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DNA Profiles of *Trichoderma* spp. in Korea

Dong-Suk Park*, Hee-Wan Kang[2], Young-Jin Park, Mi-Hee Lee, Byoung-Moo Lee, Jang-Ho Hahn and Seung-Joo Go[1]

Bioinformatics Division and [1]Molecular Physiology Division, National Institute of Agricultural Biotechnology, Rural Development Administration, Suwon 441-707, Republic of Korea
[2]Graduate School of Bio and Information Technology, Hankyoung National University, Ansung 456-749, Republic of Korea
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Molecular approaches, internal transcribed spacer (ITS) sequences of ribosomal DNA, and Universal Rice Primer Polymerase Chain Reaction (URP-PCR) were used to investigate the genetic diversity, taxonomic complexity, and relationships of *Trichoderma* species in mushroom farms. Forty-one isolates of 13 *Trichoderma* spp. were used in this study and clustered into eight groups. The DNA fingerprint patterns and ITS1 region sequence alignment data showed similar results, but not in some species, such as *T. virens*, *T. atroviride*, *T. harzianum*, and *T. aureoviride*. Results of this study have proven that the morphology-based taxonomic system has some limitations in terms of classification. The data obtained in this study would be a good index for classifying indistinguishable *Trichoderma* strains.

KEYWORDS: DNA fingerprinting, Internal transcribed spacer (ITS), *Trichoderma*, URP-PCR

The *Trichoderma* disease, commonly referred to as green mold, has been previously considered as a minor problem in mushroom production, because it typically occurred episodically in association with low-quality compost or poor hygiene (Geels *et al.*, 1988; Harvey *et al.*, 1982). Several species of the genus *Trichoderma* are potent biocontrol agents against soil-borne plant-pathogenic fungi (Chet, 1987; Jensen and Wolffhechel, 1994). The filamentous fungi included in the genus *Trichoderma* are among those considered to be important. *Trichoderma* species are mainly applied in the rhizosphere or phyllosphere, whereas, little attempts have been reported on their application for the protection of aboveground diseases (Grosclaude *et al.*, 1973; Ricard, 1983). *Trichoderma* spp. have also been investigated as biological control agents (BCAs) for over 70 years now (Hjeljord and Tronsmo, 1998), but it is only recently that strains have become commercially available. Knowledge concerning the behavior of these fungi as antagonists is essential for their effective use since they can act against target organisms in several ways (Jeffries and Young, 1994). 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). These fungi may also promote plant growth (Inbar *et al.*, 1994), or induce resistance in plants (De Meyer *et al.*, 1998). Grondona (1994) reported that controlled laboratory experiment is usually the initial step towards the identification of strains with potential biocontrol activity.

Despite many studies of various scopes, the taxonomic standard of this genus has yet to be clearly established to be able to reliably define species. *T. virens* (formerly *Gliocladium virens*) is morphologically very similar to the anamorph of *Hypocrea gelatinosa* (Rehner and Samuels, 1995), and its internal transcribed spacer (ITS) sequences have revealed that it is closely related to *T. harzianum* (Lieckfeldt *et al.*, 1998). *T. harzianum* has been divided into three, four, or five subspecific groups, depending on the strains and on the attributes considered (Grondona *et al.*, 1997). Four biotypes (Th1, Th2, Th3, and Th4) were originally proposed based on its pathogenicity on mushrooms (Seaby, 1989). Molecular studies have confirmed the distribution of *T. harzianum* in the four biotypes proposed by Seaby (1989), of which Th1 and Th3 seem to be nonpathogenic to mushrooms (Ospina-Giraldo *et al.*, 1998). Phylogenetic analysis based on ITS sequences (Ospina-Giraldo *et al.*, 1999) has revealed that Th1 is the most recent ancestor of aggressive mushroom colonizers, which was included in Th2 (Muthumeenakshi *et al.*, 1994) and Th4 (Ospina-Giraldo *et al.*, 1998) of *T. harzianum*. Th3 is consistent with *T. atroviride* Karsten, a previously described species with similarities in colony character to *T. harzianum* (Lieckfeldt *et al.*, 1998). *T. harzianum* has been neotypied by Gams and Meyer (1998) and redistributed, after molecular examination, into two major groups: *T. harzianum* sensu lato (s.l.) and *T. viride-T. atroviride* complex. However, *T. harzianum* and *T. inhamatum* are also considered as cospecific (Bissett, 1991a), and their ITS1 sequence variability ranges within the divergences described for Th1 (Kuhls *et al.*, 1997). Strains from Th2 and Th4 are not BCAs (Ospina-Giraldo *et al.*, 1999) and are also distinct from *T. harzianum* s.l. according to

*Corresponding author <E-mail: dspark@rda.go.kr>

molecular information (Gams and Meyer, 1998).

Recently, it has been found that *T. viride* is a paraphyletic group. An integrated morphological and molecular approach has confirmed the redefinition of types, and has shown that it is the true *T. viride* species, which also includes the anamorph of *Hypocrea rufa*, and is grouped together with strains of *T. atroviride* and *T. koningii* (Lieckfeldt *et al.*, 1999). The biotypes can also be distinguished by randomly amplified polymorphisms in mitochondrial DNA and ribosomal DNA, and by sequence analysis of ribosomal DNA (Castle *et al.*, 1998; Muthumeenakshi *et al.*, 1994; Muthumeenakshi and Mills, 1995; Ospina-Giraldo *et al.*, 1998, 1999).

Today, molecular methods are commonly used for identification and phylogenetic classification, and different PCR-based strategies have been used to characterize 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 obtained with randomly amplified polymorphic DNA (RAPD) have been used to differentiate numerous fungi, including *Trichoderma* spp. (Zimand *et al.*, 1994). Techniques such as isozyme analysis and serology are nonspecific at the isolate level for most fungi (Miller, 1996), including species of *Trichoderma* (Thornton *et al.*, 1994).

URPs (universal rice primers), which can be used in PCR fingerprinting of various organisms including plants, animals, and microorganisms, were developed from repetitive sequence of rice genome (Kang *et al.*, 1998, 2000). The URP-PCR technique is a useful tool for the characterization and grouping of fungal species at interspecific and intraspecific level (Kang *et al.*, 2000). In this study, URP-PCR fingerprinting was carried out to identify *Trichoderma* spp. occurring in oyster mushroom farms in Korea. DNA fingerprints were analyzed to confirm possible or impossible typing of each species strain within the genus *Trichoderma*, and to aid in understanding the taxonomic interrelationships among the confused *Trichoderma* spp. isolates.

Materials and Methods

Fungal isolates and extraction of genomic DNA. Forty-one isolates of 13 *Trichoderma* spp. were used in this study. Twenty-four isolates (T-strain) of *Trichoderma* were provided by the Chungnam National University in Korea, 13 isolates were obtained from the centraalburean voor Schimmelcultures (CBS), and 4 isolates were given by the Korean Agricultural Culture Collection (KACC: http://kacc.rda.go.kr). Detailed information about the isolates are presented in Table 1. Extraction of genomic DNA from the isolates was conducted according to the procedure described by Hong *et al.* (1998), which basically followed that by Lee and Taylor (1990).

Oligonucleotide primer and PCR amplification. The PCR reactions were performed in a 50 μl PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 2.5 mM of each dNTP, 200 ng primer URP2R (Seoulin Biotech Co., Ltd., Korea), and 2.5 unit *Taq* polymerase (Promega, USA). The total amount of genomic DNA from the isolates added into the PCR mixture was approximately 50 ng. PCR amplification was carried out in a PTC-200TM Gradient cycler (MJ Research, Inc., USA) using the following profile: one cycle for 4 min at 94°C; 35 cycles for 1 min at 94°C, 1 min at 55°C, 2 min at 72°C; one cycle of a final extension for 7 min at 72°C. DNA fragments were detected by staining with ethidium bromide. To investigate the effect of annealing temperature on URP-PCR, gradient annealing temperatures ranging from 36°C to 60°C were used in blocks of the PTC-200TM Gradient cycler. The URP-PCR products were electrophoresed on a 1.5% agarose gel in TAE buffer and visualized by staining with ethidium bromide and photographed under UV transilluminator.

rDNA amplification, sequencing, and alignment. The primers used for amplification of the rDNA internal transcribed spacer regions were primer ITS1, 5'-TCCGTAGGTGAACCTGCGG-3' (18S rDNA 3' terminal region of eukaryotic organisms) and ITS2 5'-TCCTCCGCTTATTGATATGC-3' (position 60~80 bp in eukaryotic 25S rDNA) (White *et al.*, 1990). Sequencing reactions were performed with an automatic sequence analyzer (Perkin Elmer, ABI3100). The previously determined rDNA ITS region sequences used for comparisons in this study were retrieved from the GenBank. The sequence data were aligned by using the DNASTAR 5.0 and the sequences obtained were compared with the ITS sequence of related species of *T. virens*, *T. atroviride*, *T. harzianum*, and *T. aureoviride* (Fig. 3).

Computer-assisted analysis of DNA patterns. All the DNA patterns were analyzed with the Windows version of GelCompar (version 4.0; Applied Math, Kortrijk, Belgium). The individual bands in each of the patterns produced by the PCR methods were analyzed by applying the Dice coefficient to the peaks. The similarity between pairs of linearly combined fingerprints was calculated using the product-moment correlation coefficient (r value) (Pearson, 1926), applied to the whole desitometric curves of the gel tracks (Hane *et al.*, 1993). Cluster analysis of the pairwise similarity values was performed using the unweighted pair group method with arithmetic means (UPGMA) clustering technique (Sneath and Sokal, 1973)

Table 1. Isolates of *Trichoderma* used in this study

Species	Isolate no.	GenBank accession no.	Host
T. aureoviride	T116	AF362108	compost substrate of *Pleurotus ostreatus*
T. aureoviride	T115	AF359400	compost substrate of *Pleurotus ostreatus*
T. aureoviride	T113	AF359265	compost substrate of *Pleurotus ostreatus*
T. harzianum	CBS436.95	AF359259	mushroom compost
T. aureoviride	T119	AF359408	compost substrate of *Pleurotus ostreatus*
T. inhamatum	CBS164.90	AF359261	leaf litter compost
T. aureoviride	T2	AF359395	compost substrate of *Pleurotus ostreatus*
T. inhamatum	CBS273.78	AF362101	maize-field soil
T. aureoviride	T14	AF359267	compost substrate of *Pleurotus ostreatus*
T. aureoviride	T60	AF359397	compost substrate of *Pleurotus ostreatus*
T. aureoviride	99	AF362109	compost substrate of *Pleurotus ostreatus*
T. harzianum	T4	AF359404	compost substrate of *Pleurotus ostreatus*
T. agressivum f. *europaeum*	CBS689.94	AF359258	mushroom compost
T. harzianum	CBS101525	AF359256	mushroom compost
T. harzianum	CBS450.95	AF359260	mushroom compost
T. virens	T162	AF359407	compost substrate of *Pleurotus ostreatus*
T. virens	T50	AF362110	compost substrate of *Pleurotus ostreatus*
T. virens	CBS609.95		compost substrate of *Pleurotus ostreatus*
T. virens	T1	AF501334	compost substrate of *Pleurotus ostreatus*
T. koningii	T141	AF359410	compost substrate of *Pleurotus ostreatus*
T. longibrachiatum	T153	AF359402	compost substrate of *Pleurotus ostreatus*
T. longibrachiatum	T5	AF362104	compost substrate of *Pleurotus ostreatus*
T. longibrachiatum	T152	AF362105	compost substrate of *Pleurotus ostreatus*
T. longibrachiatum	T9	AF362102	compost substrate of *Pleurotus ostreatus*
T. longibrachiatum	CBS821.91	AF369257	
T. ressei ref.	KACC40517	AF362100	*Pleurotus ostreatus*
T. citrinoviride	T7	AF362103	compost substrate of *Pleurotus ostreatus*
Trichoderma sp.	T10	AF359264	compost substrate of *Pleurotus ostreatus*
T. polysporum	CBS898.72	AF501333	
T. atroviride	T73	AF362111	compost substrate of *Pleurotus ostreatus*
T. harzianum	CBS432.95	AF362113	mushroom compost
T. atroviride	T150	AF362106	compost substrate of *Pleurotus ostreatus*
T. atroviride	KACC40556		*Pleurotus ostreatus*
T. atroviride	KACC40551	AF501327	*Pleurotus ostreatus*
T. atroviride	CBS693.94	AF359263	mushroom compost
T. atroviride	T125	AF362107	compost substrate of *Pleurotus ostreatus*
T. viride	CBS189.79		decaying wood
T. koningii	CBS989.70	AF359262	decaying angiosperm wood
T. atroviride	KACC40552	AF501328	*Pleurotus ostreatus*
T. atroviride	T123	AF359409	compost substrate of *Pleurotus ostreatus*
T. atroviride	T184	AF359406	compost substrate of *Pleurotus ostreatus*

and a band position tolerance of 1.3% was used for comparison of the DNA patterns. The analysis of the patterns was undertaken in accordance with the instructions of the manufacturer. Fingerprint patterns were constructed as described by Rademaker and De Bruijn (1997).

Results

DNA fingerprinting analysis of *Trichoderma* species. In this study, DNA fingerprinting analysis was developed for the identification and sub-typing of *Trichoderma* spp. URP2R primer characterized field isolates of *Trichoderma* spp. DNA fingerprinting patterns were evaluated using the criteria established by Gillespie *et al.* (1997). DNA fingerprint patterns were analyzed for: (i) number of DNA fragments, (ii) optical density, and (iii) size of fragments (bp). Primer URP2R defined polymorphic DNA fragments, and the number of bands was produced by amplification of the genomic DNAs ranging from 3 to 12 of a total of 293 with size ranging from 320 (*T. virens*) to 6070 bp (*T. polysporum*).

As shown in Fig. 1, DNA fingerprinting analysis of *Tri-*

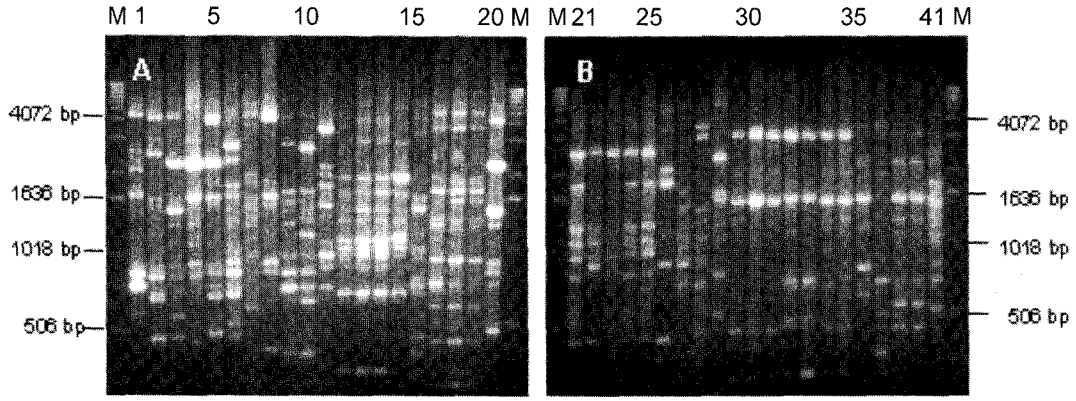

Fig. 1. PCR profiles of the 41 isolates of *Trichoderma* species generated by URP-PCR. M: 1 kb ladder. Plate A: Lanes 1~3, 5, 7, and 9~11, isolates of *T. aureoviride* (T116, T115, T113, T119, T2, T14, T60, and T99). Lanes 4, 12, 14, and 15, isolates of *T. harzianum* (CBS436.95, T4, CBS101525, and CBS450.95). Lanes 6 and 8, isolates of *T. inhamatum* (CBS164.90 and CBS273.78). Lane 13, isolate of *T. agressivum* f. *europaeum* (CBS689.94). Lanes 16~19, isolates of *T. virens* (T162, T50, CBS609.95, and T1). Lane 20, isolate of *T. koningii* (T141). Plate B: Lanes 21~25, isolates of *T. longibrachiatum* (T153, T5, T152, T9, and CBS821.91). Lane 26, isolate of *T. ressei* (KACC40517). Lane 27, isolate of *T. citrinoviride* (T7). Lane 28, isolate of *T.* sp. (T10). Lane 29, isolate of *T. polysporum* (CBS898.72). Lanes 30, 32~36, and 39~41, isolates of *T. atroviride* (T73, T150, KACC40556, KACC40551, CBS693.94, T125, KACC40552, T123, and T184). Lane 31, isolate of *T. harzianum* (CBS432.95). Lane 37, isolate of *T. viride* (CBS289.79). Lane 38, isolate of *T. koningii* (CBS979.70).

choderma spp. showed complex and confused band pattern among three species of *T. aureoviride*, *T. harzianum*, and *T. inhamatum*. Four isolates (lanes 1~3, and 5) of *T. aureoviride* appeared to have similar band pattern with each other by amplifying three common bands (4190, 1650, and 830 bp). Two isolates (lanes 7 and 11) of *T. aureoviride* showed a particularly distinct band pattern from the four isolates by form band fragments of each different size. Two isolates (lanes 9 and 10) of *T. aureoviride* appeared to have generally similar band pattern with each other.

T. harzianum (lanes 4 and CBS436.95) classified differently from *T. harzianum* cluster (lanes 12~15) showed a similar band pattern (indicated by the common band 1650 and 830 bp) with *T. aureoviride* (lanes 3 and 5), which is a different species. The *T. harzianum* cluster amplified many DNA fragments which, to a certain extent, contained a common band pattern.

Two isolates of *T. koningii* showed different band patterns except for two common band patterns (780 and 495 bp), while two isolates of *T. inhamatum* also showed two common band types (1680 and 940 bp) such as that of *T. koningii*. Three isolates of *T. virens* (lanes 17~19) amplified many common bands, whereas, an isolate of *T. virens* (lane 16: T162) did not coincide with any of the three isolates.

All *T. longibrachiatum* isolates showed generally similar band pattern, with fragments ranging approximately from 1800 to 1850 bp appearing in lanes 21, 24, and 25 but not in lanes 22 and 23. Furthermore, lane 23 did not amplify any fragment under the 980 bp.

***T. atroviride* clusters.** Two major bands in one cluster (containing lanes 30~36) were amplified as 3150 and 1610 bp, respectively, while in the other cluster (containing lanes 39 and 41), only two isolates (lanes 39 and 40) showed seven common amplicons. Lane 41 showed an almost independent band pattern except for the *T. atroviride* major band (1610 bp). The major band of *T. atroviride* appeared coincidently as 1610 bp. There was no similar band pattern that appeared in the PCR products of *T. ressei*, *T. citrinoviride*, *Trichoderma* sp., *T. polysporum*, and *T. viride*. Distinct common and variable fragments of each *Trichoderma* spp. isolate amplified by fingerprinting analysis method are shown in Table 2.

In this DNA fingerprinting analysis method of various isolates of *Trichoderma* spp., although they belong to the same species, both homology and nonhomology were presented variously by diversified band pattern according to isolates. However, in the identification of *Trichoderma* spp., fingerprinting data showed a polymorphism among the species, whereas it could not present a more accurate reciprocal relation among each other. Accordingly, the results by dendrogram using genetic tree were examined to further investigate genetic relationship (Fig. 2).

Genetic relationship analysis among *Trichoderma* spp. In this study, 41 isolates of 13 species of *Trichoderma* were classified into eight groups (Fig. 2). Genetic relationship analyzed with the Windows version of GelCompar (version 4.0; Applied Math, Kortrijk, Belgium) clustered preferentially a representative group of identical species and then groups which had similar band pattern.

Table 2. DNA fingerprint profiles of *Trichoderma* species using primer URP2R

Organism	Amplified DNA fragments (bp)		Genotype	Frequency (%)
	Primary	Variable		
T. aureoviride	1650	5710, 4500, 3680, 2000, 1900, 1580, 1500, 1310, 630	Tau1	1 (12.5)
		4190, 2550, 2090, 830, 750, 600	Tau2	1 (12.5)
		4190, 2950, 2460, 890, 830, 690, 670, 480	Tau3	1 (12.5)
		4190, 2220, 1470, 1290, 900, 830, 580	Tau4	1 (12.5)
		4190, 3790, 2220, 1560, 920, 830, 690, 500	Tau5	1 (12.5)
T. aureoviride	1000, 750	4320, 2800, 1730, 1320, 860, 420	Tau6	1 (12.5)
		4320, 2650, 1730, 1200, 860, 670, 420	Tau7	1 (12.5)
		3390, 2190, 2000, 1820, 1550, 1120, 900	Tau8	1 (12.5)
T. harzianum	–	5170, 2920, 2220, 1650, 1320, 920, 830, 470	Tha1	1 (16.7)
	–	3150, 1610, 520	Tha2	1 (16.7)
	1940, 1140,	1720, 1480, 1360, 1000, 360	Tha3	2 (33.3)
	1060,	1720, 1480, 1360, 1330	Tha4	1 (16.7)
	720	360	Tha5	1 (16.7)
T. inhamatum	1680, 940	4610, 2710, 2320, 1910, 850, 700, 550, 490	Tin1	1 (50.0)
		4200, 1890, 1560, 870, 440	Tin2	1 (50.0)
T. virens	–	5960, 4700, 4130, 3270, 1660, 1510, 1010, 770, 490, 420	Tvi1	1 (25.0)
	4550, 3500, 1750,	1870, 1620	Tvi2	1 (25.0)
	1370, 960, 460,	1870, 1620, 780, 370, 320	Tvi3	1 (25.0)
	470	780, 740	Tvi4	1 (25.0)
T. koningii	520, 780	3860, 2900, 2170, 1470, 1310, 940, 860, 660	Tko1	1 (50.)
		3280, 2060, 580	Tko2	1 (50.)
T. longibrachiatum	1260, 1100, 980	2550	Tlo1	1 (20.0)
		2550, 1850, 820, 740, 460	Tlo2	1 (20.0)
		2550, 1850, 820, 460	Tlo3	1 (20.0)
		2550, 890, 460	Tlo4	1 (20.0)
		2470, 1800, 800, 460	Tlo5	1 (20.0)
T. atroviride	1610	3250, 2310, 1370, 780, 640, 520	Tat1	2 (22.2)
		3150, 2070, 520	Tat2	2 (22.2)
		3150, 3100, 2070, 780, 520	Tat3	1 (11.1)
		3150, 780, 520	Ta4	1 (11.1)
		3150, 2070, 1030, 780, 520	Tat5	1 (11.1)
		3150, 2070, 1370, 520	Tat6	1 (11.1)
		2560, 1870, 1560, 1410, 1300, 1180, 1110, 790, 620	Tat7	1 (11.1)
T. ressei	2130, 1850, 910, 470	–	–	–
T. citrinoviride	1750, 1520, 1170,	–	–	–
Trichoderma sp.	910	–	–	–
T. polysporum	3620, 3100,	–	–	–
	1500, 1160, 760			
T. viride	6070, 2410, 1710, 1620,	–	–	–
	1300, 830, 580, 2360,			
	1620, 1470, 1260, 880,			
	690			

Group A was composed of all *T. longibrachiatum* isolates. Group B was divided into two complex groups of B1 composed of *T. oureoviride*, *T. ressei*, and *T. atroviride* (T184), and of B2 composed of *T. aureoviride*, *T. harzianum* (CBS436.95), *T. polysporum*, *T. viride*, *T. koningii* (T141), *T. inhamatum*, and *T. virens* (T162) (CBS436.95 had predescribed fingerprinting analysis data in this review). Three isolates of *T. virens* and three isolates of *T. aureoviride* constituted group C and D, respectively. Group E consisted of ab unidentifed *Trichoderma* sp. and *T. citrinoviride*. Three isolates of *T. harzianum* and *T. agressivum* f. *europaeum* (CBS689.94) constituted group F.

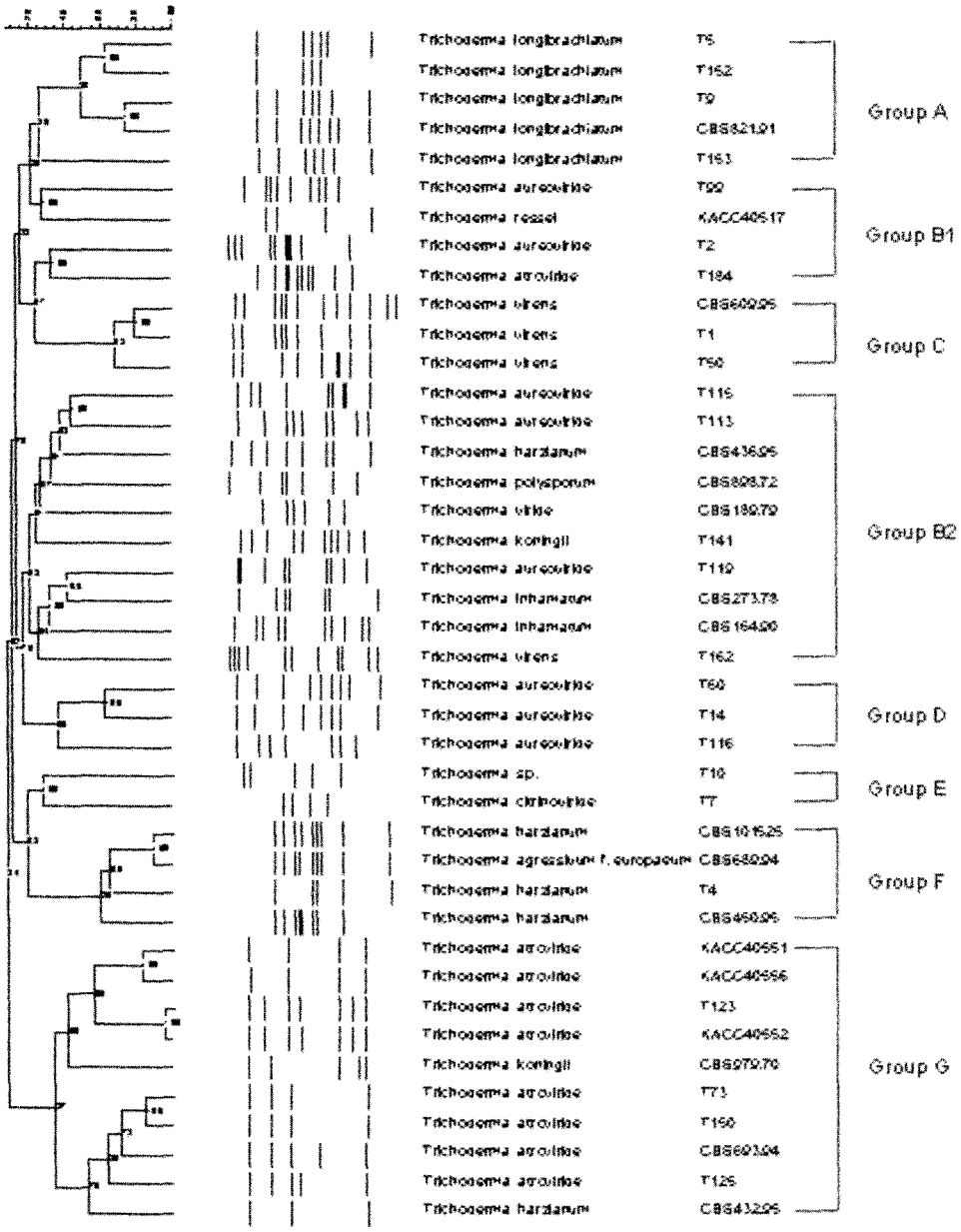

Fig. 2. UPGMA dendrogram derived from PCR profiles of genetic DNA in 41 isolates of *Trichoderma* species. Detailed information about the isolates is listed in Table 1.

Group G contained eight isolates of *T. atroviride*, an isolate of *T. koningii* (CBS979.70), and an isolate of *T. harzianum* (CBS432.95).

In these results, an interesting point noted was the distribution of *T. aureoviride* and *T. harzianum*. *T. aureoviride* showed a confused classification divided into the complex groups B1, B2, and the simple group D. *T. harzianum* was clustered into the complex group B2, the simple group F, and the *T. atroviride* group G, respectively.

As shown in Fig. 2, the total cophenetic value of 41 isolates of *Trichoderma* spp. showed common homology of 84%, while the cophenetic values (%) of isolates within each group are as follows: The highest cophenetic value of 99% was obtained from *T. aureoviride* group D of the simple groups. *T. virens* group C and *T. longibrachiatum* group A showed 98% and 89% cophenetic values, respectively. *T. harzianum* group F which included *T. agressivum* f. *europaeum* showed 89% cophenetic value. In this group, an isolate (CBS101525) of *T. harzianum* and *T. agressivum* f. *europaeum* (CBS689.94) showed considerably high homology of 100% cophenetic value. These results were consistent with the result obtained from the fingerprinting analysis. *T. atroviride* group G contained an isolate of *T. koningii*, while an isolate of *T. harzianum* showed 87% cophenetic value. In this group, *T. koningii* (CBS979.70) showed a high cophenetic value of 99%

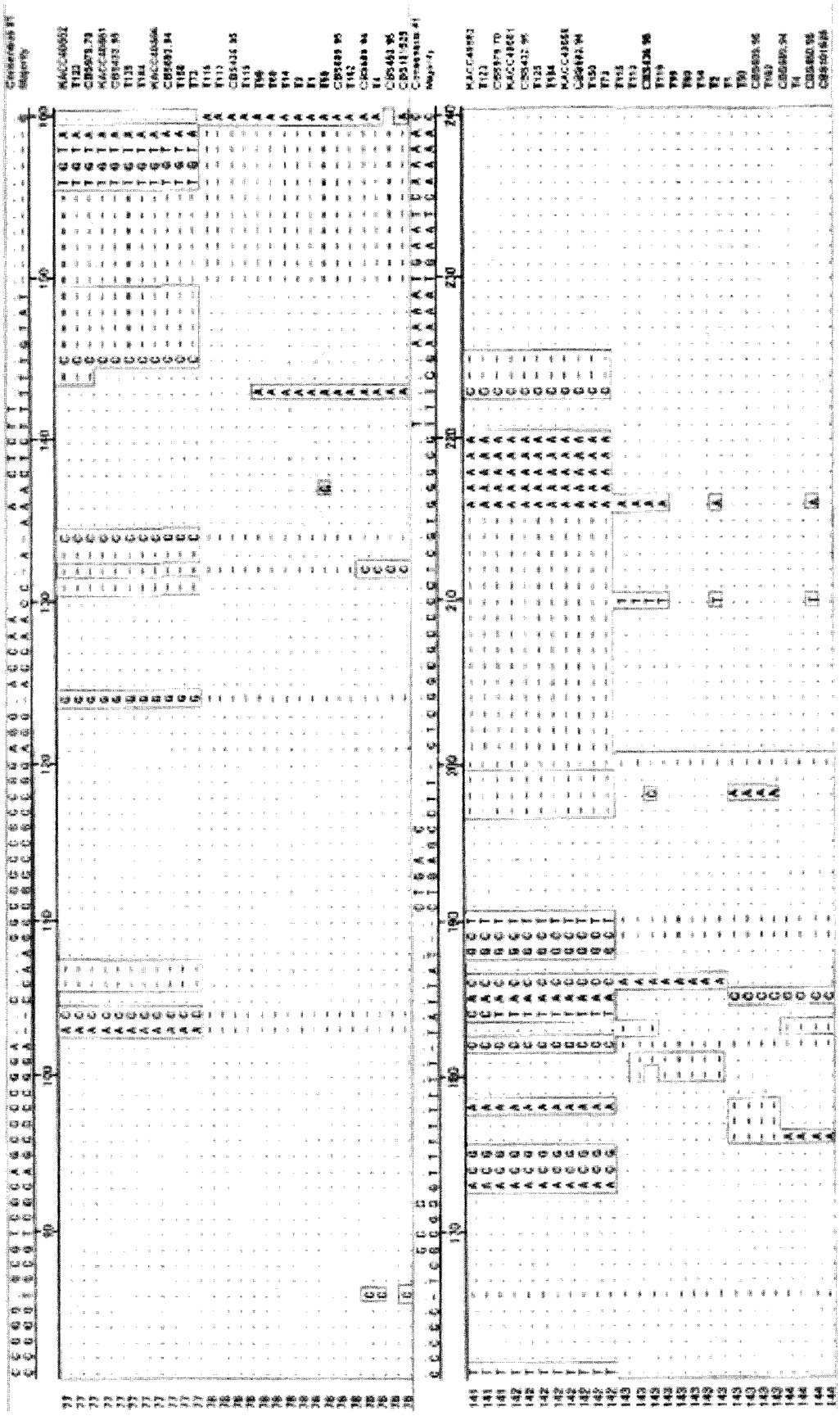

Fig. 3. Sequence alignment data comparison of IT1 regions among *T. virens*, *T. atroviride*, *T. harzianum*, and *T. aureoviride* using clustal method with weighted residue weight table.

compared with the four isolates of *T. atroviride* and *T. harzianum* (CBS432.95), which showed a cophenetic value of 70%, lower than that of CBS979.70, compared with the other four isolates of *T. atroviride*. In the complex group B1, cophenetic value was 100% between an isolate of *T. aureoviride* (T99) and an isolate of *T. ressei* (KACC40517), and 100% between an isolate (T2) of *T. aureoviride* and an isolate (T184) of *T. atroviride*. Group B2 showed a low cophenetic value of 63%. Two isolates (T115 and T113) of *T. aureoviride* which had 100% cophenetic value, showed a low cophenetic value of 58% compared with an isolate (CBS436.95) that had been called *T. aureoviride* in the past. In this group, cophenetic value among an isolate (T119) of *T. aureoviride* and two *T. inhamatum* isolates showed 100% cophenetic value with each other, while an isolate (T162) of *T. virens* had 75%.

Sequence alignment analysis of *Trichoderma* species. The ITS sequence was chosen for this analysis because it has been shown to give more information of the various sections of the genus *Trichoderma* (Kuhls *et al.*, 1996; Kuhls *et al.*, 1997; Ospina-Giraldo *et al.*, 1998) (Fig. 3).

Sequence analysis method in this study was used to distinctly compare differentiation with data by genetic tree method for classification and grouping among *Trichoderma* spp. Sequence alignment of *T. atroviride* group (containing 11 isolates ranging from KACC40552 to T73) coincided with all except for a difference of 1 gap indicated between 134 and 135 bp. In the case of *T. aureoviride*, four isolates (T115, T113, CBS436.95, and T119) showed differences with each other at around 160 bp. T2 in the remainder isolates (T99, T60, T14, and T2) of *T. aureoviride* appeared to be different from the majority sequence as base T at 185 bp and base A at 191 bp. In the *T. virens* group, T50 showed base G, which was different with the other three isolates of *T. virens* at 129 bp. In the sequence data of *T. harzianum* group, only CBS450.95 appeared to be distinct from the other isolates at 83, 13, 187, and 191 bp.

Discussion

Many *Trichoderma* strains have biotechnological potentials and are protected under patents, due to their ability to perform as BCAs against soil-borne plant pathogens. Despite significant advances in the 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. Molecular data provide a more objective measure of the genetic variability of individuals than do phenotypic characters. Classification should be performed based on a combination of sequence analysis of DNA and analysis of genomic DNA fingerprinting. This seems to be the best way to resolve species complexes for *Trichoderma* (Lieckfeldt *et al.*, 1998).

URP-PCR fingerprinting has proven to be a valuable tool for studying DNA polymorphisms in fungal organisms. It is a simple and reliable technique that can be used to detect genetic differences at the isolate level. Hence, this study used a polyphasic approach that combines data sets from fingerprinting analysis, phylogenesis, and ITS region sequencing by URP-PCR investigations to determine the relationships among strains of *Trichoderma* spp. Formal, and traditional taxonomy is based solely on morphology or, more recently, and alternatively, on molecular data. Although this has been a major point of criticism by fungal taxonomists (Seifert *et al.*, 1995), there are only a few examples of the use of combined data sets in studies of fungi (Petrini, 1992; Petrini *et al.*, 1989; Sieber-Canavesi *et al.*, 1991).

In previous studies, some approaches, based on isozyme assay (Grondona *et al.*, 1997), rDNA, and mitochondria DNA (Chen *et al.*, 1999) were used in an attempt to study phylogenic relationships among different *Trichoderma* spp. Although the methods were capable of identifying species or partial strains, they did not discriminate respective cultivar among *Trichoderma* spp. RAPD methods were used for identifying respective *Trichoderma* spp., however, these methods specifically required a complicated marker system using a number of primers, although some partial cultivars could successfully be distinguished from them. In contrast, the URP-PCR method used in this study could address each species using PCR fingerprints produced by only one URP primer, reflecting that URP-PCR can alternatively be used as a practical, rapid, and simple system to classify *Trichoderma* spp.

Lane 4 (CBS436.95: *T. harzianum*), classified differently from *T. harzianum* cluster (lanes 12~15), showed a similar band pattern (indicated by common bands such as 1650 and 830 bp) with *T. aureoviride* (lanes 3 and 5), which is a different species (Fig. 1). The band pattern of CBS436.95 in this study did not completely coincide with that of lanes 3 and 5 and a rather diverse band size was amplified except for the common band size such as 2220, 1650, and 830 bp. Therefore, it was believed that CBS436.95 should be better reclassified into sub-species close to *T. aureoviride* than to *T. harzianum*.

The dendrogram result shown in Fig. 2 matched well with the fingerprinting analysis data shown in Fig. 1. These studies showed a close genetic relationship between the species *T. inhamatum* and *T. harzianum* (Gams and Meyer, 1998, Hermosa *et al.*, 2000). In fact, Bissett (1991b) 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 from each other. Several specific results in the dendrogram obtained with the URP-PCR analysis must be taken into account. *T. harzianum* (CBS436.95) and two isolates (CBS273.78 and CBS164.90) of *T. inhamatum* were clustered into the same complex group B2 (Fig. 2). Furthermore, in Fig. 1, these isolates represented a major band around 1610 bp.

Three isolates of *T. virens* (lanes 17~19) amplified many common bands, whereas, an isolate of *T. virens* (lane 16: T162) did not coincide with any of the 3 isolates. Furthermore, T162 was clustered into complex group B2, not in the *T. virens* group in the dendrogram by genetic analysis method (Fig. 2). However, in the sequence analysis result, the T50 rather than the T162 sequence represented a few different sequence alignments compared with the other *T. virens* isolates (Fig. 3). Accordingly, it was deemed necessary to consider reclassifying T162 into the other sub-species, and not to *T. virens*.

In this study, the classification of *Trichoderma* spp. was generally presented similarly in the sequence analysis and genetic relationship analysis methods, as well as in the DNA fingerprinting analysis. Otherwise, some distinct results were obtained from the two methods.

T184 (*T. atroviride*) showed an almost independent band pattern except for a major band as that of *T. atroviride* coincidently appearing as 1610 bp. On the other hand, T184 was clustered into group B1 in the dendrogram data by genetic relationship method, whereas, it was distributed into the same population with the other *T. atroviride* isolates in the result of the sequence analysis of the ITS region. A remarkable result of the amplicon pattern in several isolates of *T. atroviride* group was that the band pattern of CBS432.95 (*T. harzianum*) revealed a similar type by coincidence with six *T. atroviride* isolates which have two common bands (Fig. 1). Also, the ITS region sequence analysis data coincided with that of other *T. atroviride* isolates (Fig. 3). In this result, confused relationship was found among *T. atroviride* (T184), other isolates of *T. atorviride*, and *T. harzianum* (CBS432.95). For this reason, CBS432.95 was considered to belong to *T. atroviride* rather than to *T. harzianum* because the CBS432.95 showed a similar band pattern with *T. atroviride*. The ITS region sequence of *T. viride* (CBS189.79) coincided with that of *T. koningii* (CBS979.70) (data not shown). This result was similar with that of the investigation of Lieckfeldt *et al.* (1999) that the *T. viride* species is grouped together with strains of *T. koningii*.

The major conclusion in this study is that molecular characters tell different stories about species definition, and that the interrelationships among species of *Trichoderma*, such as *Trichoderma* sp., are difficult to distinguish (Rifai, 1969; Bissett, 1991a). Especially, *T. harzianum*, *T. inhamatum*, and *T. atroviride* were found to be tangled into complex relationships.

Some species were closely related based on molecular characters. These results reveal that molecular characters are not completely consistent and that cultural characters may not reflect the true genetic relationships in *Trichoderma* spp. However, results of this study can be used in other investigations on the identification of *Trichoderma* spp. Therefore, it is suggested that the delimitation of species with highly similar and identical patterns of molecular characteristics be reassessed.

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