THE KOREAN JOURNAL OF MYCOLOGY
Vol. 27, No 3, p191-197 June 1999

Printed in S. KOREA

Phylogenetic Analysis of the Genus *Gliocladium* and its Related Taxa by Comparing the Sequences of Internal Transcribed Spacers and 5.8S r-DNA

Ju-Young Park, Gi-Young Kim, Myoung-Gyu Ha[1], Yong-Kook Shin[2], Yong-Ha Park[2], Tae-Ho Lee and Jae-Dong Lee*

Department of Microbiology, College of Natural Sciences, Pusan National University, Pusan 609-735, Korea
[1]Korea Basic Science Institute, Pusan 609-735, Korea
[2]Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yuseong, Taejon 305-600, Korea

Ribosomal DNA의 Internal Transcribed Spacer(ITS) 부위의 염기서열 분석에 의한 *Gliocladium* 속과 근연속에 관한 계통 분류학적 연구

박주영 · 김기영 · 하명규[1] · 신용국[2] · 박용하[2] · 이태호 · 이재동*

부산대학교 자연과학대학 미생물학과, [1]기초과학지원연구소 부산분소
[2]생명공학연구소 유전자은행

ABSTRACT: The phylogenetic position of *Gliocladium* and its related taxa were investigated, using the neighbor-joining method of the sequences from internal transcribed spacers (ITS1 and ITS2) and 5.8S ribosomal DNA (rDNA). It was focused especially on the generic concept by comparing with the related genera such as *Trichoderma, Hypocrea, Verticillium, Penicillium* and *Talaromyces*. *Gliocladium* species and its related genus were divided into three groups by the phylogenetic analysis using the neighbor-joining method. The first group includes *Penicillium*-like strains such as *Penicillium, Tararomyces, Verticillium* and one species of *Gliocladium* (*G. cibotii* JCM 9203 and JCM 9206). Especially, *Gliocladium cibotii* JCM 9203 is thought to be the similar species with *Verticillium bulbillosum* JCM 9214. Between these two species, *Gliocladium cibotii* and *Verticillium bulbillosum*, the intraspecies concept needs to examined with culture condition and morphological properties. The second group includes two species *Verticillium, Verticillium tricorpus* and *Verticillium albo-atrum which* extracted from the GenBank database in NCBI (National Center for Biotechnology Information). *Trichoderma*-like strains, such as *Trichoderma, Hypocrea* and several species of *Gliocladium are* included in the third group. Also, *Gliocladium penicillioides* IFO 5869 and *Gliocladium catenulatum* ATCC 10523 formed the subgroup of *Trichoderma*-like strains. The species of *Gliocladium* were dispersed in *Trichoderma*-like and *Penicillinum*-like group, and only one species of *Gliocladium cibotii* used in our study was located in *Penicillium*-like genus group. The species of *Verticillium* appeared in all three groups and the species of *Trichoderma* formed the monophylogeny with *Hypocrea* (telemorph). Also, *Gliocladium virens* was grouped with *Trichoderma harzianum* with a high bootstrap value, supporting that *Gliocladium virens* is to be placed in *Trichoderma*. The results suggest that *Gliocladium* is polyphyletic, and is more *Trichoderma*-like than *Penicillium*-like.

KEYWORDS: Phylogeny, rDNA, *Gliocladium, Trichoderma, Verticillium*

Damage to crops both in the agricultural field and during harvest and storage by insect is estimated about more than $3.5 billion in the United States. They have used chemical control of agricultural pest organisms in order to reduce such damage. However, chemical agent for preserving crops cause fatal damage to the human being and animals. In order to avoid such side effect, there is a great intrest in finding biological antagonist, often a natural enemy. It is reported that fundamental systematic information on species descriptions of biological antagonist, also, understanding of the relationships with other species is important to develope an effective biological control using fungi (Chet, 1987). For example, *Trichoderma* and *Gliocladium* were used to control soilborne fungal disease in temperate agricultural systems (Zimand, 1994).

The genus *Gliocladium* is well known for biological control agents to soilborne fungal disease, e.g. *G. virens* and *G. roseum* are the representative species (Samules, 1996). It is report that the genus *Gliocladium* produces fungistatic antibiotics such as gliotoxin and viridin, also, is likely to be an important biological control agent (Papavizas, 1985). The term "fungistatic antibiotics" may be defined

*Corresponding author

to a substance that are products of the normal metabolism of certain molds. These substances are toxic to other organisms even in small quantities (Weindling, 1941). The crystalline *Gliocladium* toxin, gliotoxin is similar to penicillin, purified by extraction of the cultural filtrate from *Penicillium* (Clutterbuck *et al.*, 1932), which is easily decomposed in air (Weindling, 1934). *Gliocladium*, as well as *Trichoderma*, are capable of attacking and destroying the hyphae of *Rhizoctonia solani* Kühn and other fungi during the growth of such fungi with nutrient culture media (Weindling, 1932).

Gliocladium is a promising candidate as a biological agent. Many research have vastly concentrated to collect and to screen *Gliocladium* for developing biological control agent. In oredr to classify and identify fungi, the investigation of morphological structures was carried out, showing not particular evidence of clear taxonoy and identification in *Gliocladium*. Since molecular techniques have been introduced, more objective criteria for classifying has been partially achieved (Samson, 1995). However, it still shows problems for determining their nomenclature, taxonomy, and correct identification from other similar *Gliocladium*.

The purpose of this study is to evaluate the phylogenetic relationships of *Gliocladium*, focusing particularly on the generic concept through the comparison with *Penicillium*-like genus and *Trichoderma*-like genus. Nucleotide sequences of internal transcribed spacers (ITS-1 and ITS-2) as well as the 5.8S gene of the ribosomal DNA repeat were investigated, using 11 strains of *Gliocladium* and *Verticillium*. Also, we compared the nucleotide sequences of such 11 strains with those obtained from EMBL of the related genus.

Materials and Methods

Fungal Strains and Cultivations

The 11 strains used in this study were obtained from KCTC (Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea), JCM (The Institute of Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Saitama, Japan), IFO (The Institute for Fermentation, Osaka, Japan), and ATCC (American Type Culture Collection) and are listed in Table 1. The rest strains except representing in Table 1 were extracted from the GenBank database in NCBI (National Center for Biotechnology Information). 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 benzyl chloride method (Zhu *et al.*, 1993). Approximately 0.05 g of mycelial pellets were suspended in 500 μl of extraction 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 incubated at 55°C for 30 min. Phenol : chloroform : isoamylalchol (25 : 24 : 1) was treated twice and RNase (1 mg/ml) was added. The algal cells were pelleted and the supernatant mixed with 40 μl of 3 M sodium acetate and incubated on ice for at least 1h. The DNA was precipitated from the tube at room temperature for 10 min by adding 2.5 volumes of 100% ice-cold ethanol. The pellet wes washed with 2 volumes of 70% ethanol and resuspended in distilled water. The DNA was kept at -20°C.

Table 1. List of fungal species and GenBank accession number of the genus *Gliocladium* and related taxa used in this study

Fungal species	Specimen no.[a)]	Database accession no.[b)]
Gliocladium penicillioides	IFO 5869	AF048733
Gliocladium virens	KCTC 6146	AF048734
Gliocladium roseum	KCTC 6160	AF048735
Gliocladium viride	KCTC 6161	AF048736
Gliocladium deliquencens	KCTC 6173	AF020799
Gliocladium catenulatum	ATCC 10523	AF048737
Gliocladium cibotii	JCM 9203	AF048738
Gliocladium cibotii	JCM 9206	AF048739
Verticillium bulbillosum	JCM 9213	AF048740
Verticillium bulbillosum	JCM 9214	AF048741
Verticillium dahliae	JCM 9509	AF048742

[a)]KCTC (Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea), JCM (The Institute of Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Saitama, Japan), IFO (The Institute for Fermentation, Osaka, Japan), and ATCC (American Type Culture Collection).

[b)]The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank Database under the respective accession number.

PCR Amplification and DNA Sequencing

The nuclear rDNA region spanning the ITS1, ITS2 and 5.8S rDNA was amplified by PCR from each strain as described in Table 1. Primers ITS5F (5'-GGAA GTAAAAG-TCGTAACAAGG-3') and ITS4R (5'-TCCTCCGCTTATT-GATATGC-3') were derived from the conserved regions of 18S and 28S rDNA, respectively (Fig. 1). Polymerase Chain Reaction (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 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 product from the amplification was subjected to preparative electrophoresis in a 1.6% agarose gel in TBE buffer. All PCR products yield

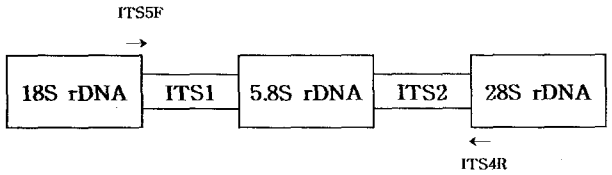

Fig. 1. A map of the ribosomal DNA region containing ITS1, ITS2 and the 5.8S rDNA gene. Arrows indicate the positions of the primers used for PCR and sequence analysis.

only a single visible band. The PCR product was 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 in 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 5F and ITS 4R, 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, U.S.A., copyright 1993).

Data Analysis

The determined ribosomal DNA sequences have been deposited in the NCBI (National Center for Biotechnology Information) data library and accession numbers are indicated in Table 1. They were initially aligned with the sequences of the related genera from the NCBI data library using the multiple alignment program Clustal W (Thompson *et al.*, 1994). The extracted sequences from the NCBI data library were as follows: *Trichoderma harzianum*, AF 055216; *T. hamatum*, U93877; *T. reesei*, Z 48933; *T. viride*, AF 127153; *Hypocera aureovidis*, Z 48819; *Penicillinum clavigerum*, L 14533; *P. vulpirum*, L 14535; *Talaromyces luteus*, L 14525: *Ta. thermophilus*, L14515; *Verticillium tricorpus*, L 28679 and *V. albo-atrum*, L 19499.

Phylogenetic relationships were inferred by the neighbor-joining method (Saitou and Nei, 1987). The strength of the internal branches from the resulting trees were statistically tested by bootstrap analysis (Felsentein, 1985) from 1,000 bootstrap replications.

Results

G+C Contents and Nucleotide Length of rDNA ITS Regions

Electrophoresis and direct sequencing of each PCR reaction confirmed that single product was amplified in accordance with each PCR reaction and the size of each product corresponded to the expected rDNA. The alignment of the DNA sequences of the internal transcribed spacers ITS1, ITS2 and 5.8S rDNA is shown in Fig. 2. There is considerable sequence variation in the ITS sequences and little in the regions of the 5.8S rDNA. The internal transcribed spacers contain most of the sequence variation. Especially, the ITS1 is more variable than ITS2 compared with all species used in this study.

G+C contents and nucleotide length of ITS1, ITS2 5.8S rRNA gene, and their total (ITS1-5.8S-ITS2) are shown in Table 2. Positive correlations in G+C contents and nucleotide length are found between ITS1 and ITS2 (Fig. 3). The total G+C contents of ITS1-5.8S-ITS2 ranged from 52.0% in *G. catenulatum* ATCC 10523 to 61.1% in *V. bulbillosum* JCM 9213. Especially, the genus *Verticillium* and two strains of *G. cibotii*, JCM 9203 and JCM 9206, were higher G+C contents than the other related taxa. Those of the 5.8S rRNA gene were stable (43.3~47.8%) among the 11 strains used in our study. On the other hand, the ITS regions showed relatively high G+C contents: 48.1~64.0% in ITS1 and 60.1~68.6% in ITS2.

The shortest size of ITS1-5.8S-ITS2 was 493 nucleotides of *G. penicillioides* IFO 5869 and the longest size of ITS regions was 549 nucleotides of *V. dahilae* JCM 9509 among 11 strains sequenced in this study, a difference of 56 nucleotides (Table 2). The size variation was derived from the variation of the ITS regions as well as 5.8S rRNA gene. Most of the tested taxa could be divided into three groups depending on the ITS length. The group with short ITS includes *G. penicillioides* IFO 5869, *G. catenulatum* ATCC 10523 and *V. bulbillosum* JCM 9213, and the group with long ITS includes *G. cibotii* JCM 9203, *G. cibotii* JCM9206, *G. virens* KCTC 6146, *G. reseum* KCTC 6160, *G. viride* KCTC 6161, *G. deliquescens* KCTC 6171, and *V. bulbillosum* JCM 9214. *V. dahliae* JCM 9509 have the longest nucleotide on the total ITS regions sequenced in our study. The length of ITS1-5.8S-ITS2 of the shortest group was between 493 to 502 nucleotides, and that of long group was from 514 to 528 nucleotides. The longest size was 549 nucleotides of *V. dahilae* JCM 9509. *G. roseum* KCTC 6160, *G. viride* KCTC 6161, and *G. deliquencens* KCTC 6173 have identical size (528 nucleotides) in the length of ITS1, 2 and 5.8S rRNA gene. Also, *G. cibotii* JCM 9203, JCM 9206, and *V. bulbillosum* JCM 9214 have identical that in the ITS1 and 5.8S rRNA gene (ITS1: 186 bp, 5.8S: 127 bp), have the variation in the that of ITS2. Also, among all the species, the DNA similarity appeared in Table 3.

Phylogenetic Analysis of the ITS1, 5.8S rDNA and ITS2 Aligned Sequences

The genus *Gliocladium* and related taxa were clearly divided into three groups in the phylogenetic tree based on the ITS1, 2 and 5.8S rDNA sequences (Fig. 4): a *Penicillium*-like genus, a *Trichoderma*-like genus group and one

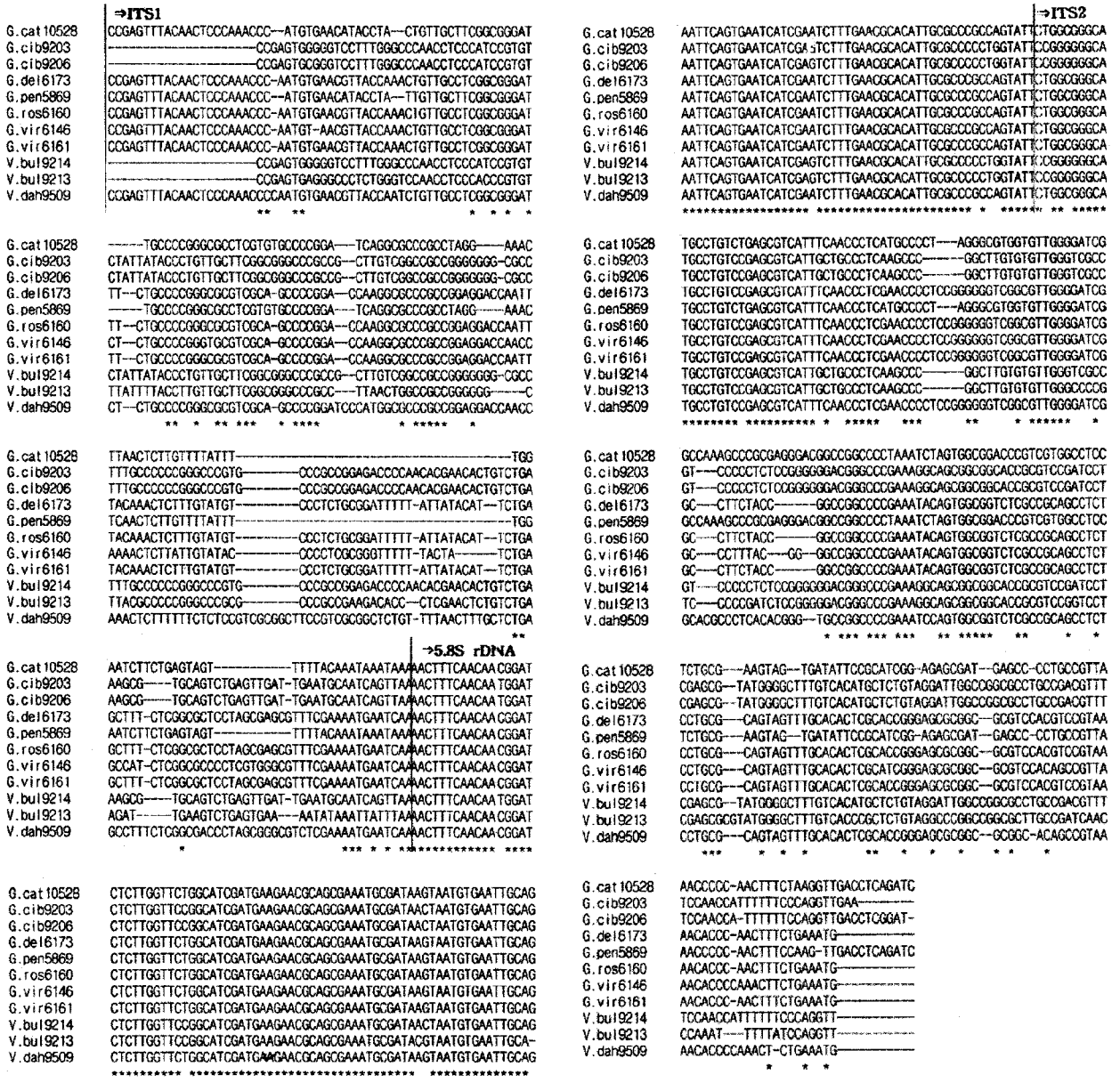

Fig. 2. Alignment of the ITS1, 5.8S rDNA and ITS2 sequences. The alignment was generated by the multiple alignment program Clustal W using a gap weight of 5.0 and a gaplength weight of 0.1. A hyphen represents a gap and a asterisk represents a base identical to that of the top sequence.

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)
G. penicillioides IFO 5869	48.1	156	46.5	157	61.1	180	52.3	493
G. virens KCTC 6146	57.4	195	46.9	162	64.9	168	56.6	525
G. roseum KCTC 6160	52.7	201	46.6	161	65.1	166	54.7	528
G. rivide KCTC 6161	53.2	201	47.2	161	65.1	166	55.1	528
G. deliquencens KCTC 6173	53.2	201	47.2	161	65.1	166	55.1	528
G. catenulatum ATCC 10523	48.7	156	45.9	157	60.1	181	52.0	494
G. cibotii JCM 9203	64.0	186	43.3	127	65.2	204	59.4	517
G. cibotii JCM 9206	64.0	186	43.3	127	64.8	210	59.2	523
V. bulbillosum JCM 9213	63.6	176	44.9	126	69.0	200	61.1	502
V. bulbillosum JCM 9214	64.0	186	44.1	127	65.7	201	59.7	514
V. dahliae JCM 9509	60.6	216	47.8	161	68.6	172	59.6	549

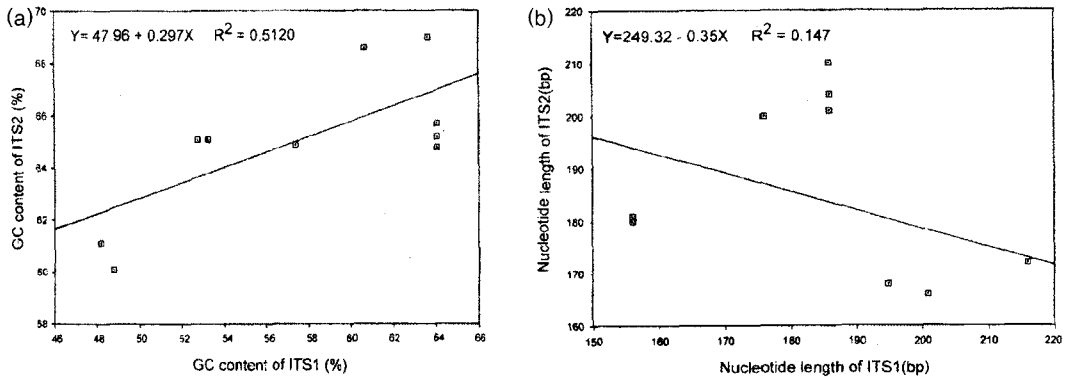

Fig. 3. Positive corelations of G+C content (a) and nucleotide length (b) between ITS1 and ITS2.

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

	1	2	3	4	5	6	7	8	9	10
1										
2	71.6									
3	71.8	99.6								
4	86.3	65.5	64.0							
5	99.4	71.8	72.2	86.0						
6	86.3	65.5	64.0	100.0	85.8					
7	85.4	67.7	66.9	93.7	85.2	93.7				
8	86.3	65.5	64.0	100.0	85.8	100.0	93.7			
9	71.7	100.0	99.8	65.6	71.8	65.6	67.5	65.6		
10	74.2	89.6	89.4	65.7	74.6	65.7	71.4	65.7	89.6	
11	84.6	65.0	64.8	93.7	84.6	93.7	94.6	93.7	65.0	69.5

The designations of number are 1. *G. catenulatum* ATCC 10528; 2. *G. cibotii* JCM 9203; 3. *G. cibotii* JCM 9206; 4. *G. deliquences* KCTC 6173; 5. *G. penicillioides* IFO 5869; 6. *G. roseum* KCTC 6160; 7. *G. viride* KCTC 6146; 8. *G. viride* KCTC 6161; 9. *V. bulbillosum* JCM 9214; 10. *V. bulbillosum* JCM 9213 and 11. *V. dahliae* JCM 9509.

other group which includes *V. tricorpus* and *V. albo-altrum*.

The first group including *Penicillium*-like strains such as *Penicillium, Talaromyces* which is teleomorph of species of *Penicillium, Verticillium* and one species of *Gliocladium*. Phylogenetic relationships among *Talaromyces* species and the relationships between *Talaromyces* and *Penicillium* in subgenus *Biverticillium* have already been discussed in a previous report (Lobuglio *et al.*, 1993). *V. bulbilosum* JCM 9213 is related to *P. vulpinum*, and *V. bulbiosum* JCM 9214 is related to two species of *G. cibotii*. *G. cibotii* was the only species of *Gliocladium* which is grouped with the *Penicillium*-like genus group. The second group included two species of *Verticillium, V. tricorpus* and *V. albo-atrum*. These two species of *Verticillium* extracted from the GenBank database in NCBI were differentiated from both the *Trichoderma*-like group and the *Penicillium*-like group. The third group including *Trichoderma*-like strains, such as *Trichoderma, Hypocrea*, that is teleomorph of species of *Trichoderma*, one species of *Verticillium, V. dahliae* JCM 9509, and several species of *Gliocladium*. *G. virens* KCTC 6146 was grouped with *T. harzianum* ATCC 60850 with a high bootstrap value. *G. roseum* KCTC 6160, *G. viride* KCTC 6161, and *G. deliquescens* KCTC 6171 formed the *Gliocladium* group, supported by high bootstrap values, in the *Trichoderma*-like genus group. Two species of *Gliocladium, G. penicillioides* IFO 5869 and *G. catenuleatum* ATCC 10523, were rooted with the *Trichoderma*-like genus group as a sister group by the neighbor-joining method.

Discussion

The morphological traits are subject to environmental influences and can vary substantially from culture to culture (Seaby, 1996). Thus, taxonomic considerations, based solely on phenotype, may be subject to ambiguities induced by environmental conditions. Combining molecular and morphological data sets has been discussed by Doyle (1992). Formerly, the taxonomy and identification of fungi have been based largely on morphological characteristics, but molecular techniques have been introduced to provide more objective criteria. The ITS regions of rDNA are useful for the analysis of phylogenetic and evolutional relationship, because the relationship usually correlates with morphological characteristics (Berbee *et al.*, 1995: Cooke and Duncan, 1997).

In the present study, the phylogenetic position of *Gliocladium* was investigated by comparing the sequences and the GC balance from internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene of the ribosomal DNA because these regions are known to be moderately variable (Bruns *et al.*, 1991). Therefore, sequence analyses of these are suitable form phylogenetic studies at both species and genus level, and complement morphological studies. ITS2 of a taxon having GC-rich ITS1 is also GC-rich. Torres *et al.* (1990) found similar phenomenon in the GC contents of ITS regions in a while range of organisms including filamentous fungi and called it "GC balance". Especially, the literature on *G. virens* reflects the taxonomic confusion

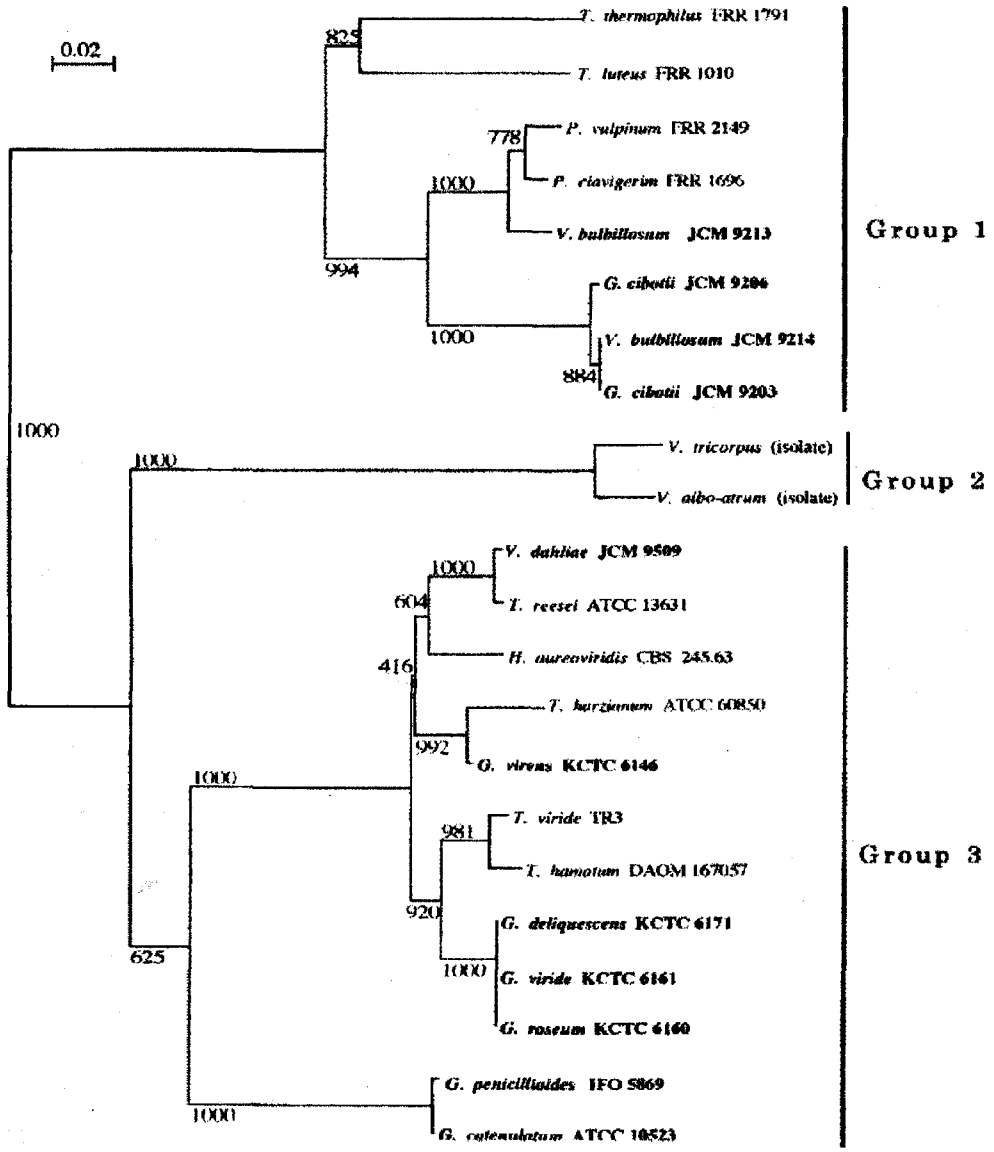

Fig. 4. Phylogenetic tree showing the relationship among the genus *Gliocladium* and related taxa. Bold represents the strain used in our study, the rest species represent the strain extracted from the GenBank database. Bar represented 2 nucleotide substitutions per 100 nucleotides in ITS1, 2 and 5.8S rDNA sequences. Bootstrap probabilities were indicated at the branch points.

surrounding the identification of this taxon. *G. virens* first received attention for its ability to produce the antifungal toxins, gliotoxin, and viridin (Brian and McGowan, 1945), and has since been referred to as *G. fimbriatum, G. virens, T. lignorum*, and *T. viride*. von Arx transferred, without providing justification, *G. virens* to *Trichoderma*. This placement was followed more recently by Bissett (1991) in a monograph on *Trichoderma*. In this study, *G. virens* was grouped with *T. harzianum* with a high bootstrap value, supporting that *G. rivens* is to be placed in *Trichoderma. T. virens* was arranged among *Hypocrea* species, and can thus be regarded as a species of *Hypocrea*. The consequence of this finding for *Trichoderma* taxonomy is that the morphological stereotype of *Trichoderma* has to be modified to accept this stereotypical *Gliocladium* morphology. In the case of *G. virens*, however, it is not too difficult to rationalize the *Gliocladium* morphology within *Trichoderma* because (i) of the intergrading *Hypocrea* anamorphs and (ii) the branching pattern of *G. virens* can be seen to be a modification of a more typical *Trichoderma*, (iii) the formation of typical *Hypocrea*-like chlamydospores, and the formation of green conidia. The results of the rDNA analysis in the present study support the taxonomic usage of von Arx and Bissett and morphological characteristic.

The results suggest (i) *Gliocladium* is polyphyletic because species of *Gliocladium* were dispersed in three group. It supports the previous report (Rehner and Samuels, 1994) suggesting that *G. virens, G. roseum* and *G. penicillioides* are clearly distinct and that *Gliocladium* is polyphyletic.

(ii) *G. penicillioides* and *G. catenulatum* form the subgroup of the *Trichoderma*-like strains. (iii) *Gliocladium* is more *Trichoderma*-like than *Penicillium*-like because all but one species of *Gliocladium* used in our study differentiated from *Penicillium*-like genus group. (iv) *G. viriens* is to be classified in *Trichoderma* because *G. virens* was grouped with *T. harzianum* with a high bootstrap value, while other species of *Gliocladium* formed the *Gliocladium* group, supported by high bootstrap values, in the *Trichoderma*-like genus group. It support the taxonomic usage of von Arx. (v) The generic concept of *Gliocladium* needs to be reexamined with molecular approach, culture condition, and morphological properties.

Acknowledgments

The authors wish to acknowledge the financial support the Korea Research Foundation made in the program year of 1998.

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