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Molecular and Cultural Characterization of *Colletotrichum* spp. Causing Bitter Rot of Apples in Korea

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***Colletotrichum* contains many important pathogens which cause economically significant diseases of crops like pepper, strawberry, tomato and apple. Forty four isolates were collected to characterize the diversity of *Colletotrichum* causing apple anthracnose in various regions of Korea. They were analyzed by random amplified polymorphic DNA (RAPD), internal transcribed spacer (ITS) of rDNA and partial β-tubulin gene DNA sequence, and culture characteristics on PDA and PDA-Benomyl. From the results of molecular analyses, 31 strains belonged to *Colletotrichum gloeosporioides*, ribosomal DNA group (RG) 4 of Moriwaki et al. (2002), 8 strains belonged to *C. acutatum*, A2 group of Talhinhas et al. (2005) and 5 strains to *C. acutatum*, A3 group of Talhinhas et al. (2005). Most isolates of *C. gloeosporioides* RG4 grew faster on PDA than strains of *C. acutatum*, A2 and A3 groups and most RG4 strains were sensitive to Benomyl. However, a few strains of RG4 grew slower and were resistant to Benomyl. On the basis of molecular characteristics, apple isolates of *C. acutatum* were clearly differentiated from red pepper isolates of the species, but apple isolates of *C. gloeosporioides* were not.**

Keywords : apple, bitter rot, anthracnose, *Colletotrichum gloeosporioides*, *C. acutatum*, taxonomy, characterization

Of the 31 apple diseases caused by fungal pathogens in Korea, bitter rot is the major one (Lee et al., 2006; The Korean Society of Plant Pathology, 2004; http://kacc.rda.go.kr/pathogen/). *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., [teleomorph: *Glomerella cingulata* (Stoneman.) Spauld. & H. Schrenk] and *C. acutatum* J. H. Simmonds have been reported as pathogens of apple bitter rot in Korea (Lee, 1994).

Characterizing the two species on the basis of their morphological differences alone is insufficient. For instance, the conidia of *C. acutatum* are elliptic-fusiform and the ends of each are more attenuated than those of *C. gloeosporioides* (Sutton, 1980; Kang et al., 2005), but this difference is not clear enough to differentiate the two species (Freeman et al., 1998; Sreenivasaprasad et al., 1996; Sutton, 1992; Kang et al., 2005). Molecular characters such as ribosomal DNA Internal Transcribed Spacer (ITS), partial β-tubulin gene and random amplified polymorphic DNA (RAPD) were reported to be useful tools in differentiating the two species. (Talhinhas et al., 2005; Brooker et al., 1991; Freeman et al., 1993; Kim et al., 2006). The growth rate on potato dextrose agar (PDA) and sensitivity to Benomyl were also proposed as available characters for differentiation (Talhinhas et al., 2005; Kim et al., 2003). The taxonomic study of *Colletotrichum* spp. from apples in Korea dated back more than 10 years ago (Lee, 1994) and only morphological characters were used for their differentiation.

In this study, we examined the diversity of *Colletotrichum* strains from apple orchards in Korea. We collected 44 strains of *Colletotrichum* spp. from various areas in Korea and used molecular characters such as rDNA-ITS, partial β-tubulin gene sequence and RAPD, and cultural characters like growth rate on PDA and PDA-Benomyl for taxonomic characterization.

Materials and Methods

***Colletotrichum* strains.** Forty four strains of *Colletotrichum* were collected from 11 apple cultivars growing in apple orchards of 16 cities in Korea. Symptomatic tissues were surface-sterilized with 10% sodium hypochlorite solution for 2 minutes, and then rinsed several times with sterile distilled water before placing on PDA at 25°C. After confirming *Colletotrichum* genus by microscope examination, two monoconidial cultures from each isolate were prepared and used in this study. Detailed information of the strains is shown in Table 1.

RAPD and nucleotide sequence analyses. Two monoconidial cultures from each strain were inoculated into

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Table 1. The list of *Colletotrichum* strains isolated from apple orchards in Korea

Isolate no.	Name by molecular analyses	Host (cultivar)/ Geographical origin	Group by RAPD-PCR analyses	Group by DNA sequence analyses	Growth on PDA* (mm)	Growth on PDA with Benomyl* (mm)	
						0.5 mg/L	2.0 mg/L
L01	*Colletotrichum gloeosporioides*	*Malus pumila* var. *dulcissima* (*Fuji*)/Uiseong	1	–	70.9	0.0	0.0
L03		*M. pumila* var. *dulcissima* (*Fuji*)/Uiseong	1	–	70.1	0.0	0.0
L05		*M. pumila* var. *dulcissima* (*Fuji*)/Pohang	1	–	78.1	0.0	0.0
L07		*M. pumila* var. *dulcissima* (*Fuji*)/Pohang	1	–	77.2	0.0	0.0
L09		*M. pumila* var. *dulcissima* (*Fuji*)/Uiseong	1	–	70.4	0.0	0.0
L11		*M. pumila* var. *dulcissima* (*Fuji*)/Uiseong	1	–	69.7	0.0	0.0
L15		*M. pumila* var. *dulcissima* (*Fuji*)/Uiseong	1	–	80.5	0.0	0.0
KACC42494 (L21)		*M. pumila* var. *dulcissima* (*Misima-fuji*)/Cheongsong	1	RG4	75.2	0.0	0.0
KACC42497 (L31)		*M. pumila* var. *dulcissima* (*Saenara*)/Gunwi	1	RG4	76.5	0.0	0.0
L33		*M. pumila* var. *dulcissima* (*Saenara*)/Gunwi	1	–	72.4	0.0	0.0
KACC42498 (L35)		*M. pumila* var. *dulcissima* (*Sansa*)/Yeongju	1	RG4	70.9	0.0	0.0
L49		*M. pumila* var. *dulcissima* (*Tsugaru*)/Seoul	1	–	67.7	30.8	26.3
L53		*M. pumila* var. *dulcissima* (*Tsugaru*)/Cheongsong	1	–	74.1	0.0	0.0
L57		*M. pumila* var. *dulcissima* (*Tsugaru*)/Gunwi	1	–	82.4	0.0	0.0
L59		*M. pumila* var. *dulcissima* (*Hongro*)/Jangsu	1	–	77.6	0.0	0.0
L69		*M. pumila* var. *dulcissima* (*Hongro*)/Mungyeong	1	–	73.9	0.0	0.0
L71		*M. pumila* var. *dulcissima* (*Hongro*)/Mungyeong	1	–	70.2	0.0	0.0
L73		*M. pumila* var. *dulcissima* (*Hongro*)/Mungyeong	1	–	71.5	0.0	0.0
L75		*M. pumila* var. *dulcissima* (*Hongro*)/Gunwi	1	–	71.9	0.0	0.0
KACC42506 (L77)		*M. pumila* var. *dulcissima* (*Hongro*)/Paju	1	RG4	80.8	0.0	0.0
L79		*M. pumila* var. *dulcissima* (*Hongro*)/Yeongcheon	1	–	65.4	0.0	0.0
L81		*M. pumila* var. *dulcissima* (*Hongro*)/Yeongcheon	1	–	69.6	0.0	0.0
KACC42499 (L37)		*M. pumila* var. *dulcissima* (*Sansa*)/Gunwi	2-1	RG4	63.0	56.7	47.5
KACC42507 (L83)		*M. pumila* var. *dulcissima* (*Hongro*)/Jeongeup	2-1	RG4	77.6	0.0	0.0
L89		*M. pumila* var. *dulcissima* (*Hongok*)/Yeongcheon	2-1	–	70.6	12.8	12.1
KACC42501 (L45)		*M. pumila* var. *dulcissima* (*Tsugaru*)/Yongin	2-2	RG4	81.2	0.0	0.0
L85		*M. pumila* var. *dulcissima* (*Hongro*)/Jangseong	2-2	–	74.7	0.0	0.0
L87		*M. pumila* var. *dulcissima* (*Hongro*)/Jangseong	2-2	–	69.3	0.0	0.0
KACC42503 (L55)		*M. pumila* var. *dulcissima* (*Tsugaru*)/Yeongju	3	RG4	72.0	0.0	0.0
L14		*M. pumila* var. *dulcissima* (*Fuji*)/Uiseong	4	–	71.6	0.0	0.0
KACC42390 (L17)		*M. pumila* var. *dulcissima* (*Fuji*)/Sangju	4	RG4	60.4	0.0	0.0
KACC42403 (L20)	*C. acutatum*	*M. pumila* var. *dulcissima* (*Fuji*)/Andong	5	A2	62.0	25.7	20.7
L23		*M. pumila* var. *dulcissima* (*Samdogye-fuji*)/Bonghwa	5	–	59.3	26.1	22.0
KACC42496 (L27)		*M. pumila* var. *dulcissima* (*Sunhong*)/Gunwi	5	A2	61.5	28.6	25.0
L41		*M. pumila* var. *dulcissima* (*Tsugaru*)/Yongin	5	–	63.6	26.1	23.2
KACC42502 (L47)		*M. pumila* var. *dulcissima* (*Tsugaru*)/Seoul	5	A2	60.8	30.1	28.7
L51		*M. pumila* var. *dulcissima* (*Tsugaru*)/Seoul	5	–	75.1	0.0	0.0
KACC42505 (L65)		*M. pumila* var. *dulcissima* (*Hongro*)/Gunwi	5	A2	42.6	28.9	24.6
L67		*M. pumila* var. *dulcissima* (*Hongro*)/Gunwi	5	–	40.6	28.4	23.4
KACC42508 (L91)		*Capsicum annuum*/Uiseong	5	A2	54.2	21.3	20.9
L93		*Capsicum annuum*/Uiseong	5	–	50.6	22.8	22.7
KACC42509 (L95)		*Capsicum annuum*/Gunwi	5	A2	46.6	22.5	21.5
KACC42495 (L25)		*M. pumila* var. *dulcissima* (*Hirosaki*)/Bonghwa	6	A3	57.4	24.9	17.8
L29		*M. pumila* var. *dulcissima* (*Chukwang*)/Gunwi	6	–	59.2	20.8	19.7
KACC42500 (L43)		*M. pumila* var. *dulcissima* (*Tsugaru*)/Yongin	6	A3	51.2	19.2	20.0
KACC42504 (L61)		*M. pumila* var. *dulcissima* (*Hongro*)/Jangsu	6	A3	48.1	23.8	22.3
L63		*M. pumila* var. *dulcissima* (*Hongro*)/Jangsu	6	–	56.4	24.9	22.4

*Each number of the column is the mean value of three repetitions.

potato dextrose broth (PDB) and incubated at 180 rpm for 3 days at 25°C. Mycelia were harvested and freeze-dried. Dried mycelia were ground to powder and from which DNA was extracted using the DNeasy kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Extracted DNAs were preserved at -20°C.

For RAPD-PCR of all strains, each primer PELF (5'-ATA TCA TCG AAG CCG C-3'), URP1F (5'-ATC CAA GGT CCG AGA CAA CC-3') (Kang et al., 2002) was used for each. The PCR was performed in 30 μl reaction mixture containing 2 μl of 2.5 mM dNTP, 3 μl of 10X buffer, 0.3 μl of 100 uM primer, 0.3 μl of 5 unit/μl Taq polymerase (SolGent Co., Ltd.), 1 μl of genomic DNA and distilled water. The PCR conditions consisted of predenaturation for 4 min at 95°C followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 58°C and extension for 2 min at 72°C with a final extension for 7 min at 72°C. The PCR products were electrophoresed on a 1.2% agarose gel.

Eighteen representative strains were chosen for DNA sequence analyses by geological location, cultivar of host and RAPD pattern. rDNA-ITS and partial β-tubulin were respectively amplified using the primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') & ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al., 1990) and T1 (5'-AAC ATG CGT GAG ATT GTA AGT-3') (O'Donnell and Cigelnik, 1997) & βt2b (5'-ACC CTC AGT GTA GTG ACC CTT GGC-3') (Glass and Donaldson, 1995). The reaction mixture for the PCR contained 3 μl of 2.5 mM dNTP, 5 μl of 10X buffer, 0.4 μl of 100 uM of each primer, 0.3 μl of 5 unit/μl *Taq* DNA polymerase, 1 μl of genomic DNA in a 50 μl reaction volume. The PCR conditions for ITS amplification were predenaturation for 4 min at 95°C followed by 35 cycles at denaturation for 1 min at °C, annealing for 1 min at 58°C and extension for 2 min at 72°C with a final extension for 7 min at 72°C. In the case of β-tubulin, the conditions were the same as that used for ITS except for the annealing temperature which was set at 64°C. The primers ITS1 and ITS4 amplified to about 570 bp of ITS region contained partial 18S, 28S and full ITS1, 5.8S and ITS2 regions. The primers T1 and βt2b amplified to about 750 bp of β-tubulin gene included exon 1 to partial exon 6 regions. The PCR products were then purified by PCR_{96} Cleanup Plates (Millipore Corp., Bedford., MA 01730) and sequencing reaction was done using BigDye Terminator v3.1 Cycle Sequence Kit (ABI0401041) and the same primers for the amplification. Then the sequencing PCR products were purified by Montage SEQ_{96} Sequencing Reaction Cleanup Kit (Millipore Corp., Bedford., LSKS 096 24) and were determined by ABI 3100 DNA Sequencer.

DNA sequences were edited with Seqman of DNASTAR computer package (DNASTAR Inc.) and aligned using CLUSTAL W method (Thompson et al., 1994). For the phylogenetic tree, we used full ITS1, 5.8S and ITS2 regions for ITS analysis and partial exon 3 to partial exon 6 regions for β-tubulin analysis. For Neighbor-Joining (NJ) analysis, MEGA v3.1 (Kumar et al., 2004) was employed, and sequence distance was first calculated using the Tamura-Nei parameter model, which was then used to construct the NJ tree. Bootstrap analysis was performed with 1,000 replications to determine the support for each clade.

Cultural characteristics on PDA and PDA-Benomyl. All strains were cultured on potato dextrose agar (PDA) at 25°C in the dark for 5 days, and the diameter of mycelial growth was measured (Table 1). To determine the sensitivity of the isolates to Benomyl, each isolate was cultured in PDA at 25°C in the dark, and mycelial disks, 3 mm in diameter, were cut from the margins of colonies and transferred to PDA medium with 0.5 and 2.0 mg/L Benomyl (Benlate wettable powder; Syngenta Korea Co., Ltd.). The set-up was maintained at 25°C in the dark for 5 days; after which, the diameter of each colony was measured (Table 1).

Results

RAPD and nucleotide sequence analyses. Each primer, PELF and URP1F, produced many polymorphic bands (Fig. 1). Forty-four strains were divided into 6 groups by the two RAPDs. Twenty-two strains were gathered in RAPD group 1 and 6 strains in RAPD group 2, 1 strain in RAPD group 3 and 2 strains in RAPD group 4. Eight strains were gathered into RAPD group 5 and 5 strains into RAPD group 6. In the case of Fuji cultivar strains, 7 strains were in RAPD group 1, 2 strains in RAPD group 4 and 1 strain was in RAPD group 5. Three pepper strains (KACC 42508, L93, KACC 42509) showed different band patterns from apple strains within RAPD group 5.

Eighteen strains were selected for the representative of 6 groups of RAPD. The strains from RAPD group 1, 2, 3 and 4 were clustered into ribosomal DNA group (RG) 4 (Moriwaki et al., 2002) of *C. gloeosporioides* in rDNA-ITS phylogenetic tree (Fig. 2). The strains from RAPD group 1, 2 and 4 were intermixed within the RG4 of *C. gloeosporioides* and each strain showed quite high-diversity in each other. RAPD group 3, KACC 42503 and CBS 145.29 were separated from RAPD group 1, 2 and 4 with 82% bootstrap value. The strains of RAPD group 5 and 6 were grouped into A2 and A3 of *C. acutatum* (Talhinhas et al., 2005), respectively (Fig. 2). Phylogenetic tree based on partial β-tubulin gene sequence showed the identical topology with rDNA-ITS tree except minor position changes of several strains and bootstrap values (Fig. 3). Pepper isolates of *C. acutatum* were located separately from apple isolates of the species, but pepper isolates of *C. gloeosporioides*

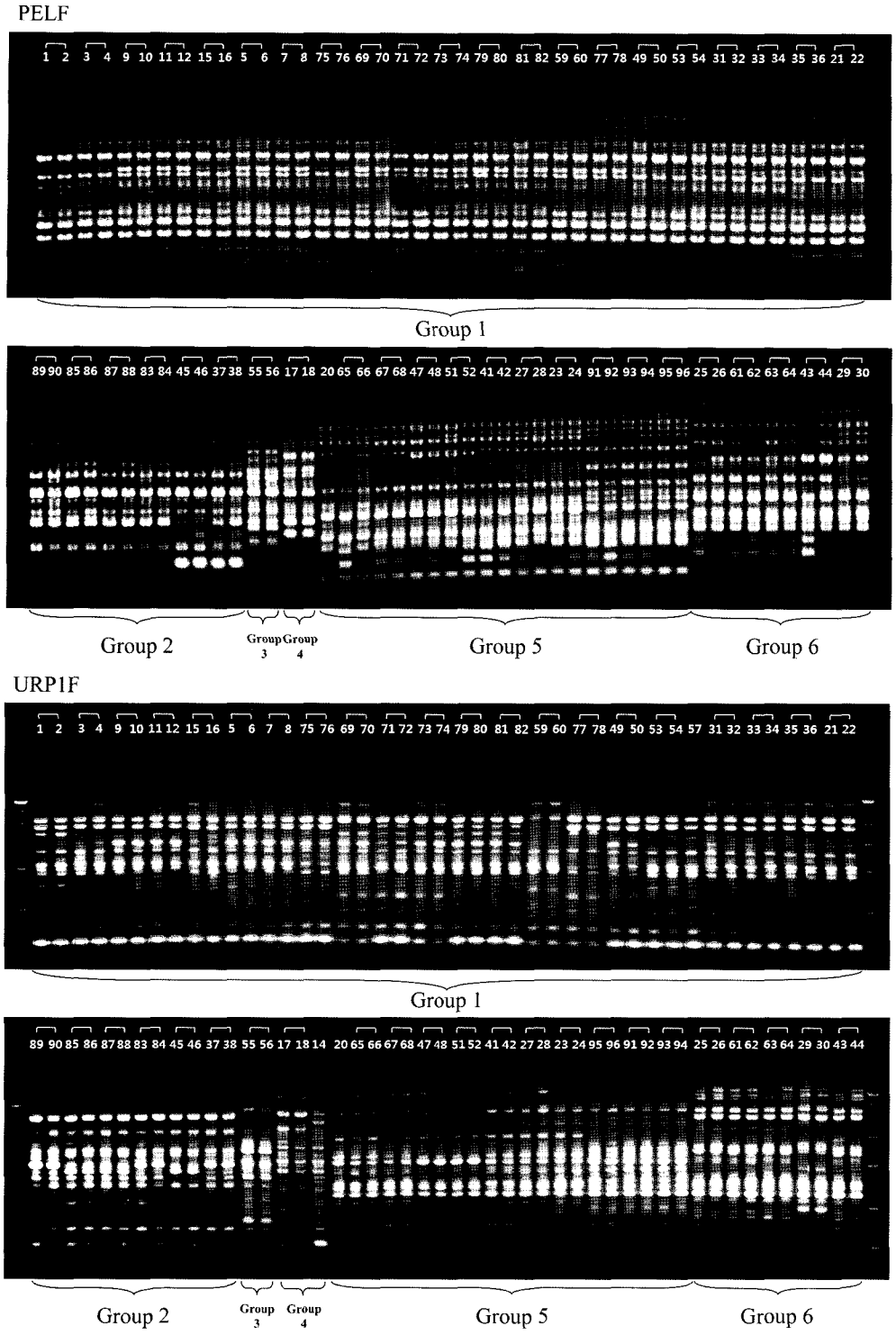

Fig. 1. RAPD patterns of *Colletotrichum* strains from apple orchards by primers, PELF (upper) and URP1F (lower). The number above the lane means L number of Table 1. The two strains bound are monoconidial cultures from the same isolate.

were not differentiated from apple isolates of the species in the two phylogenetic trees (Fig. 2, 3).

Growth rate on PDA and PDA-Benomyl. The growth size on PDA and PDA-Benomyl were described in Table 1. The strains of RG4, *C. gloeosporioides* grew 60.4-82.4 (average 72.8) mm and strains of A2 and A3 of *C. acutatum* grew 40.6-63.6 (average 54.3) mm on PDA.

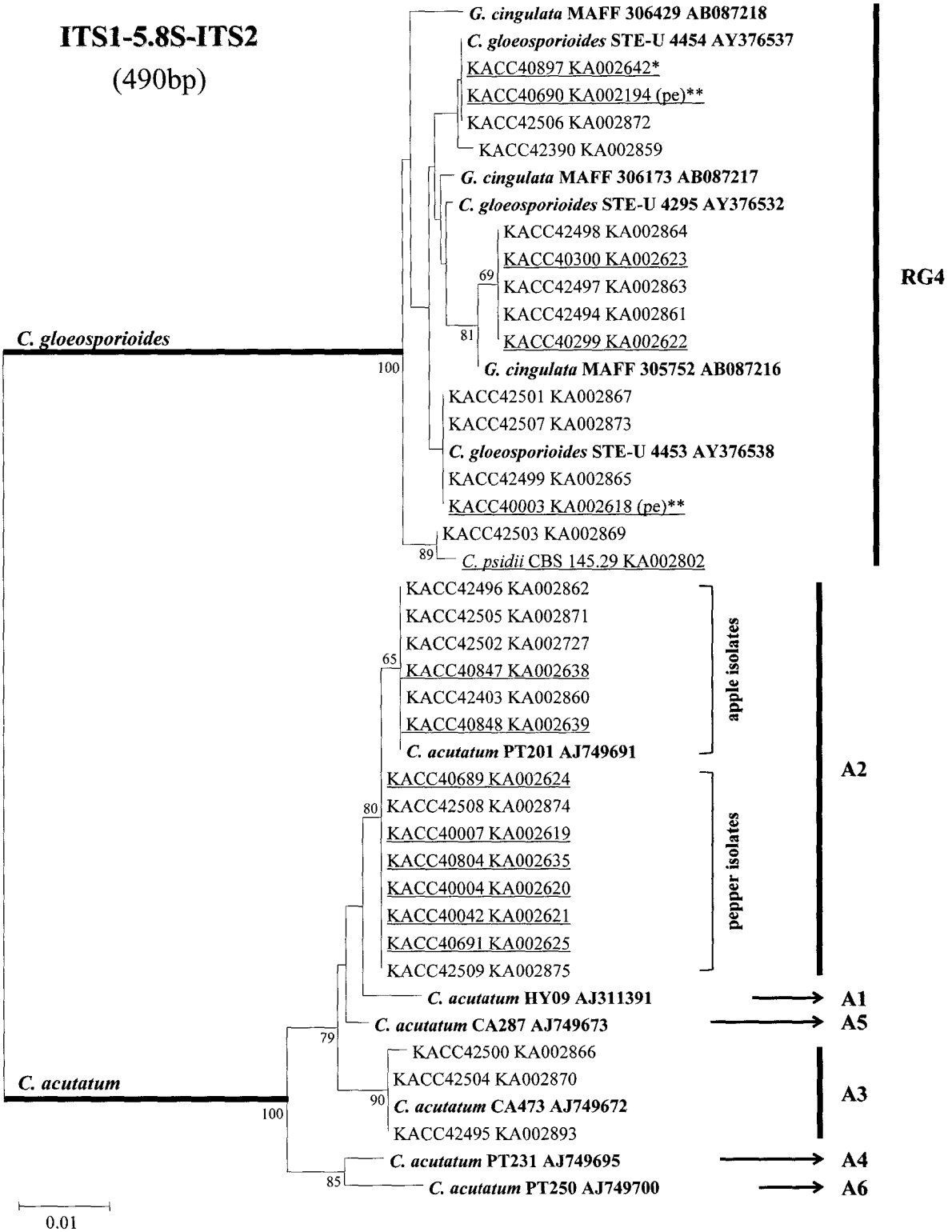

Fig. 2. Neighbor-Joining tree of *Colletotrichum* strains from apple orchards in Korea based on rDNA-ITS sequence. The sequences of strains written with bold were obtained from Thalhinhas et al. (2005), Thalhinhas et al. (2002), Lubbe et al. (2004) and Moriwaki et al. (2002) and the sequences of underlined strains were obtained from Kim et al. (2006). The numbers above the nodes represent bootstrap values of >60% (out of 1,000 bootstrap replication). *AB087218, AY376537, AJ749691, KA002642, etc. are DNA sequence accession number. AB, AY, AJ except KA, numbers are from NCBI GenBank, and KA number are DNA sequence accession number of Korean Agricultural Culture Collection, KACC (http://kacc.rda.go.kr). **pe: The strain was isolated from red pepper.

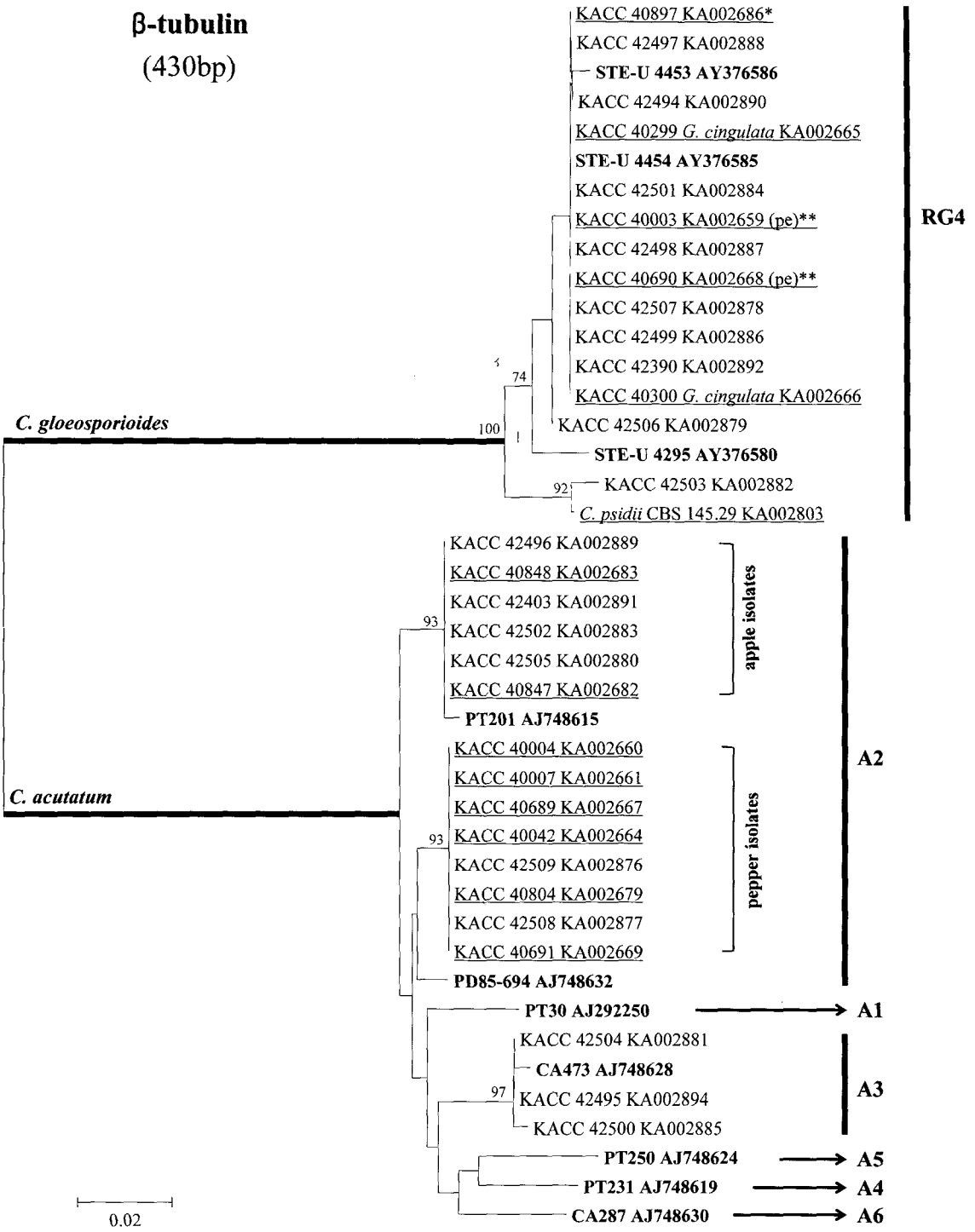

Fig. 3. Neighbor-Joining tree of *Colletotrichum* strains from apple orchards in Korea based on partial β-tubulin sequence. The sequences of strains written with bold were obtained from Thalhinhas et al. (2005), Thalhinhas et al. (2002) and Lubbe et al. (2004) and the sequences of underlined strains were obtained from Kim et al (2006). The numbers above the nodes represent bootstrap values of >60% (out of 1,000 bootstrap replication). * KA002889, AJ748615, AY376580, etc. are DNA sequence accession number. AJ and AY numbers are from NCBI GenBank, and KA number are DNA sequence accession number of Korean Agricultural Culture Collection, KACC (http://kacc.rda.go.kr). ** pe: The strain was isolated from red pepper.

However, L51 of *C. acutatum* A2, showed unusual growth of 75.1 mm. Most strains of RG4 did not grow on PDA containing 0.5 g/ml and 2.0 g/ml Benomyl, but most strains of A2 and A3 grew 19.2-30.1 (average 24.9) mm on PDA

containing 0.5 g/ml Benomyl and 20.0-28.7 (average 22.3) mm on PDA containing 2.0 g/ml Benomyl (Table 1). Three strains of RG4 (L49, KACC 42499, L89), however, grew on PDA-Benomyl while L51 of A2 did not grow.

Discussion

Based on molecular characters including rDNA-ITS, β-tubulin gene sequences and RAPD, apple isolates of *Colletotrichum* were divided into three genotypes, *C. gloeosporioides*, RG4 of Moriwaki et al. (2002) and *C. acutatum* A2 and A3 of Talhinhas et al. (2005). On the basis of RAPD, the strains of *C. gloeosporioides*, RG4 were divided into 4 subgroups, but the grouping was not supported by rDNA-ITS and β-tubulin gene analyses. RAPD group 3, composed of KACC 42503 and CBS 145.29, was separated from the other 3 groups in both DNA sequences and RAPD. The CBS 145.29 is authentic strain of *C. psidii* Curzi and is a casual pathogen of guava anthracnose. However, the species is usually considered as synonym of *C. gloeosporioides* because the species was not clearly differentiated from *C. gloeosporioides* by morphological characteristics except by host range (Singh and Sharma, 1982). It is interesting that *C. psidii* showed different molecular characteristics from *C. gloeosporioides* in this study. However, this study used restricted numbers of *C. gloeosporioides* and *C. psidii* strains. For the clear conclusion of taxonomic position of the *C. psidii*, more studies are needed. In this study, although the two strains positioned separately from *C. gloeosporioides* strains, we did not consider them as separate genotype, because they showed quite high similarity with *C. gloeosporioides* and *C. psidii* is generally considered as *C. gloeosporioides*.

Out of forty-four strains from Gyeongbuk, Jeonbuk and Gyeonggi, 31 strains (70%) were *C. gloeosporioides*, 8 strains (18%) were *C. acutatum* A2, and 5 (11%) strains were *C. acutatum* A3. Although 44 strains are not sufficient to represent all apple bitter rot in Korea, there is a high possibility that *C. gloeosporioides* is the dominant pathogen in Korea. This result is consistent with the previous report (Lee, 1994), in which apple bitter rot from Daegu and Yesan, accounted for by *C. gloeosporioides* and *C. acutatum*, were 92.3% and 7.7%, respectively. Pathogenecity and virulence in apples of the three genotypes have to be examined in future.

Although, out of 10 strains from Fuji cultivar, 9 strains were *C. gloeosporioides* and only 1 isolate was *C. acutatum* A2, the composition of genotypes of apple anthracnose was generally not correlated with apple cultivars. From Gyeongbuk and Gyeonggi provinces, all genotypes, *C. gloeosporioides* RG4, *C. acutatum* A2 and A3 were isolated. However, only *C. gloeosporioides* RG4 and *C. acutatum* A3 were isolated from Jeonbuk province. Considering the small number of sampling sites in Jeonbuk province, the occurrence of *Colletotrichum* genotypes were not influenced by geographical location within Korea.

We compared apple anthracnose strains with pepper ones based on molecular characters. In the case of *C. acutatum*, apple and pepper strains were clustered into each subgroup within A2 on rDNA-ITS and β-tubulin phylogenetic trees (Figs. 2 and 3). In addition, apple and pepper strains showed clearly different band patterns on RAPD by each of the two primers (Fig. 1). However, the strains of *C. gloeosporioides* RG4 showed diverse subclades without correlation of hosts. For example, KACC 40003 from pepper were clustered into the same subclade with strains from apple, KACC 42507, KACC 42499 and KACC 42501 within RG4 (Figs. 2 and 3). Pepper strain (KACC 42508) and apple strains (L9 and L11) were isolated from the same orchard in Uiseong, Gyeongbuk. The pepper strain L93 and apple strain L1 were also isolated from the same orchard in Uiseong. However, the 2 pepper strains were included in *C. acutatum* A2 and the 3 apple strains were included in *C. gloeosporioides* RG4. These data showed that there is a low possibility for *Colletotrichum* strains, especially *C. acutatum* to cross-infect apple and red pepper. In the case of *C. gloeosporioides*, we need more strains and further studies.

The strains of *C. gloeosporioides* grew faster than that of *C. acutatum* on PDA. *C. gloeosporioides* strains were sensitive to Benomyl but *C. acutatum* strains were resistant. However, growth characteristics on PDA and PDA-Benomyl were not consistent according to the two species. L51, *C. acutatum* showed more rapid growth than the average of *C. gloeosporioides* growth. In sensitivity to Benomyl, 3 strains of *C. gloeosporioides* RG4 were resistant to Benomyl and one strain of *C. acutatum* A2 was sensitive to Benomyl. Furthermore, growth sizes of some strains were overlapped between the two species. Therefore, it is thought that growth characters on PDA and PDA-Benomyl can not differentiate the two species and they can only be used as reference data for the differentiation. These results are consistent with the study of Kim et al. (2006).

C. gloeosporioides RG4 dominantly occurred in apple orchards in Korea. However, we collected the pathogens only from Gyeongbuk, Jeonbuk and Gyeonggi provinces and there is still a need to collect *Colletotrichum* from other provinces. In addition, the pathogenecity and virulence of the three genotypes of *Colletotrichum* in apple have to be further studied.

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