Molecular Phylogenetic Analyses of Biological Control Strains of *Trichoderma harzianum* and Other Biotypes of *Trichoderma* spp. Associated with Mushroom Green Mold

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ABSTRACT

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A polymerase chain reaction-amplified DNA containing the internal transcribed spacer (ITS)-1, 5.8S, and ITS-2 regions of the nuclear ribosomal DNA transcriptional unit was sequenced for 81 isolates of *Trichoderma* spp. associated with mushroom culture or used for biological control of plant pathogens. Phylogenetic analyses revealed that the biocontrol isolates were more closely related to an isolate of *T. harzianum* biotype 1 (Th1) than to the aggressive biotypes 2 and 4. Th1 has been isolated from mushroom compost but is not the cause of widespread green mold epidemics that have occurred during the last 12 years in Europe and North America. Three isolates of *T. harzianum* obtained from shiitake (*Lentinula edodes*; Shi1B and S3-96) and maitake (*Grifola frondosa*; Mai1) substrates were placed within the biocontrol group. We also found evidence suggesting that some isolates of *T. harzianum* originally identified as Th4 from Pennsylvania are more closely related to Th2 from Europe. Finally, considering the wide range in sequence distribution of our samples, we propose that the consensus sequence found in this investigation be used as the reference sequence for further studies involving the identification and taxonomy of *T. harzianum*.

Additional keywords: *Agaricus bisporus*, ITS sequences, systematics.

Trichoderma harzianum is the cause of green mold, a disorder that affects the common cultivated mushroom *Agaricus bisporus.* The disease is characterized by a rapid infestation of the compost by *T. harzianum* and subsequent inhibition of *A. bisporus* fructification. Although green mold has been associated with *A. bisporus* since it was first cultivated over 300 years ago, the malady caused only minor crop losses for growers. During the last 12 years, however, new, aggressive forms of the pathogen have led to severe crop losses in the British Isles (7,17,18,27–31). In the early 1990s, growers in Ontario (23), British Columbia (23), and Pennsylvania (24) experienced outbreaks of green mold similar to those in the British Isles. Since then, losses are estimated to have exceeded $30 million dollars in North America alone.

Four biotypes of *T. harzianum*, designated Th1, Th2, Th3, and Th4, have been found associated with mushroom compost. Biotype Th3, previously identified as *T. harzianum*, recently was recognized as a strain of *T. atroviride* (5,20). Biotypes Th2 and Th4 are the most aggressive, causing the majority of crop losses. In Pennsylvania, the predominant biotype associated with the green mold epidemic is Th4, as shown by random amplified polymorphic DNA (RAPD) analysis (21). Biotype Th4 also is the major cause of crop loss in Canada (23).

Several species of *Trichoderma* are well-documented mycoparasites and have been used successfully against certain pathogenic fungi (6,34). Among these, *T. harzianum* is the species most often used for biological control of pathogens. It is commercially available as F-Stop (Eastman Kodak Co., Rochester, NY) for control of several soilborne plant-pathogenic fungi and, in combination with *T. polysporum* (as BINAB T; Binab USA, Inc., Bridgeport, CT), for control of wood decay (1). The genetic relationship among these strains and biotypes Th2 and Th4 has not been examined. Concern has emerged regarding the potential role of biological control strains in the development of mushroom green mold epidemics. Phylogenetic analyses would help clarify these relationships and may provide useful information about strategies to control the disease.

We compared nucleotide sequences from the ribosomal DNA transcriptional unit (rDNA) of 81 isolates, comprising green mold- and biocontrol-related isolates of *T. harzianum* and other species of *Trichoderma*. We found that, although the isolates known for biological control properties and green mold-associated isolates share a recent ancestor, they constitute different phylogenetic groups.

MATERIALS AND METHODS

Cultures. A total of 81 isolates of *Trichoderma* spp., representing *T. harzianum* Th4 as well as other green mold-associated biotypes, were used in this study. These isolates constitute a wide, geographically distributed sample. All biological control strains of *Trichoderma* spp. available in the American Type Culture Collection (ATCC) were included in this analysis (Table 1).

DNA extraction. Fungal genomic DNA was extracted using the Puregene kit (Gentra Systems, Inc., Minneapolis, MN), following the manufacturer's directions. Fungal tissue, ground in liquid nitrogen, was lysed with the cell lysis solution provided. After incubation at 55°C, an RNase treatment was performed, followed by protein extraction. DNA was precipitated with isopropanol, washed with ethanol (70%), and air-dried for 15 min. DNA was rehydrated with 50 µl of DNA hydration solution (10 mM Tris and 1 mM EDTA).

Polymerase chain reaction (PCR) amplification of internal transcribed spacer (ITS)-1 and -2 regions and 5.8S rDNA. DNA (10 ng) was amplified in 15-µl PCR mixtures containing 0.4 U of *Taq* DNA polymerase (Promega Corp., Madison, WI), 0.2 mM each dNTP, 0.5 µM primers ITS-1 (5′ TCTGTAGGTGAACCTG-CGG 3′) (26) and ALR0 (5′ CATATGCTTAAGTTCAGCGGG 3′),

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and 2 mM $MgCl_2$. Reactions were performed in capillary tubes using a Rapidcycler (Idaho Technology, Idaho Falls, ID) with the following program: 1 cycle at 94°C for 15 s; 40 cycles at 94°C for 0 s, 56°C for 15 s, and 72°C for 45 s; and 1 cycle at 72°C for 2 min.

Sequencing of PCR products. PCR products from eight 15-µl reactions of each sample were purified with the Wizard PCR system (Promega Corp.), and the concentration was adjusted to 20 ng/µl. DNA sequences were obtained by automated DNA sequencing with fluorescent terminators using an ABI 377 Prism Sequencer (Applied Biosystems, Inc., Foster City, CA) at the Nucleic Acid Facility, Biotechnology Institute, The Pennsylvania State University. Each sample was sequenced in both directions with the same primers that were used in the amplification step. Consensus sequences were determined using the Seqman module from the Lasergene software package (DNASTAR Inc., Madison, WI).

Data analysis. Sequences were aligned following the Clustal W algorithm (33) included in the Megalign module (DNASTAR Inc.). Multiple alignments parameters used were gap penalty = 10 and gap length penalty = 10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters (K-tuple = 2, gap penalty = 5, window = 4, and diagonals saved = 4) were used for the pairwise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 1.01; Institute of Molecular Evolutionary Genetics, University Park, PA) (26). Neither gaps (due to insertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Nucleotide distances were estimated by the Jukes-Cantor model (12), and phylogenetic inference was performed by the neighbor-joining (NJ) method (26). Bootstrap tests with 1,000 replications (8) were conducted to examine the reliability of the interior branches and the validity of the trees obtained. An additional standard error test was performed with the data set using the same characters in order to evaluate the statistical confidence of the inferred phylogeny.

RESULTS

Amplification of the rDNA region with primers ITS-1 and ALR0 yielded products of approximately 600 bp as estimated by agarose gel electrophoresis. The sequences used for the final phylogenetic analysis were 532 to 548 bp after manual contig trimming. In addition to ITS-1, 5.8S rDNA, and ITS-2, such sequences contained the last base of the 18S rDNA and the first 12 bases of the 28S rDNA.

Isolates of *Trichoderma* spp. used as biocontrol agents showed the highest nucleotide variation in the ITS-1 region (31.0%), followed by the ITS-2 (14.0%) and the 5.8S rDNA (11.3%) regions, respectively (Table 2). Within ITS-1, most of the substitutions (38)

TABLE 1. List of species, isolate code, source, geographic origin, and GenBank accession number for *Trichoderma* spp. analyzed in this study

Species	Isolate code	Source[a]	Geographic origin	GenBank accession no.	Species	Isolate code	Source[a]	Geographic origin	GenBank accession no.
T. atroviride[b,c]	N/A[d]	ATCC 32173	Israel	Z48811	*T. harzianum*	Th4-85	Romaine/Royse	Berks Co., PA	AF057620
T. atroviride[b]	36042	ATCC 36042	N/A	Z48812	*T. harzianum*	Th4-89	Beyer/Royse	Berks Co., PA	AF057621
T. atroviride[b,c]	N/A	ATCC 42831	Hungary	Z48811	*T. harzianum*	Th4-90	D. Rinker	Canada	AF057622
T. atroviride[b]	95-41	ATCC 28036	North Carolina	AF057604	*T. harzianum*	Th4-91	D. Rinker	Canada	AF057623
T. harzianum	95-40	CBS 227-95	N/A	AF057605	*T. harzianum*	Th4-92	D. Rinker	Canada	U78882
T. harzianum	95-43	CBS 226-95	N/A	AF057606	*T. harzianum*	Th4-96	D. Royse	Chester Co., PA	AF057624
T. harzianum	20691	ATCC 20691	N/A	AF057571	*T. harzianum*	Th4-105	D. Beyer	Chester Co., PA	AF057625
T. harzianum	20846	ATCC 20846	N/A	AF057572	*T. harzianum*	Th4-108	D. Beyer	Chester Co., PA	AF057626
T. harzianum	20847	ATCC 20847	N/A	AF057573	*T. harzianum*	Th4-109	D. Beyer	Chester Co., PA	AF057627
T. harzianum	20848	ATCC 20848	N/A	AF057574	*T. harzianum*	Th4-111	D. Beyer	Berks Co., PA	AF057628
T. harzianum	20873	ATCC 20873	N/A	AF057575	*T. harzianum*	Th4-112	D. Beyer	Berks Co., PA	AF057629
T. harzianum	20902	ATCC 20902	New York	AF057576	*T. harzianum*	Th4-113	D. Beyer	Berks Co., PA	AF057630
T. harzianum	20907	ATCC 20907	New York	AF057577	*T. harzianum*	Th4-114	D. Beyer	Berks Co., PA	AF057631
T. harzianum	52424	ATCC 52424	Ohio	AF057578	*T. harzianum*	Th4-116	D. Beyer	Berks Co., PA	AF057632
T. harzianum	52443	ATCC 52443	Georgia	AF057579	*T. harzianum*	Th4-117	D. Beyer	Berks Co., PA	AF057633
T. harzianum	52444	ATCC 52444	N/A	AF057580	*T. harzianum*	Th4-118	D. Beyer	Berks Co., PA	AF057634
T. harzianum	52445	ATCC 52445	N/A	AF057581	*T. harzianum*	Th4-119	D. Beyer	Berks Co., PA	AF057635
T. harzianum	56678	ATCC 56678	New York	AF057582	*T. harzianum*	Th4-120	D. Beyer	Berks Co., PA	AF057636
T. harzianum	58673	ATCC 58673	Maryland	AF057583	*T. harzianum*	Th4-121	D. Beyer	Berks Co., PA	AF057637
T. harzianum	58674	ATCC 58674	Maryland	AF057584	*T. harzianum*	Th4-122	D. Beyer	Chester Co., PA	AF057638
T. harzianum	60850	ATCC 60850	Colombia	AF057585	*T. harzianum*	Th4-123	D. Beyer	Chester Co., PA	AF057639
T. harzianum	64263	ATCC 64263	Hungary	AF057586	*T. harzianum*	Th4-169	C. P. Romaine	Chester Co., PA	AF057640
T. harzianum	Th1-64	P. Mills	Northern Ireland	AF057599	*T. harzianum*	Th4-170	C. P. Romaine	Chester Co., PA	AF057641
T. harzianum	Th1-65	P. Mills	Northern Ireland	U78881	*T. harzianum*	Th4-171	C. P. Romaine	Chester Co., PA	AF057642
T. harzianum	Th2-62	P. Mills	Northern Ireland	AF057600	*T. harzianum*	Th4-172	C. P. Romaine	Chester Co., PA	AF057643
T. harzianum	Th2-63	P. Mills	Northern Ireland	U78880	*T. harzianum*	Th4-178	D. Beyer	Chester Co., PA	AF057644
T. harzianum	Th3-60	P. Mills	Northern Ireland	AF057601	*T. harzianum*	A5-96	D. J. Royse	Chester Co., PA	AF057587
T. harzianum	Th3-61	P. Mills	Northern Ireland	U78879	*T. harzianum*	A6-96	D. J. Royse	Chester Co., PA	AF057588
T. harzianum	Th4-12	G. J. Samuels	Berks Co., PA	U78877	*T. harzianum*	A9-96	D. J. Royse	Chester Co., PA	AF057589
T. harzianum	Th4-23	D. J. Royse	Berks Co., PA	AF057609	*T. harzianum*	Mai1	D. J. Royse	Maitake, MRC	AF057590
T. harzianum	Th4-24	D. J. Royse	Berks Co., PA	AF057610	*T. harzianum*	Ple1	D. J. Royse	Pleurotus, MRC	AF057591
T. harzianum	Th4-31	C. Fordyce	N/A	AF057611	*T. harzianum*	Shi1	D. J. Royse	Shiitake, MRC	AF057595
T. harzianum	Th4-32	C. Fordyce	N/A	AF057612	*T. harzianum*	Shi2	D. J. Royse	Shiitake, MRC	AF057596
T. harzianum	Th4-33	C. Fordyce	N/A	AF057613	*T. harzianum*	Shi3	D. J. Royse	Shiitake, MRC	AF057597
T. harzianum	Th4-35	C. P. Romaine	Chester Co., PA	AF057614	*T. harzianum*	Shi4	D. J. Royse	Shiitake, MRC	AF057598
T. harzianum	Th4-36	D. J. Royse	MRC	U78878	*T. harzianum*	S1-96	D. J. Royse	Shiitake, Chester Co., PA	AF057592
T. harzianum	Th4-66	D. J. Royse	Chester Co., PA	AF057615	*T. harzianum*	S3-96	D. J. Royse	Shiitake, Chester Co., PA	AF057593
T. harzianum	Th4-69	D. Beyer	Berks Co., PA	AF057616	*T. harzianum*	S5-96	D. J. Royse	Shiitake, Chester Co., PA	AF057594
T. harzianum	Th4-72	Romaine/Royse	Berks Co., PA	AF057617	*T. inhamatum*	95-39	G. J. Samuels	N/A	AF057602
T. harzianum	Th4-78	Romaine/Royse	Berks Co., PA	AF057618	*T. virens*	Tvir2	Growth promoter	N/A	AF057603
T. harzianum	Th4-79	Romaine/Royse	Berks Co., PA	AF057619					

[a] ATCC = American Type Culture Collection; CBS = Centraalbureau voor Schimmelcultures, Baarn, the Netherlands; and MRC = Mushroom Research Center, The Pennsylvania State University.
[b] These strains appear in the ATCC catalogue under different names. New names determined by W. Gams (13).
[c] DNA sequences from these strains are identical and are reported in the GenBank with the same accession number.
[d] N/A = not available.

were located in a 87-nucleotide (nt) range between sites 112 and 198. No substitutions were found within the last 13 positions of ITS-1. Within ITS-2, more than half of the substitutions (13) were clustered in a 23-nt range between sites 415 and 438 near the region's center. There were only two changes in the first 43 base positions of ITS-2. A similar trend was observed in the 5.8S rRNA gene, in which eight changes occurred in a central 26-nt segment but only three changes were found in the last 59 positions of the sequence (Fig. 1). Transitions accounted for most of the substitutions and the transition/transversion ratios were as high as 8.0.

DNA sequence analysis of biocontrol-related *T. harzianum* isolates only revealed that ITS-1 contained most of the nucleotide changes (Table 2). The substitution percentage in this region (19.1%) is almost four times that of ITS-2 (5.8%). With the sole exception of the absence of base changes in the last 15 positions, no major trend was observed in the substitution pattern in ITS-1. In the case of ITS-2, only two base changes were found in the first 44 positions of the region. Likewise, only two substitutions occurred within the last 73 nt of ITS-2. Six additional changes were observed in a 55-nt segment spanning positions 417 through 471 of the sequence alignment. For the 5.8S rDNA, the substitution pattern was very similar to the one described above, with most changes observed toward the middle of the gene. The transition/transversion ratio ranged from 0.33 to 5.0.

For Th4 isolates, the ITS-1 region showed the highest percentage of nucleotide substitutions (20.6%) (Table 2). For the 5.8S rDNA, 20 changes (12.6%) were found, 14 of them in the first 50 positions of the gene and only 6 in the last 109 nt sites. The ITS-2 region had a percentage of substitutions (12.9%) similar to that of the 5.8S rRNA gene, but none were observed within the first 48 bases.

Sequence comparison among all isolates used in this study revealed that most of the nucleotide variation occurred in ITS-1 (Table 2). Within this region, a significant number of substitutions were found near the 3′ end. The remaining substitutions were spread evenly throughout the rest of ITS-1. Out of 160 sites evaluated in the 5.8S rDNA, 29 were different from the consensus sequence. Differences were mainly located in the first 65 nt of the gene, with only 11 differences found in the last 95 positions of the 5.8S rDNA. ITS-2 accounted for 10% of the total variation among all fungal sequences examined. Only three substitutions were found in the first 37 nt of the ITS-2 sequence.

The phylogenetic tree of all isolates (Fig. 2) revealed the presence of multiple clusters. One cluster contained *T. atroviride* (Th3 isolates) and the biocontrol strain ATCC 64263. This cluster shares the oldest ancestor of the isolates studied. At least six other subgroups are derived from this ancestor. Isolates obtained from shiitake substrate and *A. bisporus* compost clustered together and share the most recent ancestor with an isolate of Th1. A specimen of *T. virens*, isolated from a commercial growth promoter, is closely related to this cluster. Biocontrol- and green mold-related strains that share a very recent common ancestor formed two other subgroups. This distinction, however, was not very well supported by bootstrap tests. The biocontrol subgroup included 16 isolates (out of the 17 used in this study) of *T. harzianum* and 1 isolate of *T. inhamatum*, known for their antagonistic properties. However, this subgroup also included one specimen of Th1 and several samples obtained from green mold-infested shiitake, maitake, and *A. bisporus* substrates. The sister subgroup, constituted by the aggressive Th4 isolates, contained further subdivisions. Isolates 120, 122, 123, and 169 of Th4 are part of an independent lineage. The other subdivision contained the remaining confirmed Th4 isolates and one additional sample from *Pleurotus ostreatus* and *A. bisporus* substrates, respectively.

DISCUSSION

The genus *Trichoderma* poses a major challenge for systematists because the phylogenetic relationships of many of its members still are unclear (11). The concepts of "species aggregate" and "section" introduced by Rifai (22) and Bissett (2) have helped clarify placement of conflictive species such as *T. harzianum*, *T. viride*, and *T. atroviride* within the genus. However, the influence

TABLE 2. Internal transcribed spacer (ITS)-1, 5.8S rDNA, and ITS-2 nucleotide variation in *Trichoderma* spp. and *T. harzianum* used as biocontrol agents, in Th4 biotype isolates associated with mushroom culture, and in all isolates used in this study[a]

		ITS-1			5.8S rDNA			ITS-2		
Specimen	Sequences[b]	Sites[c]	Total[d]	V (%)[e]	Sites	Total	V (%)	Sites	Total	V (%)
Trichoderma spp.[f]	21	65	210	31.0	18	160	11.3	25	179	14.0
T. harzianum	17	40	210	19.1	15	160	9.4	10	173	5.8
Th4 biotype[g]	39	42	204	20.6	20	159	12.6	23	179	12.9
All isolates[h]	80	92	215	42.8	29	160	18.1	56	185	30.3

[a] Gaps and equivocal sites were removed from the data set before analysis.
[b] Number of sequences.
[c] Number of different sites.
[d] Total number of sites compared.
[e] V = sequence variation.
[f] Species included are *T. atroviride*, *T. harzianum*, *T. inhamatum*, and *T. virens*.
[g] Th4 = *T. harzianum* biotype 4 from Pennsylvania and Canada.
[h] Two strains of *T. atroviride*, ATCC 32173 and ATCC 42831, have identical DNA sequence and share the same GenBank accession number.

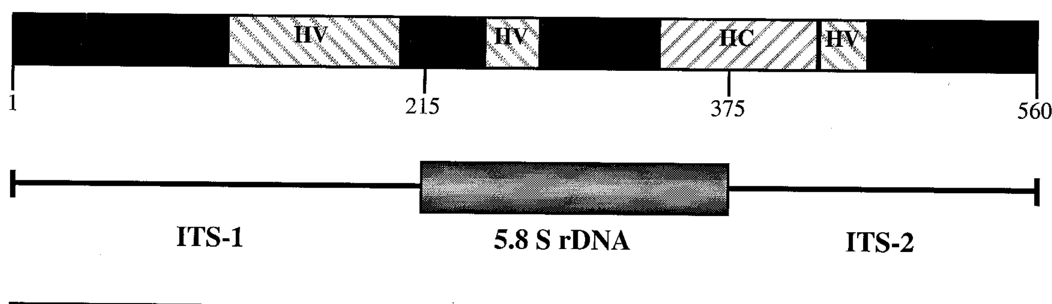

Fig. 1. Highly variable (HV) and highly conserved (HC) domains within the internal transcribed spacer (ITS)-1, 5.8S, and ITS-2 of the rDNA transcriptional unit of biocontrol species of *Trichoderma*. A similar pattern was observed for all isolates of *Trichoderma* spp. examined in this study. Scale: bar = 100 bp.

of environmental conditions on morphological and physiological characteristics have made accurate identification of new biotypes difficult (16). Such is the case with some green mold-associated isolates, in which initial identifications classified them as members of *T. harzianum*. For example, molecular phylogenetic analysis has demonstrated that Th3 is *T. atroviride* rather than *T. harzianum* (5,20).

rRNA genes from closely related species are highly evolutionarily conserved and share significant sequence similarity (4). Their slower evolutionary rate increases the usefulness of these genes for phylogenetic studies of distantly related organisms (35). In contrast, ITS and intergenic region domains are much more variable and, therefore, more useful for phylogenetic studies of members of the same species or genus (35). In this study, however, comparisons involving biocontrol- or green mold-related isolates of *T. harzianum* only revealed that the 5.8S rRNA gene is as variable as, or even more so than, ITS-2. For example, green mold isolates show almost equal rates of substitutions (12.6 versus 12.9% for the 5.8S rDNA and ITS-2, respectively). Furthermore, in *T. harzianum* biocontrol isolates, the 5.8S rRNA gene is more variable than ITS-2 (9.4 and 5.8%, respectively). This result appears to be the consequence of a substantially lower degree of variation within ITS-2 for these isolates.

The sequence alignment using Clustal W revealed that, in all cases, the ITS-1 region accounted for the highest amount of nucleotide variation. A segment encompassing approximately 50 nucleotides near the 3′ end contained the highest number of substitutions when compared with other segments of similar length. These results suggest the presence of a "hot spot" within ITS-1, where substitutions occur more frequently. In addition, very few changes were found in the last 15 bases of ITS-1. This region fuses with the 5′ end of the 5.8S rRNA gene, also a domain with relatively little variation. The most conserved segment of the rDNA region examined is located between the last 35 bases of the 5.8S rDNA and the first 35 to 45 sites of ITS-2, where virtually no variation occurs. Such conservation could be related to the necessity of a conserved sequence for ribonuclease recognition during further processing of the heterogeneous rRNA transcript. However, since only partial information is known regarding the processing of the rRNA primary transcript in eukaryotes (14), it is difficult to determine the biological significance of this finding.

Phylogenetic analysis indicates that green mold-causing biotypes and biocontrol isolates are very closely related and share the most recent common ancestor (Fig. 2). Bootstrap analysis, using 1,000 replications, of the NJ tree provides weak evidence to place both groups in distinct clades. However, no biocontrol strain was ever placed as the most recent ancestor of an aggressive biotype. Alternative tree topologies, using different bootstrap cutoff values, also appear to support such a hypothesis (data not shown). A non-aggressive isolate of the Th1 biotype was almost identical in sequence to biocontrol strain ATCC 20873. This strain was reportedly obtained by protoplast fusion of ATCC 20737 and ATCC 60850. In addition, three other green mold-associated isolates (Mai1, Shi1B, and S3-96), found on substrates of the specialty mushrooms shiitake and maitake, were placed within the biocontrol group. The standard error analysis, a statistically robust test in which the null hypothesis of branch-length-equal-to-zero is tested, also supported the results obtained after bootstrapping (data not shown).

Biotype Th1 represents another example whereby members of one group are not genetically homogeneous. Whereas isolate 65 undoubtedly is a member of the *T. harzianum* biocontrol group, isolate 64 was consistently placed within the *T. virens* clade. RAPD and beta-tubulin gene sequence analyses have shown that all Th1

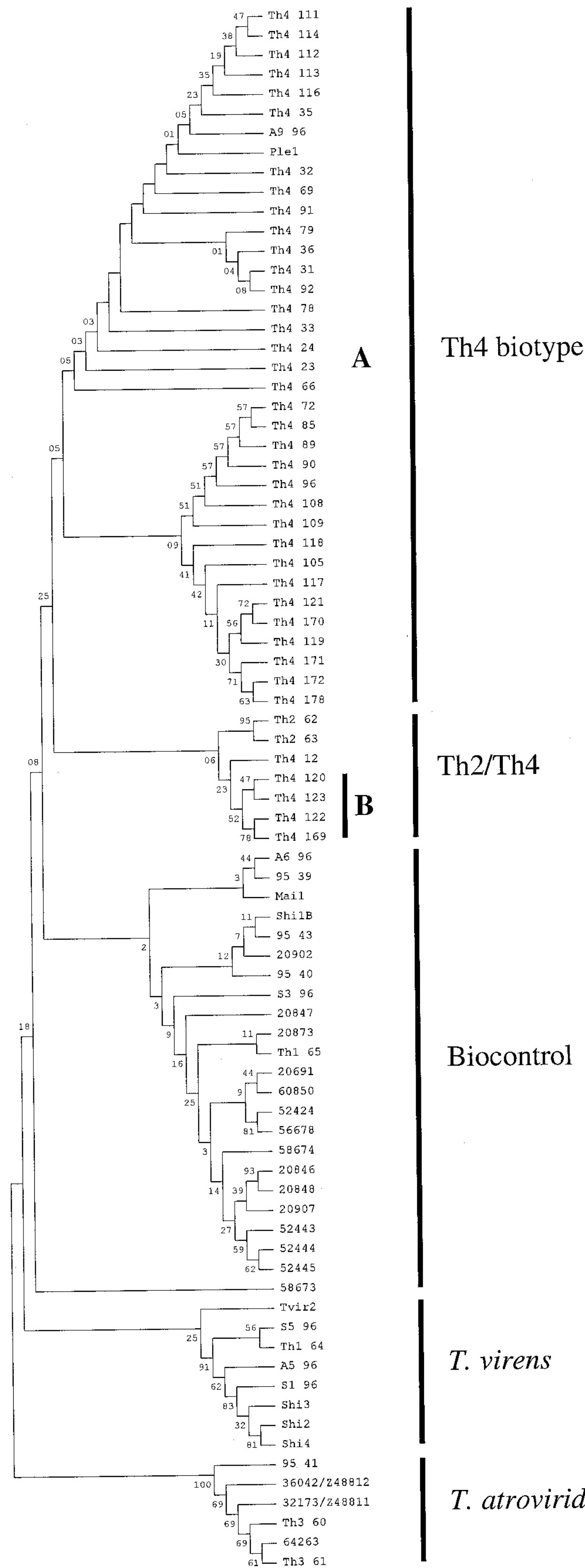

Fig. 2. Phylogenetic relationships of 81 isolates of *Trichoderma* spp. inferred from rDNA sequence analysis. The phylogenetic tree was obtained by the neighbor-joining method using the Jukes-Cantor distance. The number of nucleotides in the subset was 572. Gaps and equivocal sites were excluded from the analysis. The numbers above the branches represent the values obtained after a bootstrap test with 1,000 replications. GenBank accession no. Z48811 represents ATCC 32173 and ATCC 42831 strains of *T. atroviride*. Clusters within Th4 and Th2/Th4 subgroups are labeled A and B, respectively.

isolates, with the exception of isolate 65, are genetically uniform (X. Chen, C. P. Romaine, M. D. Ospina-Giraldo, and D. J. Royse, *unpublished data*). Therefore, it is possible that the true Th1 biotype is *T. virens* and not *T. harzianum*, as previously classified. Since only two isolates of the Th1 biotype were included in this analysis, however, it is not possible to determine with certainty whether the Th1 biotype is part of *T. virens* or *T. harzianum*.

Our results also suggest that two isolates used for biological control were misidentified. Phylogenetic analysis placed isolate ATCC 64263 (*T. harzianum*) as most closely related to biotype Th3 and ATCC strains 28036, 32173, and 36042, all identified as strains of *T. atroviride*. The independence of this group is supported by the highest bootstrap value (100%) in our analyses (Fig. 2). Misidentification with members of these species may frequently occur because of the extreme similarity in their conidia (3). Likewise, bootstrap tests suggest that isolate 95-39, identified as *T. inhamatum*, is part of the *T. harzianum* group rather than constituting an independent lineage. Considering the wide range in sequence distribution of our samples, we propose that the consensus sequence found in this study be used as a reference sequence for further studies involving the identification and taxonomy of *T. harzianum*.

Most of the Th4 isolates appear to be very closely related to each other, confirming the results obtained by Qi et al. (21), who found very similar RAPD patterns in a population of Th4 isolates collected in Pennsylvania and Canada. Furthermore, all of these isolates were identified as Th2/Th4 biotypes using specific PCR primers (X. Chen, C. P. Romaine, M. D. Ospina-Giraldo, and D. J. Royse, *unpublished data*). The presence of several subgroups within the Th4 biotype (cluster A) suggests that some members of this biotype may have diverged along the evolutionary pathway (Fig. 2). In this group, it should be noted that the evolutionary rate is not constant for all isolates and several show an increased evolutionary distance. This suggestion is supported by bootstrap values as high as 72% (Fig. 2). Cluster B includes isolates 120, 122, 123, and 169, all of which came from different geographic locations. When compared with all isolates, this subgroup appears to be more closely related to the Th2 biotype, cause of green mold epidemics in Europe (Fig. 2). However, bootstrap values do not fully support this relationship (Fig. 2). In addition, DNA fingerprinting obtained by arbitrarily primed PCR indicated that isolates 120, 122, 123, and 169 are similar to the Th4 biotype (data not shown). It would be helpful, therefore, to analyze other parts of these isolates' genome and additional Th2 isolates in order to further examine their genetic relatedness to Th2 and Th4.

Depending on the group analyzed, nucleotide variation within the ITS regions and the 5.8S rDNA appeared to occur in different locations. Hence, independent phylogenetic analyses were also conducted for *Trichoderma* spp., *T. harzianum* used as biocontrol agents, and Th4 biotype isolates. In any case, phylogenies obtained confirmed the internal topology and distribution observed when all samples were analyzed simultaneously (data not shown).

Multiple alignment algorithms, such as Clustal, rely on similar computational approaches. Initially, these methods align and score all sequence pairs in the data matrix. Subsequently, they evaluate and score all sequences until the final alignment is reached. This "progressive" approach utilizes the Needleman and Wunsch (19) pairwise algorithm iteratively to achieve the multiple alignment of a set of sequences. The essence of the progressive method, as developed by Feng and Doolittle (9,10), is based on the rule "once a gap, always a gap." Therefore, the final alignment is usually dependent on the order of the pairwise alignments (9). To minimize this effect, a "clustering" alternative has been introduced in the method. First, the most closely related pair is determined. Subclusters that may exist in the set are revealed by the preliminary set of pairwise measurements. These subclusters are treated as units during the alignment process, without altering the relative positions of the nucleotides within the cluster. Thus, all subclusters are prealigned before the final alignment is performed (10). This method is very accurate with closely related sequences (33). However, more recent versions of pairwise alignment-based algorithms, such as Clustal W, have introduced changes (mostly related to penalties due to gap number and location) that dramatically improved the sensitivity of the progressive alignment method for difficult alignments involving numerous or highly diverged sequences (33). Furthermore, Clustal W generates an output file in which the sequences are in the alignment order instead of maintaining the input file order. Finally, if unjustified gaps remain in the final alignment, they can be removed by manual editing. For these reasons, we selected Clustal W as the most appropriate algorithm for the multiple alignment of our highly complex data set (80 operational taxonomic units). In addition, the increased speed of distance methods allows more thorough testing of alternative tree topologies when large data sets are in use (32). Hard copies of the final sequence alignments are available upon request.

The selection of Clustal W for the multiple alignment was in accordance with the NJ method as the preferred choice for phylogenetic tree reconstruction. The NJ method does not assume a constant rate of nucleotide substitution (15) and, consequently, is more robust regarding the effects of unequal evolutionary rates in different lineages (33). Additional reasons supporting the use of the NJ method over other available methods are computational time and consistency when handling large data sets (15), as well as previous results from simulation studies (25). Furthermore, phylogenetic trees obtained by the NJ method can be tested with a statistically robust and very conservative standard error test (15).

In conclusion, we have found phylogenetic evidence suggesting that aggressive biotypes Th2 and Th4 are very closely related to biocontrol strains. However, the biocontrol strains do not appear to be responsible for the emergence of the green mold-causing biotypes of *T. harzianum*. Instead, Th2 and Th4 have evolved from a recent common ancestor for both biocontrol and green mold-related biotypes.

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