, were identified by molecular methods. Arbitrarily-primed PCR (ap-PCR) using repeat motif primers was performed on DNA from a *Trichoderma* spp. collection of 76 isolates, and representative isolates were further characterized into three main clades by internal transcribed spacer (ITS) sequence analysis. Consequently, a reliable phylogenetic tree was constructed containing isolates belonging to the *T. harzianum* clade (comprising *T. aureoviride, T. inhamatum,* and *T. virens*), the *T. longibrachiatum* and *T. saturnisporum* cluster, and that including the species *T. asperellum*, *T. atroviride*, *T. koningii* and *T. viride*.
KEY WORDS: Anthracnose; *Fragaria* × *ananassa*; gray mold; internal transcribed spacer (ITS) region; arbitrarily-primed polymerase chain reaction (ap-PCR); ribosomal DNA.

Isolates of *Trichoderma* species are known for their ability to control plant pathogens (4). In a recent study it was shown that various isolates of *Trichoderma* spp. that originated from a *Trichoderma* collection of 76 biocontrol isolates, were effective in controlling anthracnose (*Colletotrichum acutatum* Simmonds) and gray mold (*Botrytis cinerea* Pers.;Fr.) in strawberry, under laboratory and greenhouse conditions (5,7). This collection contained isolate T-39, which was formulated and registered under the trade name Trichodex, as well as isolates T-105, T-161 and T-166, which were shown specifically to reduce disease incidence (7).

Characterization of *Trichoderma* isolates to species is based mainly on criteria such as conidial size, color and shape, mycelial branching patterns and frequency, and other morphological characteristics that are not very reliable. Therefore, recent studies have resorted to molecular methods for species identification (2,9). Sequence analysis of the internal transcribed spacers (ITS) 1 and 2 of the ribosomal DNA (rDNA) has been especially reliable for characterization of *Trichoderma* to the species level. For example, *Trichoderma* biocontrol agents have been differentiated from the pathogenic biotypes Th2 and Th4 of *T. harzianum*, causal agents of mushroom green mold, by ITS 1 sequence analyses (8,14). Likewise, additional molecular techniques based on restriction fragment length polymorphism (RFLP) analysis of functional genes, random amplified polymorphic DNAs (RAPDs) and other PCR-fingerprinting methods, have been used to distinguish between species of this genus (1,10,12,17).

In this study a collection of 76 *Trichoderma* strains (Table 1), with different biocontrol capabilities, were identified to species by arbitrarily primed-PCR (ap-PCR) and ITS 1-2 sequence analyses. The monoconidial *Trichoderma* cultures used in this study included 70 Israeli isolates collected by the authors from strawberry plants growing in cultivated plots in the Kadima area of the central Sharon region, isolate T-39 Trichodex (3), and five other reference cultures kindly provided by E. Monte, Universidad de Salamanca, Spain (8) (Table 1). All fungi were

Received Dec. 10, 2003; accepted Feb. 15, 2004; http://www.phytoparasitica.org posting July 14, 2004.
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TABLE 1. *Trichoderma* isolates used in this study

Isolate	Species	Source[z]	GenBank accession
[x]T-39 (Trichodex)	*T. harzianum*	(Ref. 17)	[y]AY222351
[w]T-105	*Trichoderma* sp.	Crown	[y]AY222340
[v]T-115	*Trichoderma* sp.	Root	[y]AY222348
T-127	*Trichoderma* sp.	Rhizosphere	[y]AY222350
T-137	*Trichoderma* sp.	Crown	[y]AY222341
T-146	*Trichoderma* sp.	Petiole	[y]AY222349
[u]T-151	*Trichoderma* sp.	Fruit	[y]AY222346
[t]T-160	*Trichoderma* sp.	Fruit	[y]AY222342
[s]T-161	*Trichoderma* sp.	Fruit	[y]AY222344
[r]T-162	*Trichoderma* sp.	Fruit	[y]AY222343
T-166	*Trichoderma* sp.	Fruit	[y]AY222347
T-170	*Trichoderma* sp.	Fruit	[y]AY222345
260	*T. atroviride*	(Ref. 8)	AJ224007
2925	*T. harzianum*	(Ref. 8)	AJ224011
2927	*T. harzianum*	(Ref. 8)	AJ224013
2930	*T. inhamatum*	(Ref. 8)	AJ224016
2932	*T. longibrachiatum*	(Ref. 8)	AJ224018
ThVA	*T. asperellum*	(Ref. 8)	AJ224021
DAOM 167057	*T. hamatum*	GenBank	Z48816
DAOM 175924	*T. aureoviride*	GenBank	AF191039
NR6883	*T. aureoviride*	GenBank	AF194010
Th1 64	*T. virens*	GenBank	AF057599
95 39	*T. inhamatum*	GenBank	AF057602
CBS 226.95	*T. harzianum*	GenBank	AJ222720
CBS 240.63	*T. viride*	GenBank	X93979
CBS 227.95	*T. harzianum*	GenBank	AF057605
CBS 886.72	*T. saturnisporum*	GenBank	X93974
CBS 978.70	*T. harzianum*	GenBank	AJ222723
Th 4c	*T. harzianum*	GenBank	U78882
GL-20	*T. virens*	GenBank	AF099007
Tr 22	*T. viride*	GenBank	AJ230678
ATCC 36042	*T. atroviride*	GenBank	AF278796
Tr 75	*T. koningii*	GenBank	Z95495
ATCC 52326	*T. longibrachiatum*	GenBank	Z48935
ATCC 13631	*T. reesei*	GenBank	Z31016
ATCC 28019	*T. parceramosum*	GenBank	Z31015

[z]All Israeli *Trichoderma* isolates (T- series) were collected by the authors from strawberry plants growing in cultivated fruiting fields in the Kadima area of the central Sharon region, Israel.
[y]Submitted to GenBank by the authors.
[x]Additional isolates belonging to this genotype based on ap-PCR: T-118, T-119, T-121, T-123, T-129, T-131, T-132, T-133, T-134, T-147.
[w]Additional isolates belonging to this genotype based on ap-PCR: T-101, T-102, T-103, T-104, T-106, T-107, T-108, T-109, T-110, T-111, T-112, T-113, T-114, T-120, T-122, T-126, T-128, T-130, T-136, T-138, T-140, T-141, T-142, T-143, T-144, T-145.
[v]Additional isolates belonging to this genotype based on ap-PCR: T-116, T-117, T-124, T-125, T-148, T-149.
[u]Additional isolates belonging to this genotype based on ap-PCR: T-151, T-152, T-154, T-155, T-156, T-157, T-158, T-159, T-163.
[t]Additional isolates belonging to this genotype based on ap-PCR: T-168, T-169.
[s]Additional isolates belonging to this genotype based on ap-PCR: T-164, T-165, T-167.
[r]Additional isolates belonging to this genotype based on ap-PCR: T-171, T-172, T-173.

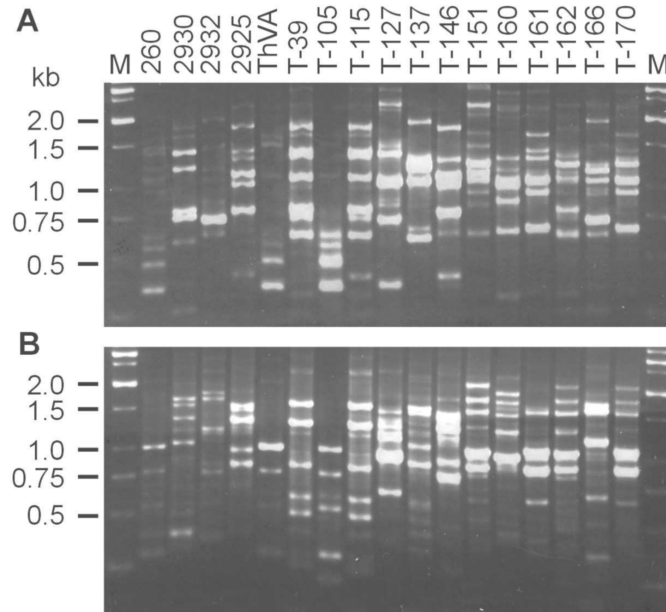

Fig. 1. Band patterns of arbitrarily-primed PCR amplified genomic DNA of the representative *Trichoderma* spp. isolates (260 = *T. atroviride*), (2930 = *T. inhamatum*), (2932 = *T. longibrachiatum*), (2925 = *T. harzianum*), (ThVA = *T. asperellum*), (T-39 = *T. harzianum*); (T-115, T-127 and T-146 = *T. harzianum* clade comprising *T. aureoviride, T. inhamatum* and *T. virens*); (T-105, T-137, T-151, T-160, T-161, T-162, and T-170 = clade comprising *T. asperellum*, *T. atroviride*, *T. koningii* and *T. viride*) (T-166 = *T. longibrachiatum* and *T. saturnisporum* clade) using primers $(GACA)_4$ (A) and $(GACAC)_3$ (B). Lane M: DNA markers with sizes in kb.

cultured in the dark at 25°C on potato dextrose agar supplemented with 250 mg l^{-1} chloramphenicol.

For DNA extraction, liquid cultures comprising 100 ml of potato dextrose broth in 250-ml Erlenmeyer flasks were inoculated with five mycelial disks of each of the cultures (Table 1) derived from colony margins. DNA was extracted and purified as described previously (6), dissolved in 0.5 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) to an approximate concentration of 500–1500 ng μl^{-1} and diluted to a final concentration of 10–100 ng μl^{-1} for PCR reactions, as described previously (6).

For ap-PCR, primers were derived from microsatellite or repeat sequences as follows: CAGCAGCAGCAGCAG, AGGAGGAGGAGGAGG, GACACGACACGACAC and GACAGACAGACAGACA, and conditions for amplification were as described previously (6). In the text, these primers have been designated $(CAG)_5$, $(AGG)_5$, $(GACAC)_3$ and $(GACA)_4$, respectively. Universal PCR primers were used (ITS 1, TCCGTAGGTGAACCTGCGG and ITS 4, TCCTCCGCTTATTGATATGC) for amplification of the ITS 1 and ITS 2 regions between the small and large nuclear rDNA, including the 5.8S rDNA (as described, ref. 6), and resulting amplified products were used for sequence analysis. PCR reactions were performed as described previously (6). All PCR experiments were conducted at least four times, with identical results being observed. Variation according to ap-PCR was interpreted according to comparisons between isolates based on overall band patterns. Isolates were grouped according to band patterns and a representative from each genotype was then selected for sequence analysis. ITS sequence data of each genotype were analyzed for species designation, using the ARB program package

(15), as described previously (6). Complete ITS 1-2 sequences of the isolates were submitted to the GenBank, with accession numbers appearing in Table 1. Additional *Trichoderma* sequences retrieved from the GenBank were included in this study for comparison (Table 1).

Amplification products were obtained from all 76 *Trichoderma* isolates tested with the microsatellite derived primers $(CAG)_5$, $(AGG)_5$, $(GACA)_4$ and $(GACAC)_3$ (data not shown). After comparison of band patterns, representative Israeli *Trichoderma* isolates were selected according to identical ap-PCR genotype (Table 1). The representative isolates from Israel T-39, T-105, T-115, T-127, T-137, T-146, T-151, T-160, T-161, T-162, T-166, T-170 and those representing previously characterized *Trichoderma* species (260 = *T. atroviride*, 2930 = *T. inhamatum*, 2932 = *T. longibrachiatum*, 2925 = *T. harzianum* and ThVA = *T. asperellum*) were further compared by ap-PCR. Gels showing diversity among and between representative isolates of each genotype or defined species, using the respective primers $(GACA)_4$ and $(GACAC)_3$, are presented (Fig. 1). As can be noted, certain isolates possessed similar banding patterns such as ThVA and T-105; T-39 and T-115; and T-151, T-162 and T-170, indicating that ap-PCR may be able to associate between unknown isolates to representatives of known, defined species or clades. Similar degrees of diversity within and among populations were obtained using primers $(CAG)_5$ and $(AGG)_5$ (data not shown).

In order to verify isolate identification to associated clades, the 13 representative *Trichoderma* isolates (T-39, T-105, T-115, T-127, T-137, T-146, T-151, T-160, T-161, T-162, T-166, T-170 and ThVA) were analyzed by comparing ITS 1, ITS 2 and ITS 1-2 sequences of these isolates, with previously reported sequences of other *Trichoderma* species. ITS 1 was reliable and accurate for the purpose of identification of isolates to defined species (8). Sequence of the ITS 1 region therefore permitted a population study, grouping the representative *Trichoderma* isolates to defined clades compared with characterized isolates (Fig. 2). Phylogenetic analyses of the ITS 1 sequence performed by applying ARB parsimony, distance matrix, and maximum-likelihood methods, produced similar tree topologies. Removing highly variable positions from the sequence analysis did not affect tree topology. Isolate T-115 was grouped within the *T. harzianum* clade (including *T. aureoviride, T. inhamatum,* and *T. virens* species) represented by isolate T-39, which was also verified by identical ap-PCR products (Fig. 1). Likewise, isolates ThVA (representative of the *T. asperellum* clade) and T-105 were closely grouped by sequence analysis and shared similar ap-PCR banding patterns (Figs. 1 and 2). Other isolates, which were grouped into clades according to ap-PCR and ITS 1 sequence, included T-166 as *T. longibrachiatum* or *T. saturnisporum*. Other representative isolates could not be defined to clades by both ap-PCR and ITS sequence analysis. However, ITS sequence alone grouped T-127 and T-146 within the *T. harzianum* clade and isolates T-137, T-151, T-160, T-161, T-162 and T-170 within the *T. atroviride*, *T. viride* and *T. koningii* clades (Fig. 2).

Molecular characterization and identification has gained popularity over the past decade and reliable differentiation between and among *Trichoderma* isolates has been reported. It should be noted, however, that more than one molecular method should be used in combination to attain reproducible and accurate results. For example, Cumagun *et al.* (2) and Lübeck *et al.* (11) used universally-primed (UP)-PCR and RFLPs of rDNA-ITS1 amplification for characterization of *Trichoderma* isolates from rice fields in the Philippines and from building materials, respectively. Arisan-Atac *et al.* (1) used RAPD analysis for identifying groups of *Trichoderma* capable of chestnut blight control; however, in our study, ap-PCR alone was not sufficient for characterization of the Israeli isolates. ITS sequence analysis has been very useful for speciation of certain species of fungi such as *Colletotrichum* (6) but not for *Fusarium* spp. (13). In this work, all the isolates were pre-screened by ap-PCR before ITS sequence analysis, which was used reliably for phylogenetic studies in *Trichoderma* (9,10). In our study, representative isolates were accurately grouped into the recognized clade of *T. harzianum* (comprising *T. aureoviride, T. inhamatum* and *T. virens*), the *T. longibrachiatum* and *T. saturnisporum* cluster and that of *T. asperellum*, *T. atroviride*, *T. koningii* and *T.*

viride, similar to the work conducted by Hermosa *et al.* (8). The isolates used in biocontrol studies (7) belong to each of the different sections listed above, indicating the taxonomic diversity of potential *Trichoderma* agents for biocontrol.

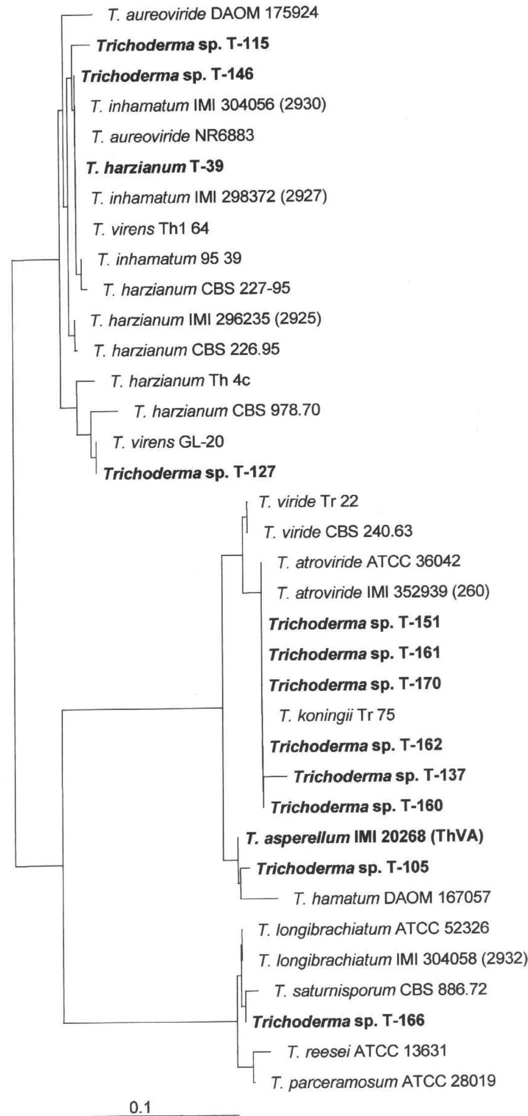

Fig. 2. ITS 1-based phylogenetic tree of *Trichoderma* isolates and published sequences. The tree was produced using the neighbor-joining algorithm. The orders of branching were similar in all tree construction approaches used. *Trichoderma* isolates that appear in bold text were sequenced in this study. Bar indicates 10% sequence divergence.

ACKNOWLEDGMENTS

Contribution No. 527/03 from the Agricultural Research Organization, Institute of Plant Protection, Bet Dagan, Israel, We thank Prof. Enrique Monte from Universidad de Salamanca, Spain, for kindly providing the reference *Trichoderma* strains. The research was supported by the EU within the framework of FAIR project number CT 98-4140 - IPM in strawberry.

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