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Intrageneric Relationships of *Trichoderma* Based on Internal Transcribed Spacers and 5.8S rDNA Nucleotide Sequences

Gi-Young Kim, Goang-Jae Lee[1], Myung-Gyu Ha[2], Tae-Ho Lee and Jae-Dong Lee*

Department of Microbiology, College of Natural Sciences, Pusan National University, Pusan 609-735, Korea
[1]Research Center, Hansung Enterprise CO. LTD., Kimhae, 159-16, Korea
[2]Korea Basic Science Institute, Pusan 609-735, Korea

The nucleotide sequences of the internal transcribed spacer (ITS) regions of the ribosomal DNA including the 5.8S ribosomal RNA gene (rDNA) have been determined for 11 species in order to analyze their intrageneric relationships. The total length of these sequences ranged from 530 nucleotides for *Trichoderma reesei* KCTC 1286 to 553 nucleotide for *Trichoderma koningii* IAM 12534. Generally speaking, the length of ITS1 region was about 30 nucleotides longer than that of the ITS2 region. Also, the sequences of 5.8S rDNA were more conserved in length and variation than those of ITS regions. Although the variable ITS sequences were often ambiguously aligned, the conserved sites were also found. Thus, a neighbor-joining tree was constructed using the full sequence data of the ITS regions and the 5.8S rDNA. The *Trichoderma* genus used to be grouped on the basis of the morphological features and especially the shape of phialides needs to be reexamined. The phylogenetic tree displayed the presence of monophylogeny in the species of *Trichoderma*. Therefore, it was difficult to distinguish the intrageneric relationships in the *Trichoderma* genus.

KEYWORDS: Phylogeny, rDNA, *Trichoderma*, ITS

Trichoderma is a genus of filamentous Deuteromycotina. Its members are generally found in all soils including forest humus layer (Wardle *et al.*, 1993) as well as in agricultural and orchard soils (Chet, 1987; Roiger *et al.*, 1991). *Trichoderma* species are rarely reported to occur on living plants and have not been found as endophytes of living plants (Petrini, 1986). *Trichoderma* species are not involved in plant parasitism or in postharvest crop loss (Samules, 1996). However, because of its mycoparasitic abilities, *T. harzianum* cause serious losses in commercial mushroom production such as *Lentinus edodes* and *Agaricus* mushrooms cultivation in the world (Muthumeenakshi *et al.*, 1994, Speranzismi *et al.*, 1995). *Trichoderma harzianum* has most frequently been associated with aggressive colonization of mushroom compost, although *T. viride*, *T. pseudokoningii*, *T. hamatum* and *T. longibrachiatum* have also been isolated from compost (Seaby, 1989). Several fungal species of the genus *Trichoderma* have attracted a strong interest because of the ability producing large quantities of cellulases (Kubieck *et al.*, 1990) and other hydrolytic enzymes (Lorito *et al.*, 1994), and the potent biocontrol agents against soil-borne plant-pathogenic fungi.

The morphological concept of *Trichoderma* is not completely settled (Rifai, 1969; Bisset, 1991a). The *Trichoderma* morphology is usually mistakable, because there is morphological intergrade with other hyphomycetes genera (Carmichael *et al.*, 1980). *Tolypocladium* could be a synonym of *Trichoderma*, possibly because of a similarity in arrangement of phialides of *T. inflatum* (*To. niveum*) to that of *T. polysporum*. However, the morphological comparison is only superficial as *To. niveum* is now known to be the anamorph of *Cordyceps facis* (Hodge, 1995), a member of the Clavicipitales. The inclusion of *Gliocladium virens*, one of the most frequently cited as the biological control fungi, in *Trichoderma* by Arx (1987) has only been accepted by using the DNA sequence analysis (Rehner 1994, 1995). The penicillus of phialides, combined with conidia held in slime, all on a more or less discrete conidiophore are generally characteristic of *Gliocladium*. *Trichoderma virens* is not the only *Trichoderma* to have a generalized *Gliocladium*-type conidiophore, which is found among anamorphs of *Hypocera* species such as *H. gelatinosa* and various other *Hypocera* species (Doi, 1972).

The purpose of this study is to evaluate the extent of sequence variation and further our knowledge of the phylogenetic relationships within *Trichoderma*. We amplified and sequenced the DNA fragments coding for the 5.8S and the internal transcribed spacers. We report the phylogenetic analysis obtained by the comparison of these rDNA sequences.

Materials and Methods

Fungal strains and cultivations

The 12 strains used in this study were obtained from KCTC (Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea), JCM (Institute of Japan Collection of Microorganisms, the Institute of Physical and Chemical Research, Saitama, Japan),

*Corresponding author <E-mail: leejd@hyowon.cc.pusan.ac.kr>

Table 1. List of fungal species and GenBank accession number of the genus *Trichoderma* in this study

Fungal species	Specimen no.[a)]	Database accession no.[b)]
T. hamatum	IAM 12505	U93877
T. harzianum	IAM 12506	AF030395
T. pseudokoningii	KCTC 6046	AF140046
T. koningii	KCTC 6042	AF031420
T. koningii	IAM 12534	AF027216
T. viride	KCTC 6047	AF140045
T. viride	IAM 5141	AF030396
T. reesei	KCTC 1285	AF140047
T. reesei	KCTC 1286	AF140048
T. reesei	KCTC 6045	AF140049
T. saturisporum	KCTC 6510	AF048744
T. saturisporum	JCM 1884	AF048743

[a)]KCTC (Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea), JCM (The Institute of Japan Collection of Microorganisms, and IAM (Microbial and Microalgal Research Center, Institute of Applied Microbiology, The University of Tokyo).[b)]The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank Database under the respective accession number.

IAM (Microbial and Microalgal Reseach Center, Institute of Applied Microbiology. The University of Tokyo), and IFO (Institute for Fermentation, Osaka, Japan), and are listed in Table 1. All cultures were incubated at 25°C with no agitation for one week.

DNA extraction

Fungal DNA was extracted from each sample according to the procedure adapted from the benzyl chloride method (Zhu and Zhu, 1993). Approximately 0.05 g of fungal pellets were suspended in 500 μl of Tris buffer (100 mM Tris-HCl, pH 8.0, 40 mM EDTA), 150 μl of 10% (w/v) sodium dodecyl sulphate (SDS) and 300 μl of benzyl chloride, and then incubated at 55°C for 30 min. The treatment of phenol : chloroform : isoamylalchol (25 : 24 : 1) and RNase (1 mg/m*l*) was carried out for the purification of DNA. The DNA was precipitated by adding 2.5 volumes of 100% ice-cold ethanol. The pellet was washed with 2 volumes of 70% ethanol and resuspended in distilled water. The purified DNA was kept at -20°C.

PCR amplification and DNA sequencing

The nuclear rDNA region spanning the ITS1, ITS2 and 5.8S rRNA gene was amplified by polymerase chain reaction (PCR) from each strain as described in Table 1. Primers ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) were derived from the conserved regions of 18S and 28S rDNA, respectively (Fig. 1). PCR was carried out with Perkin-Elmer model 480 thermocycler using the following program: initial denaturation for 3 min at 95°C, 30 cycles of

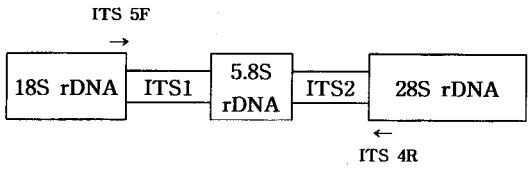

Fig. 1. A map of the ribosomal DNA region containing ITS1, ITS2 and the 5.8S rDNA gene. Arrows indicate the positions of the primers used for PCR and sequence analysis.

amplification (denaturation for 30 sec at 95°C, annealing for 30 sec at 50°C, and extention for 1 min at 72°C) and final extention of 5 min at 72°C. The PCR products from the amplification were subjected to preparative electrophoresis in a 1.6% agarose gel in TBE buffer. All PCR products yielded only a single visible band. The PCR products were excised from the ethidium bromide-stained gel and purified using a QIAGEN gel elution kit (Qiagen, Wartworth CA). Direct sequencing of PCR products was done by an Perkin-Elmer Applied Biosystems ABI 377A sequencer using a PRISM Dye Dideoxi Terminator Cycle Sequencing kit (Perkin Elmer) following the manufacture's protocol. Two primers, ITS 4F and ITS 5R, were used for sequencing in both directions and DNA sequences were edited and assembled with the program CLONE MANAGER version 4.0 (Scientific Educational Software, Stateline, PA.).

Data analysis

The determined ribosomal DNA sequences have been deposited in the European Molecular Biology Laboratory (EMBL) data library (Heidelberg, FRG) and accession numbers are indicated in Table 1. They were initially aligned with the sequences of the related genera from the EMBL data library using the multiple alignment program Clustal W (Thompson *et al.*, 1994). Phylogenetic relationships were inferred by the neighbor-joining method (Saitou and Nei, 1987). The strength of the internal branches from the resulting trees was statistically tested by bootstrap analysis (Felsentein, 1985) from 1,000 bootstrap replications. The distance matrix was calculated using NucML, and the initial tree based on the neighbor-joining method was reconstructed by NJdist of the PHYLIP 3.5 software package.

Result

G+C contents and nucleotide length of rDNA ITS regions

The G+C contents and nucleotide length of ITS1, ITS2, 5.8S rDNA, and their total length (ITS1-5.8S-ITS2) were shown in Table 2. Positive correlations in G+C contents and nucleotide length are found between ITS1 and ITS2 (Fig. 2), ITS2 of a taxon having GC-rich ITS1 is also GC-rich, and

Table 2. G+C contents and nucleotide length of the ITS1, ITS2, and 5.8S rDNA sequence

Fungal species	ITS1		5.8S rDNA		ITS2		Total	
	GC content (%)	Length (nt)	GC content (%)	Length (nt)	GC content (%)	Length (nt)	GC content (%)	Length (nt)
T. hamatum IAM 12505	53.5	200	44.6	166	63.5	170	53.9	536
T. harzianum IAM 12506	55.5	200	47.2	161	63.2	171	55.5	532
T. pseudokoningii KCTC 6046	56.0	207	44.4	162	67.7	164	56.0	533
T. koningii KCTC 6042	58.6	220	49.7	153	62.6	179	57.4	552
T. koningii IAM 12534	58.4	221	49.0	153	62.6	179	56.9	553
T. viride KCTC 6047	54.4	180	43.8	160	64.6	163	54.2	503
T. viride IAM 5141	54.1	185	41.1	185	62.1	177	54.5	547
T. reesei KCTC 1285	58.1	215	45.0	160	67.1	164	57.1	539
T. reesei KCTC 1286	59.1	203	46.0	161	73.5	166	59.6	530
T. reesei KCTC 6045	58.6	215	46.9	162	67.7	164	57.9	541
T. saturisporum KCTC 6510	59.2	218	45.3	161	68.3	167	57.9	546
T. saturisporum JCM 1884	59.2	218	44.1	161	67.3	165	57.3	544

the length of ITS1 sequences is longer than that of ITS2 sequences. Torres *et al.* (1990) found a similar phenomenon in the G+C contents of ITS regions in a wide range of organisms including filamentous fungi and called it "GC balance". The total G+C contents of ITS1-5.8S-ITS2 ranged from 53.9 in *T. hamatum* IAM 12505 to 59.6% in *T. reesei* KCTC 1286. Those of the 5.8S rRNA gene were variable (41.1~49.9%) and low among the 12 strains investigated. Also, the ITS regions showed relatively high G+C contents: 53.5~59.2% in ITS1 and 62.1~73.5% in ITS2.

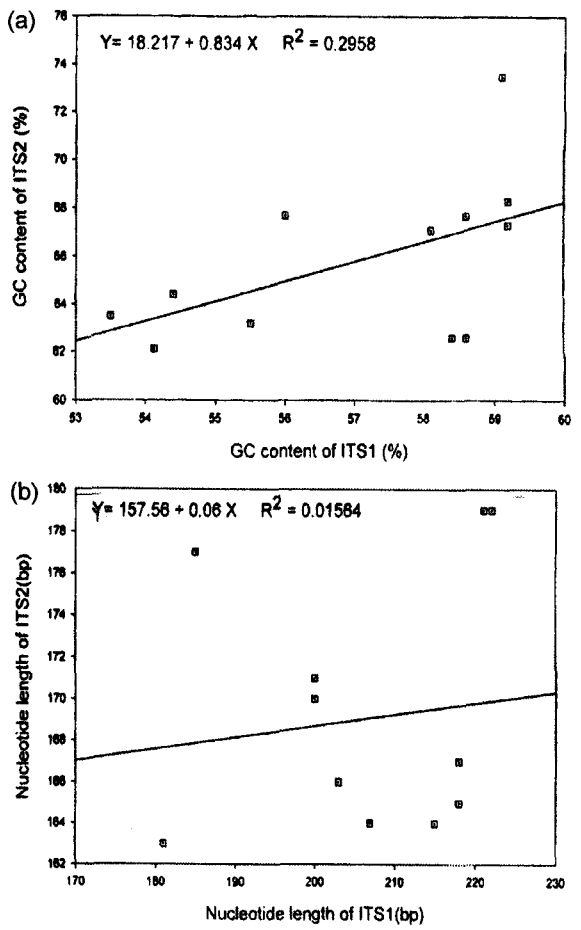

Fig. 2. Positive correlations of G+C content (A) and nucleotide length (B) between ITS1 and ITS2.

The shortest size in ITS1-5.8S-ITS2 was 503 nucleotides of *T. viride* KCTC 6047, and the longest was 553 nucleotides of *T. koningii* IAM 12534 among 12 strains with a difference of 51 nucleotides (Table 2). The size variation was derived from the ITS regions as well as 5.8S rRNA gene. Most of the tested taxa could be divided into two groups depending on the ITS length. The group with short ITS includes *T. viride* KCTC 6047, and the group with long ITS includes *T. hamatum* IAM 12505, *T. harzianum* IAM 12506, *T. viride* IAM 5141, *T. pseudokoningii* KCTC 6046, *T. reseei* KCTC 1285, *T. reesei* KCTC 1286, *T. koningii* KCTC 6042, *T. reesei* KCTC 6045, *T. saturisporum* KCTC 6510, and *T. saturisporum* JCM 1884. *Trichoderma koningii* IAM 12534 has the longest nucleotide on the total ITS regions sequenced in our study. The length of ITS1-5.8S-ITS2 of the former group was 503 nucleotides, and that of the latter was from 530 to 553 nucleotides. *Trichoderma koningii* IAM 12534 and KCTC 6042 have one different nucleotide in the length of ITS1. Two strains of the *T. viride* IAM 5141 and KCTC 6047 have much different nucleotides (20 nucleotices) in the total length. Two starins of *T. saturisporum* have ranged from 544 to 546 nucleotides. *Trichoderma reesei* has various length.

Phylogenetic analyses and DNA similarity

The nucleotide sequence data set obtained from the 12 taxa in Table 1 gave 569-nucleotide aligned sequence, including many ambiguously aligned site due to the variable nucleotide sequence of the ITS regions. However, since some conserved sites were found in the ITS regions and the 5.8S rDNA were used for the current analysis. Figure 3 shows a neighbor-joining tree obtained by use of PHYLIP

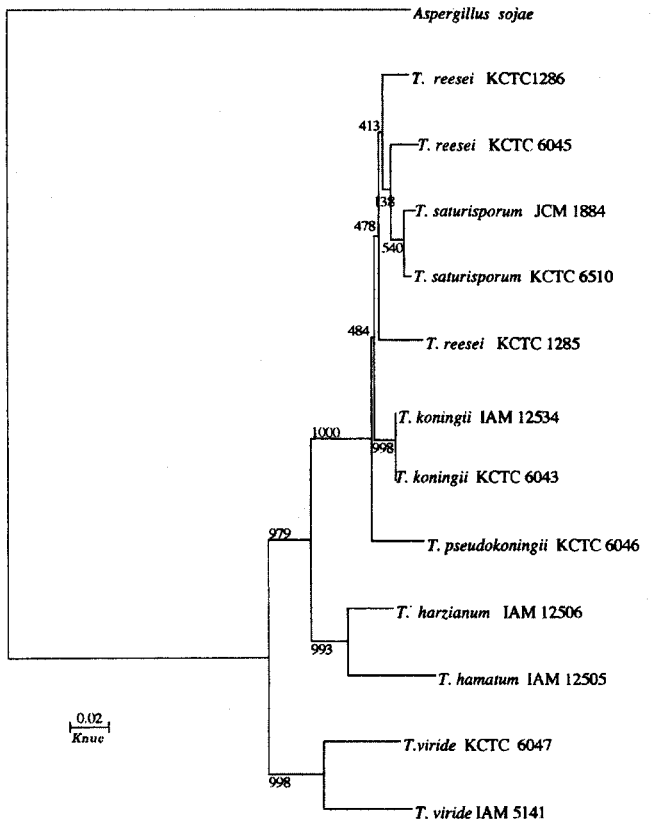

Fig. 3. Phylogenetic tree showing the relationship among the genus *Trichoderma* and *Aspergillus sojae* extracted from the GenBank is formed outgroup. Bar represented 2 nucleotide substitutions per 100 nucleotides in ITS1, 2 and 5.8S rDNA sequences. Bootstrap values were indicated at the branch points.

3.5. The 12 tested taxa were divided into three groups. All taxa included in group 1 belonged to the long-ITS 1 group. On the other hand, those included in group 2 and 3 belonged on the short-ITS 1 group.

The first group included *T. reesei*, *T. saturisporum*, *T. koningii*, and *T. pseudokoningii*. Two starins of *T. koningii*, IAM 12534 and KCTC 6043, had 100% of DNA similarity that excluded 1 gap in ITS1 sequences (Table 3), and also, the 998 bootstrap value supported the identical species. *Trichoderma reesei* and *T. saturisporum* were colsely related, but the bootstrap value was very low. In group 1, *T. psedokoningii* formed the subgroup. The second was grouped with *T. harzianum* and *T. hamatum*, the third group formed *T. viride*. The bootstrap values of two groups were as high as 993 and 998 respectively. The species including these two groups were promising candidates for biological agents, forming another group compared with many *Trichoderma* species in this study.

Discussion

The genus *Trichoderma* is effective biocontrol agents against plant pathogens (Chet, 1987). Because of this growing interest, a reliable and precise system for species and strain identification is very difficult. The genus monographed by Rifai (1969) and the *Trichoderma* species are mostly assigned to his nine species aggregates differentiated primarily by conidiophore branching patterns and conidium morphology. Rifai (1969) recognized that significant variation remained to be defined in each of these nine aggregate taxa and considered his revision as a preliminary investigation of variation in the genus. The genus has been partly revised by Domsch *et al.* (1980) and later by Bissett (1984, 1991a, 1991b, and 1991c).

Table 3. DNA similarity matrix (excluded gaps) for ITS1-5.8S rDNA-ITS2 sequences of the genus *Trichoderma*

	1	2	3	4	5	6	7	8	9	10	11
1											
2	97.3										
3	92.6	93.9									
4	92.0	93.4	100.0								
5	92.4	93.5	98.5	98.5							
6	91.7	92.9	98.2	98.2	99.3						
7	87.1	90.2	90.6	90.0	89.7	89.1					
8	87.2	87.8	90.7	90.9	88.7	87.4	92.8				
9	90.7	92.3	97.8	97.8	97.0	96.6	88.0	90.2			
10	90.0	92.5	98.3	98.3	98.5	98.1	88.8	88.4	97.7		
11	89.1	91.2	96.3	96.3	96.7	96.5	87.9	87.2	96.1	98.5	
12	89.3	91.2	96.6	96.6	96.2	95.5	87.3	88.3	96.8	96.6	95.1

1, *T. hamatum* IAM 12505; 2, *T. harzianum* IAM 12506; 3, *T. koningii* KCTC 6043; 4, *T. koningii* IAM 12534; 5, *T. saturisporum* KCTC 6510; 6, *T. saturisporum* JCM 1884; 7, *T. viride* IAM 5141; 8, *T. viride* KCTC 6047; 9, *T. reesei* KCTC 1285; 10, *T. reesei* KCTC 1286; 11, *T. reesei* KCTC 6045; 12: *T. pseudokoningii* KCTC 6046.

Several new approaches including molecular techniques have been tested to distinguish among isolates of *Trichoderma* (Zimand *et al.*, 1994; Fujimori and Okuda, 1994; Muthumeenaksi *et al.*, 1994). Isoenzyme profiles have been suggested for strain identification by Zamir and Chet (1985), but this comparison is useful only under standardized conditions, because extracellular enzymes are highly substrate dependent. An assay based on the recognition of *Trichoderma* antigens by specific antisera was used by Carter and Lynch (1991), who concluded that the reactivity of the antisera to *T. harzianum* depends on the type of medium on which the fungus has been cultured. This also renders the serological assay problematic for identification purposes. Although the introduction of new molecular tools in taxonomy will be extremely helpful, it is necessary to correlate molecular phylogeny with those base on morphological and other biochemical and physiological characters. The rapid decline in the number of systematic mycologists working with these fungi represents a major problem.

In our study, the sequences of ITS1, 5.8S rDNA, and ITS2 distinguished among strains of *Trichoderma*. Nine

strains were identified as *T. reesei*, *T. saturisporum*, *T. koningii*, and *T. pseudokoningii*. Between *T. reesei* and *T. saturisporum*, the intra-species concept needs to be reexamined. Between the strains of *T. viride*, the sequences of ITS regions yield similar patterns. Zimand *et al.* (1994) reported that none of the strains identified as *T. hamatum* showed any similarities. However, *T. hamatum* was grouped with *T. harzianum* with high boothstrap value in our study. Our conclusion was same to that of Bissett (1991b) and Rifai (1969), which supported *T. hamatum* and *T. harzianum* species aggregates.

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