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Morphological Variations, Genetic Diversity and Pathogenicity of *Colletotrichum* species Causing Grape Ripe Rot in Korea

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Ripe rot was frequently observed on fruits, leaves and stems of grape growing in eight locations in Korea from 2004 to 2006. All 30 isolates of *Colletotrichum* sp. were obtained from lesions of the ripe rot on grape plants. Out of the isolates, 19 isolates were identified as *Colletotrichum acutatum* and the others as *Colletotrichum gloeosporioides* based on morphological and cultural characteristics. Inter and intra specific variations of the *Colletotrichum* spp. isolates were investigated using RAPD and sequences of rDNA ITS and β-tubulin-2. Isolates of *C. acutatum* and *C. gloeosporioides* were distinctly differentiated by molecular analyses. Phylogenetic trees of ITS and β-tubulin-2 showed that Korean isolates of *C. acutatum* were clustered into groups A2 and A3 among the eight global groups. A2 included non-chromogenic isolates and A3 chromogenic ones. Both *C. acutatum* and *C. gloeosporioides* isolates were tested for pathogenicity to grape leaves. All isolates tested induced lesions on the leaves of grape by artificial inoculation. There was no difference in pathogenicity between *C. acutatum* and *C. gloeosporioides* isolates. This is the first report that *C. acutatum* except *C. gloeosporioides* causes grape ripe rot in Korea.

Keywords : *Colletotrichum*, grape, ITS, pathogenicity, ripe rot, β-tubulin-2

Grape (*Vitis* spp.) is one of the most widely planted fruit trees from temperate to tropical regions, and has been used as a material for production of wine, brandy and juice in the world. The plant was first introduced to Korea at 1900s and has been grown all over the country. Ripe rot of grape is a serious disease occurring in most vineyards and leading to yield loss and deterioration. Incidence of the disease in Korea usually appears at late June as the rainy season begins and reaches the peak at August as the fruits mature. It has been reported that the disease severity is related to rainfall days and ripening time of the fruits (Kim et al., 2001).

Grape ripe rot was first reported in USA in 1891 (Southworth, 1891). *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. was reported to be a pathogen of the disease. *Colletotrichum acutatum* Simmonds was also reported as a pathogen of the disease in USA (Daykin and Milholland, 1984), Japan (Yamamoto et al., 1999) and Australia (Melksham et al., 2002). The two species are not clear enough to separate based on only conventional taxonomical methods because of morphological similarity, extensive variability of isolates in culture and overlapping host ranges. *C. gloeosporioides* and *C. acutatum* have been recognized as a species complex or group species that encompasses a wide range of morphological and genetic diversity. Molecular techniques coupled with conventional methods have been applied for differentiating the two species and provided new insight for their identification (Johnston and Jones, 1997).

C. acutatum and *C. gloeosporioides* have been reported to be common anthracnose pathogen of fruit trees and chili pepper in Korea (Kim et al., 2007; Kim and Hong, 2008; Kim et al., 2008). However, there is no information available on *Colletotrichum* species causing ripe rot on grape except a few records in relation to *C. gloeosporioides* (Cho and Shin, 2004). The objective of present work was to accurately identify *Colletotrichum* spp. associated with grape ripe rot, to clarify genetic variations within the species based on molecular aspects, and to ascertain pathogenicity of the fungal species to grape.

Materials and Methods

Field survey and isolation of pathogen. Diseased tissues showing grape ripe rot were obtained from fruits, leaves and stems of grape grown in vineyards at eight locations in Korea during disease survey from 2004 to 2006. Pieces of the diseased tissues were sterilized in 1% NaOCl for 1 minute followed by several rinses with sterile distilled water. The pieces were placed on water agar (WA) in petri plates at 25°C for two to three days. Conidia produced on

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the pieces were suspended with sterile distilled water, streaked on WA, and then incubated at 25°C for 18hr. Monoconidial isolates were obtained from germinated spores on WA.

Examination of morphological and cultural characteristics. The isolates were cultured on potato dextrose agar (PDA) in darkness at 25°C for a week. The culture plates were transferred into an incubator with alternating cycles of 12 h NUV light and 12 h darkness for another week to induce conidial production. The conidia from each isolates were mounted in water and their size was measured under a light microscope. To observe appressorial formation, a drop of conidial suspension (1×10^5 conidia/ml) were dropped on sterilized slide glass in petri plates with water-soaked paper towel, and then kept at 25°C for a day. Mycelial growth and colony features of the isolates were examined on PDA plates kept in darkness at 27°C for a week. Colony colors were recorded using Munsell renovation color system today's color/300 (Nippon Shikisai co., Ltd.).

RAPD-PCR and nucleotide sequences analyses. The isolates were cultured on potato dextrose broth (PDB) at 25°C for five days. Mycelia harvested from the PDB were lyophilized and homogenized in a microtube using toothpick and genomic DNA was extracted using a DNeasy™ kit (QIAGEN, Valencia, CA, USA) following the manufacture's instruction.

RAPD-PCR was carried out using URP-2R among the twelve universal rice primers (URPs) (Kang et al., 2002). The PCR reactions were performed in a total volume of 20 μl, containing 50 ng of genomic DNA, 10 mM of Tris-HCl, 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.01% gelatin, 0.2 mM each of dNTP, 1 μM of primer, and 1 unit of *Taq* polymerase (Promega, USA). The cycle parameters for PCR amplification were programmed for an initial denaturation at 94°C for 4 min, 35 cycles at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min and one cycle of a final extension at 72°C for 7 min. Amplification products were separated in 1.5% of agarose gel. Presence or absence of RAPD fragments was scored as 1 or 0, respectively. The fingerprint profile was used to construct a dendrogram by the unweighted pair group method by arithmetic averages (UPGMA) using NTSYS 2.0. Software (Rohlf, 1993).

The rDNA ITS region was amplified by PCR with primers ITS1 and ITS4 (White et al., 1990) and the exon 3 to 6 of β-tubulin-2 gene with primer Bt2a and Bt2b (Glass and Donaldson, 1995). PCR amplification and its cycle parameters were performed under the same condition as those of RAPD-PCR. The PCR products were electrophoresed on 1% agarose gel, purified using a Wizard PCR clean-up system (Promega, USA) and cloned into the plasmid pGEM T easy vector (Promega). Inserts of plasmid were sequenced on both strands. Sequences were edited using DNASTAR computer package (DNASTAR Inc.) and aligned by CLUSTAL W method (Thompson et al., 1994). Phylogenetic tree for ITS and β-tubulin-2 was obtained from the data by neighbor-joining methods using MEGA v4.0 (Tamura et al., 2007), and sequence distance was calculated with Tamura-Nei parameter model. Bootstrap analysis was performed with 1,000 replications to determine the support for each clade.

Pathogenicity test. Leaves detached from grape varieties Campbell Early, Muscat Bailey A (MBA), and Tamnara grown in vineyards were used for testing pathogenicity of four isolates of *C. acutatum* and two isolates of *C. gloeosporioides*. The leaves were wounded with a blunt pencil point or left intact. Each of the leaf was dropped with 20 μl of conidial suspensions adjusted to 5×10^5 conidia/ml, and then placed in plastic boxes where two sheets of paper towel soaked in water. Twenty μl of water as the control was dropped on wounded and unwounded leaves. The boxes were kept at 27°C for five days and formation of lesions on the leaves was observed. Disease rating was made based on the lesion development on the leaves five days after inoculation. The inoculation test was performed with three replicates.

Results

Disease incidence and symptoms. During disease survey from 2004 to 2006, grape ripe rot was observed in vineyards in eight locations surveyed in Korea. The symptoms on the fruits appeared initially as circular, reddish brown spots (Fig. 1A). The spots were sunken and gradually enlarged to entire surface of fruits. Salmon-colored conidial masses on surface of diseased fruits under moistened condition were produced and resulted in dried and mummified fruits as they decayed (Fig. 1B). Symptoms were also appeared in the form of black spots on leaves and resulted in reddish brown lesions with purple margin (Fig. 1C). Symptoms on stems appeared as a black-colored and cracked canker (Fig. 1D).

Cultural and morphological characteristics. All 30 isolates of *Colletotrichum* sp. were isolated from lesions of grape ripe rot. Of these, nineteen isolates were identified as *C. acutatum* and eleven isolates as *C. gloeosporioides* based on cultural and morphological characteristics (Table 1). The isolates of two species showed difference in mycelial growth on PDA. Most isolates of *C. acutatum* grew slowly less than 60 mm for 7 days and *C. gloeosporioides* isolates

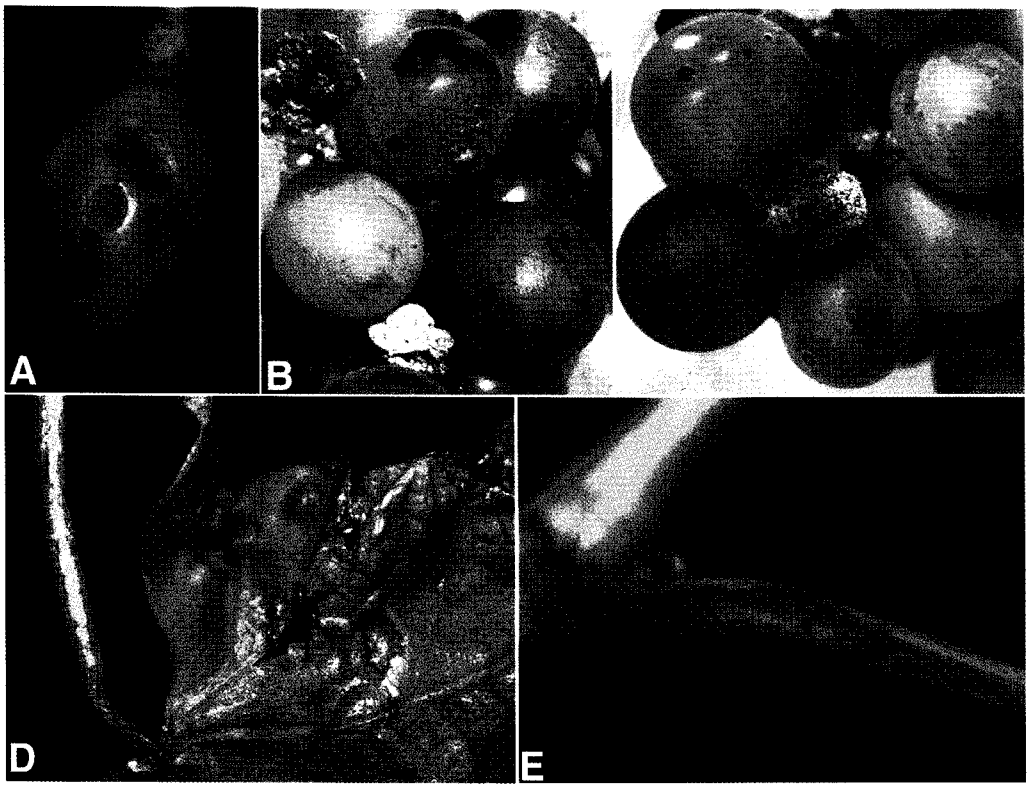

Fig. 1. Symptoms of grape ripe rot. A, sunken blackish brown spots on a diseased fruit at early stage; B, rotting fruit with salmon-colored conidial masses on a cluster of grape at middle stage; C, mummified fruit with yellow-colored conidial masses on a cluster of grape at later stage; D, reddish brown spots with purple margin on a leaf; E, a elongated lesion with black-colored and cracked canker on a stem.

fast more than 60 mm. However, some isolates of *C. acutatum* grew more than 60 mm, whereas some isolates of *C. gloeosporioides* grew less than 60 mm. The colonies of *C. acutatum* isolates were assigned to two types. Eleven out of the fungal isolates were chromogenic form producing pigment, and eight isolates non-chromogenic form. The chromogenic isolates usually showed pale pink or pale orange in top color, and light yellow orange, reddish yellow or yellowish red in bottom color (Figs. 2A and 2B). The non-chromogenic isolates showed light olive gray or yellowish white in top color, and dark green, light olive gray, yellowish white or pinkish white in bottom color (Figs. 2C and 2D). *C. gloeosporioides* isolates showed commonly light olive gray in top color, and pinkish or yellowish white in bottom color (Figs. 2E and 2F). However, some isolates such as C0562 and C0624 showed light olive gray or olive in top color, and brown or olive in bottom color (Figs. 2G and 2H).

Conidial shape and size revealed significant differences between *C. acutatum* and *C. gloeosporioides*. Conidia of the *C. acutatum* isolates had fusiform with their both end pointed, sometimes tapering towards one end or rarely cylindrical with both end blunted (Fig. 3A to 3D). Conidial size of the fungus was described variably as 8.3-14.4× 2.5-4 μm (Simmonds, 1965), 8-16×2.5-4 μm (Dyko and Mordue, 1979) and 12.5-20(~22.5)×3-5 μm (Gunnell and Gubler, 1992). Korean isolates of the fungus in conidial size was 7.5-21.7×3.4-7.0 μm, with a mean size range of 11.4-16.5×4.2-4.7 μm (Table 1). This was almost consistent with description of Gunnell and Gubler (1992). Conidial shape of *C. gloeosporioides* isolates had typically cylindrical with both end blunted, rarely tapering towards one end (Fig. 3E to 3H). Conidial size of the fungus was described as 12-22×4-6 μm (von Arx, 1957) and 9-24×3-6 μm (Mordue, 1971). Korean isolates of the fungus in conidial size was variable among the isolates and 9.5-29.8× 3.6-9.1 μm, with a mean range of 14.4-18.0×4.5-6.5 μm. Conidial size of most Korean isolates of *C. gloeosporioides* was consistent with descriptions of previous workers, but size of two isolates C0562 and C0622 remarkably deviated from the range. From the above results, the mean conidial length and width of *C. acutatum* isolates showed smaller than those of *C. gloeosporioides* isolates.

Appressoria produced directly from conidia were investigated (Table 1 and Fig. 3). *C. acutatum* isolates were constantly ovate in shape and 5.3-10.5×4.1-8.5 μm in size, with a mean range of 6.6-8.7×5.1-6.8 μm. Appressoria of *C. gloeosporioides* isolates variable in shape were ovate,

Table 1. Morphology of *C. acutatum* and *C. gloeosporioides* isolates from grapes

Isolate	Origin	Colony			Conidium			Appressorium		
		Diameter[c] (mm)	Color[d]		Shape[f]	Size (μm)		Shape[g]	Size (μm)	
			Top	Bottom		Range	Mean		Range	Mean
Ca[a] C0599	Suwon	48-50	PP (12)[e]	LYO(93)	F (Ta)	10.3-20.3×3.9-5.2	15.7×4.5	OV, CL	6.6-8.5×4.9-6.5	7.7×5.9
C0600	Suwon	48-52	YO(62)	LYR(23)	F (Ta)	10.2-18.1×3.5-6.7	14.6×4.7	OV, CL	6.6-8.7×4.6-6.4	7.7×5.5
C0601	Suwon	55-57	PO(65)	RY(84)	F (C, Ta)	9.1-16.1×3.4-5.9	12.1×4.3	OV, CL	6.7-9.1×4.6-6.5	7.9×5.6
C0608	Suwon	51-63	R(24)	YR(28)	F (OB)	8.5-20.6×3.5-5.0	11.4×4.3	O,V CL	6.8-9.2×5.2-6.5	8.1×5.8
C0609	Suwon	57-60	PP(12)	YR(27)	F	11.6-17.7×4.0-5.4	15.2×4.7	OV, CL	6.3-8.8×5.1-6.8	7.6×6.0
C0612	Suwon	48-61	PO(65)	LYO(93)	F (Ta)	7.5-20.8×3.1-5.9	13.3×4.6	OV, CL	6.7-9.4×5.0-6.4	7.7×5.8
C0616	Suwon	56-60	PP(12)	YR(28)	F (Ta)	9.9-19.0×3.9-5.6	14.8×4.7	OV, CL	6.2-8.0×4.5-6.5	6.9×5.5
C0617	Suwon	58-60	PO(65)	LYO(93)	F (C)	9.1-16.5×3.5-5.4	13.4×4.5	OV, CL	7.3-9.5×6.0-7.5	8.3×6.8
C0618	Suwon	57-60	PP(12)	YR(28)	F	9.7-17.7×3.7-5.4	13.5×4.4	OV, CL	7.4-10.0×5.6-7.4	8.7×6.3
C0619	Suwon	52-54	PO(65)	LYO(93)	F (C, Ta)	8.7-19.1×3.5-5.9	11.8×4.5	OV, CL	7.3-9.7×5.1-7.2	8.4×6.3
C0623	Suwon	44-50	PO(65)	LYO(93) PO(65)	F (Ta)	9.6-17.3×3.6-5.3	14.1×4.6	OV, CL	6.7-9.6×4.8-8.5	8.2×6.3
C0417	Milyang	57-65	LOG (122)	DG(161)	F	9.3-19.0×3.6-5.2	16.0×4.4	OV, CL	6.2-8.4×4.8-7.1	7.4×5.9
C0569	Okcheon	54-60	YW (109)	YW(97)	F (C)	12.0-21.7×3.7-4.8	16.5×4.3	OV, CL	6.2-9.8×4.7-7.5	8.0×5.9
C0570	Daejeon	52-54	LOG(118)	YW(97) PO(65)	F (C, Ta)	10.7-21.5×3.5-5.2	16.0×4.2	OV, CL	6.9-10.5×5.6-7.2	8.6×6.4
C0602	Suwon	56-60	LOG(118)	LOG(122)	F (C)	10.4-15.8×3.8-4.8	12.2×4.3	OV, CL	5.3-8.3×4.2-6.0	6.6×5.1
C0613	Suwon	61-63	YW (109)	YW(97)	F	11.2-18.9×3.7-5.1	14.5×4.4	OV, CL	6.0-8.1×5.0-6.8	6.9×6.0
C0615	Suwon	63-65	LOG (122)	YW(97) LB(59)	F (C, OB)	8.5-21.0×3.6-7.0	12.5×4.7	OV, CL	6.2-8.4×5.0-6.3	7.2×5.8
C0620	Suwon	58-62	YW (109)	YW(97)	C (Ta)	9.7-15.5×3.9-5.9	12.0×4.7	OV, CL	5.9-9.3×4.1-7.1	7.4×5.8
C0621	Incheon	41-57	YW (109)	YW(97) PW(50)	F (C)	11.6-17.2×4.0-5.7	14.5×4.6	OV, CL	6.5-9.1×5.2-7.0	7.3×6.2
Cg[b] C0568	Okcheon	72-77	LOG(122)	YW(97) PW(50)	C (Ta)	12.9-17.1×4.3-5.5	14.9×4.8	OV, CL	8.2-11.2×4.2-7.5	9.5×5.9
C0607	Daegu	75-81	LOG(122)	YW(97) LOG(122)	C	11.7-20.0×4.5-6.7	16.9×5.6	OV, CL, IR	8.3-11.4×5.4-9.3	9.8×6.2
C0614	Suwon	73-82	LOG(122)	YW(97) PW(50)	C (Ta)	11.8-20.0×4.2-6.1	16.2×5.4	OV, CL	8.1-12.8×5.5-9.6	10.0×7.1
C0622	Incheon	80-82	W(293)	YW(97)	C	11.7-29.8×3.8-6.9	18.0×5.1	LC	8.6-16.9×6.4-11.2	12.4×7.9
C0624	Uisung	81-82	LOG(122) GO(120)	O(105)	C (Ta)	10.8-17.9×5.1-8.0	15.3×6.5	CL, IR	8.1-15.2×6.2-8.8	10.6×7.1
C0562	Cheonan	57-61	O(105)	B(74)	C (Ta)	11.4-25.9×4.6-7.1	16.3×5.5	LC	8.0-14.7×5.5-8.1	10.9×7.1
C0563	Cheonan	69-73	W(293)	YW(97) PP(12)	C (Ta)	11.6-18.5×4.5-5.9	15.8×5.4	CL	6.9-12.8×5.1-7.1	8.7×6.1
C0564	Cheonan	57-62	W(293)	YW(97) PW(50)	C	15.0-19.9×4.5-5.8	17.7×5.1	CL, IR	8.6-11.7×5.6-8.6	10.0×7.4
C0566	Cheonan	61-70	W(293)	YW(97)	C	13.0-19.1×3.6-5.2	15.8×4.5	LC, IR	8.3-15.2×5.0-7.7	10.4×6.3
C0610	Suwon	55-58	LOG(122)	PW (50)	C (Ta)	9.5-17.3×4.6-9.1	14.4×5.8	CL	7.3-10.4×5.4-7.7	8.7×6.7
C0611	Suwon	61-63	LOG(122)	YW(97)	C (Ta)	13.4-21.8×3.8-6.4	16.7×5.2	CL	6.7-9.1×5.3-6.9	7.7×6.3

[a]Ca, *C. acutatum*; [b]Cg, *C. gloeosporioides*; [c]colony diameter measured 7 days after inoculation; [d]B, brown; DG, dark green; GO, grayish olive; LB, light brown; LOG, light olive gray; LYO, light yellow orange; LYR, light yellowish red; PO, pale orange; PP, pale pink; PW, pinkish white; O, olive; R, red; RY, reddish yellow; YO, yellowish orange; YR, yellowish red; YW, yellowish white; W, white; [e]a serial number of Munsell renovation color system today's color/300; [f]F, fusiform; C, cylindrical; Ta, tapering toward one end; OB, obtuse; [g]CL, clavate; IR, irregular; LC, long clavate; OV, ovate.

clavate, long clavate or irregular, and their size was 6.7-16.9×4.2-11.2 μm, with a mean range of 7.7-12.4× 6.1-7.9 μm. Accordingly, appressoria of *C. acutatum* and *C. gloeosporioides* isolates were differentiated in shape and size.

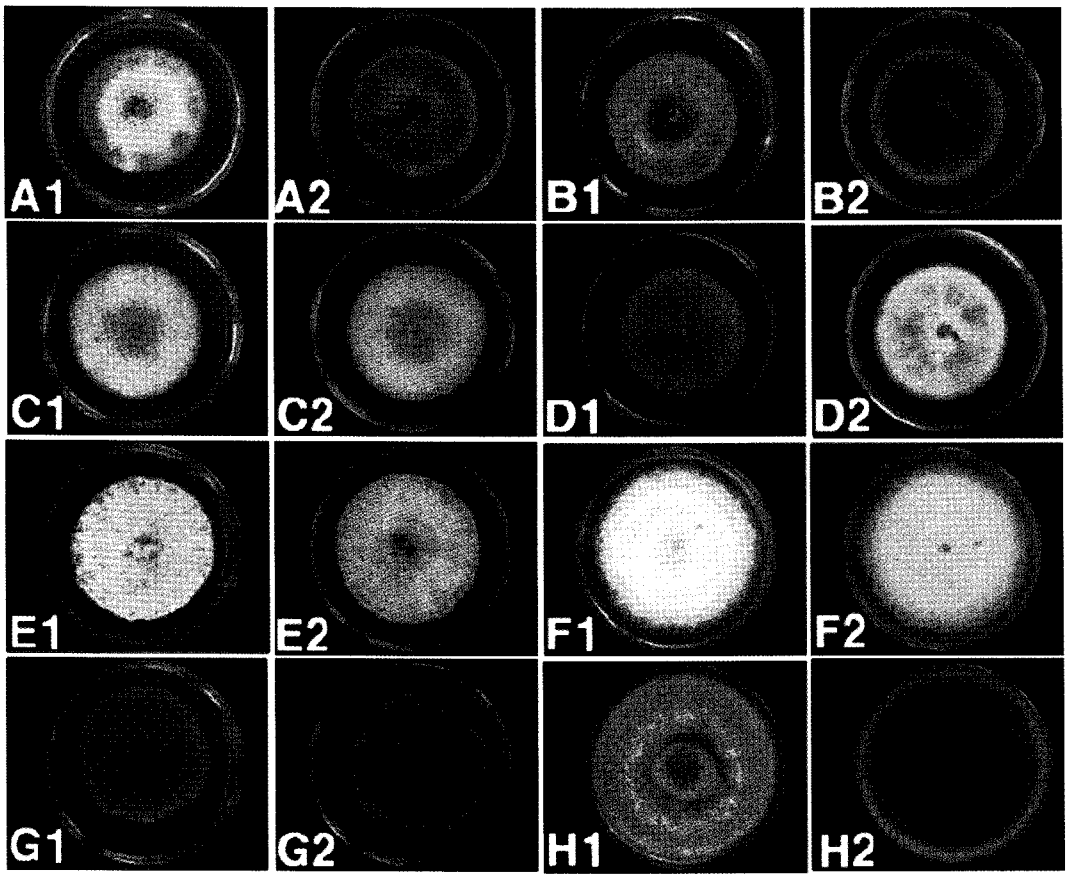

Fig. 2. Colonial appearance of *C. acutatum* and *C. gloeosporioides* isolates from grape on PDA plates incubated in darkness at 27°C for a week. Number 1 and 2 following English alphabet indicate top and bottom of culture, respectively. A and B, chromogenic forms of *C. acutatum* (isolates C0599 and C0609); C and D, non-chromogenic form of *C. acutatum* (isolates C0569 and C0602); E to H, *C. gloeosporioides* (isolates C0564, C0614, C0562, and C0624, respectively).

RAPD-PCR. RAPD-PCR analysis with primer URP-2R generated a sufficient profile to differentiate 19 isolates of *C. acutatum* and eleven isolates of *C. gloeosporioides* (Figs. 4 and 5). *C. acutatum* isolates from the profile were obviously divided to two groups. The one group included eleven isolates of chromogenic and the other group eight isolates of non-chromogenic. *C. gloeosporioides* isolates were also divided to two groups except isolate C0614.

ITS and b-tubulin-2 sequences analysis. Size of entire ITS region for *C. acutatum* and *C. gloeosporioides* isolates was 495 bp and 482 bp, respectively, and yielded 508 aligned nucleotide positions for all species included in the alignment. In all of the species, 173 variable nucleotide sites (34.1%) were observed, and 149 (29.3%) of these were phylogenetically informative. Nucleotide divergence in the ITS sequences between *C. acutatum* and *C. gloeosporioides* was observed up to 10.5%, and that within Korean isolates of the two species up to 2.1% and 1.4%, respectively. Phylogenetic analysis based on ITS sequences clearly separated *C. acutatum* and *C. gloeosporioides* isolates (Fig. 6). The nineteen Korean isolates of *C. acutatum* were assigned to A2 and A3 among the *C. acutatum* groups described by Sreenivasaprasad and Talhinhas (2005). *C. acutatum* group A2 with a bootstrap value of 65% included non-chromogenic isolates, whereas group A3 with a bootstrap value of 91% included chromogenic isolates. The eleven Korean isolates of *C. gloeosporioides* were clustered into two groups except isolates C0568 and C0614. The one group, G1 included three Korean isolates and three isolates of other countries, and the other group, G2 six Korean isolates and one isolate from other country.

Nucleotide sequences of partial β-tubulin-2 gene from *C. acutatum* and *C. gloeosporioides* isolates were 463-467 bp and 466-469 bp, respectively and yielded 472 aligned nucleotide positions for all species included in the alignment. One hundred and ninety six variable nucleotide sites (41.5%) were observed and 170 (36.0%) of these were phylogenetically informative. Nucleotide divergence in the β-tubulin-2 sequences between *C. acutatum* and *C. gloeosporioides* was observed up to 12.5% and that within

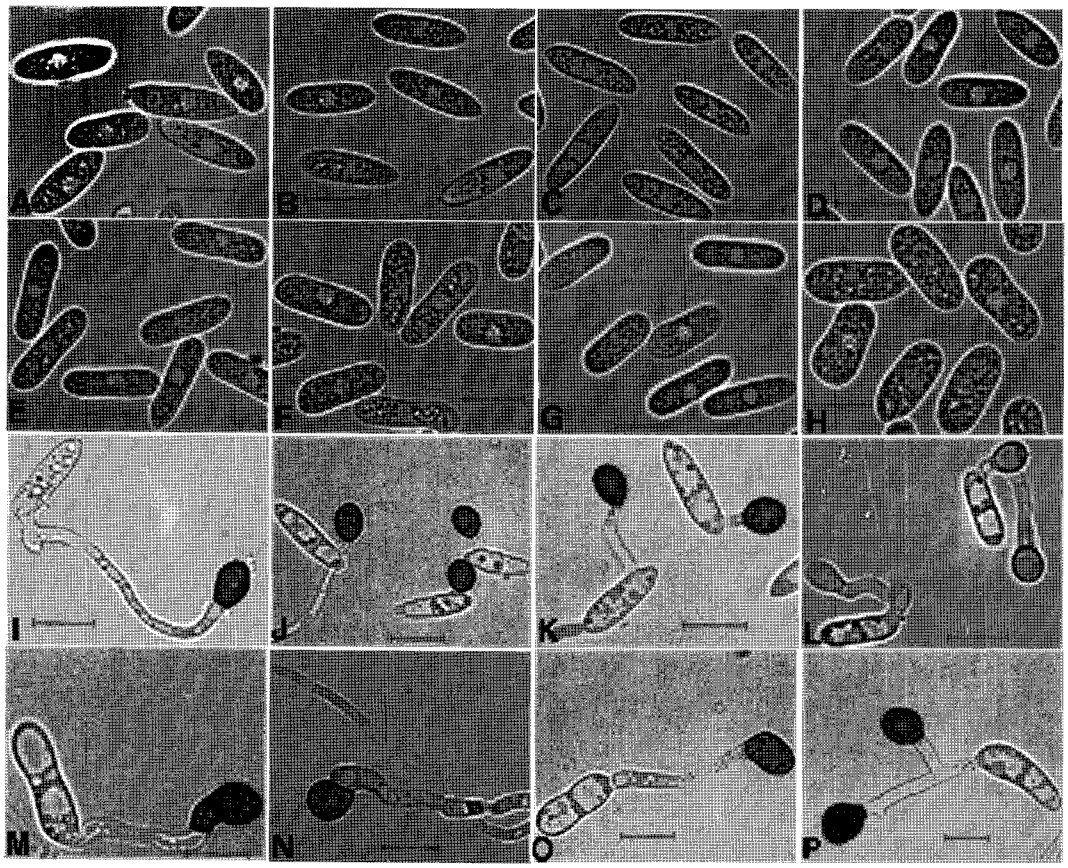

Fig. 3. Morphology of conidia and appressoria of *C. acutatum* and *C. gloeosporioides* isolates from grape. A to D, conidia of *C. acutatum* (isolates C0599, C0609, C 0569 and C0602, respectively); E to H, conidia of *C. gloeosporioides* (isolates C0564, C0614, C0562, and C0624, respectively); I to L, appressoria of *C. acutatum* (isolates C0599, C0609, C 0569 and C0602, respectively); M to P, appressoria of *C. gloeosporioides* (isolates C0564, C0614, C0562, and C0624, respectively). All scale bars represent 10 μm.

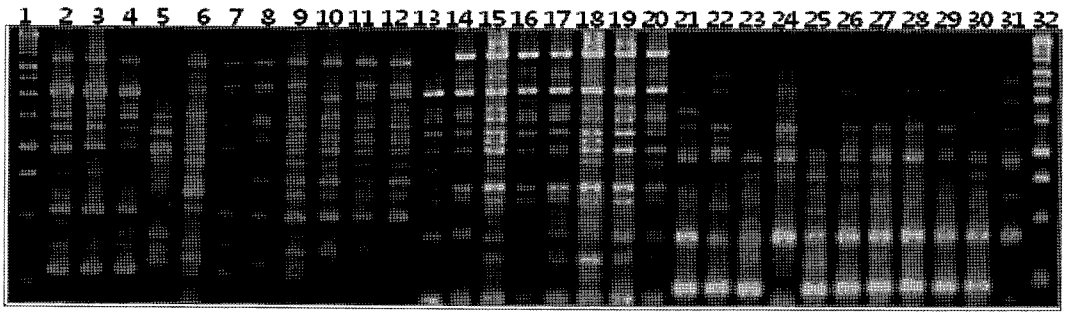

Fig. 4. RAPD-PCR fingerprint profiles obtained from primer URP-2R for *C. gloeosporioides* and *C. acutatum* isolates from grape. Lane 1 and 32, 1 kb DNA ladder; lanes 2-12, *C. gloeosporioides* (isolates C0607, C0622, C0624, C0614, C0562, C0563, C0564, C0566, C0568, C0610, and C0611, respectively); lanes 13-20, non-chromogenic isolates of *C. acutatum* (isolates C0417, C0569, C0570, C0602, C0613, C0615, C0620, and C0621, respectively); lanes 21-31, chromogenic isolates of *C. acutatum* (isolates C0599, C0600, C0601, C0608, C0609, C0612, C0616, C0617, C0618, C0619, and C0623, respectively).

Korean isolates of the two species up to 1.6% and 11.5%, respectively. High divergence of nucleotides within *C. gloeosporioides* isolates was due to isolates C0622 and C0614. β-tubulin-2 tree for Korean isolates of *C. acutatum* further divided groups A2 and A3 into subgroups and produced one group for those of *C. gloeosporioides* except isolates C0622 and C0614 (Fig. 7). β-tubulin-2 sequences in *C. acutatum* isolates showed therefore further improved resolution than the ITS sequences but not in *C. gloeosporioides*. It is interesting that isolate C0608 assigned into *C. acutatum* group A3 in ITS tree is assigned to group A2 in β-tubulin-2 tree.

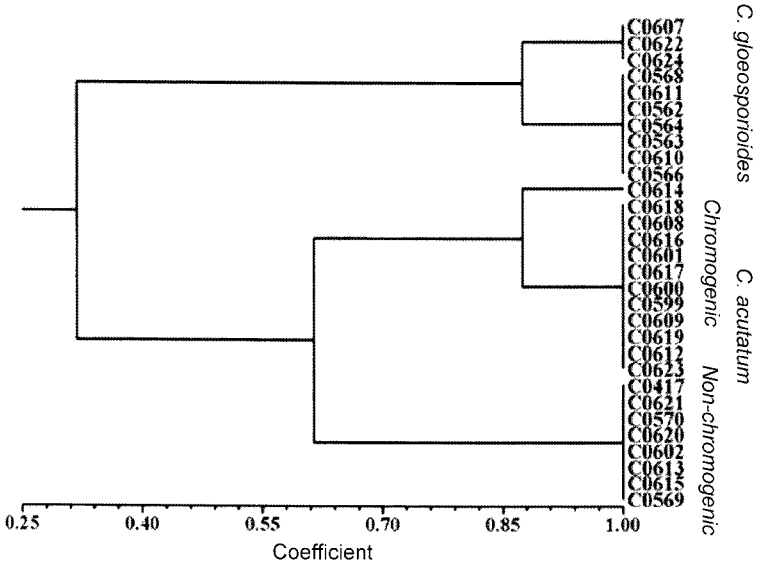

Fig. 5. UPGMA dendrogram using RAPD-PCR polymorphic bands of *C. acutatum* and *C. gloeosporioides* isolates from grape.

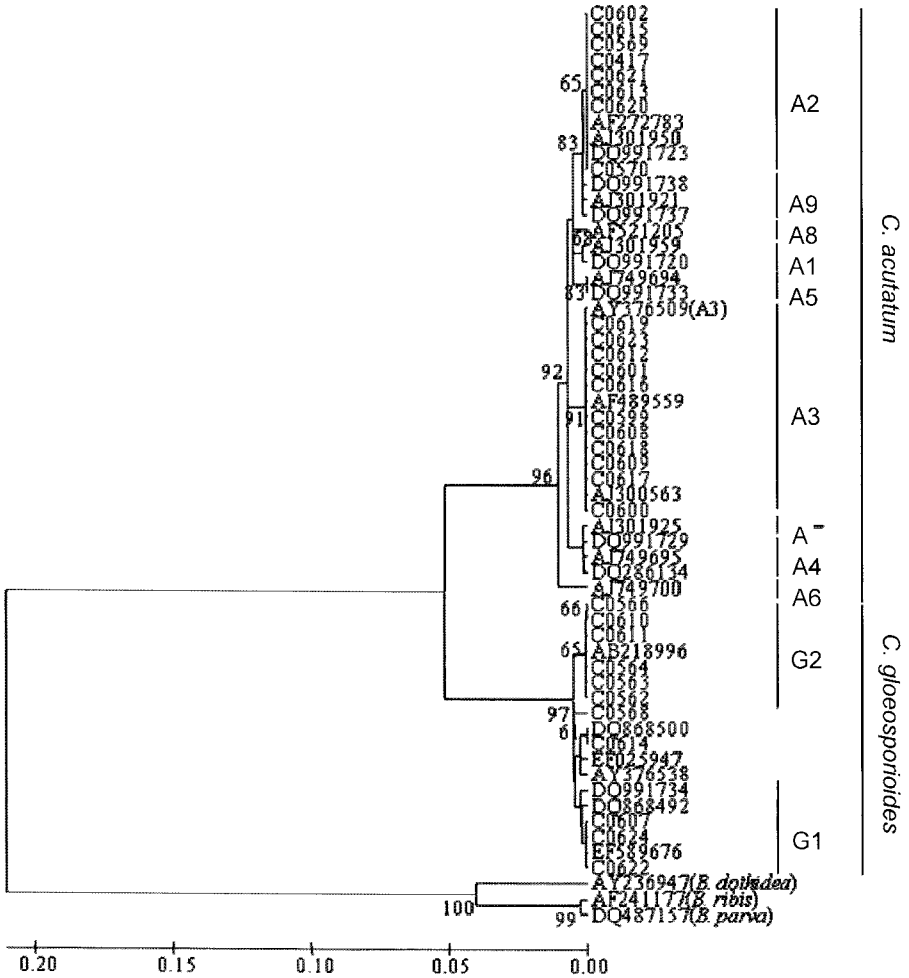

Fig. 6. Phylogenetic tree of *C. acutatum* and *C. gloeosporioides* isolates from grape based on ITS1-5.8S and ITS2 rDNA sequences. The tree was generated using Neighbour-Joining analysis and Tamura-Nei option. The marks at the branch nodes denote bootstrap values more than 60% using 1,000 replications. Marks AJ, AF, AY, DQ and EF are DNA sequence accession number from NCBI GenBank.

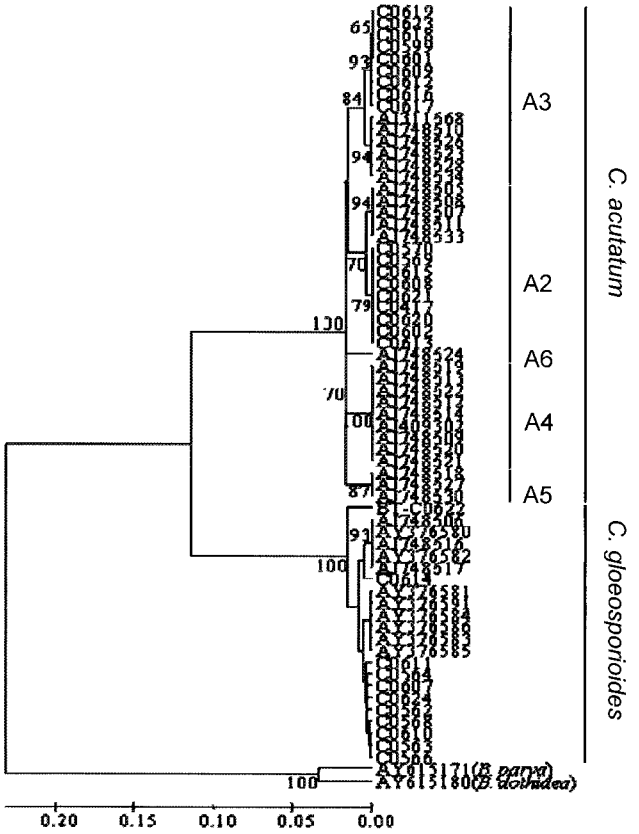

Fig. 7. Phylogenetic tree of *C. acutatum* and *C. gloeosporioides* isolates from grape based on exon 3 to 6 sequences of β-tubulin-2. The tree was generated using Neighbour-Joining analysis and Tamura-Nei option. The marks at the branch node denote bootstrap values more than 60% using 1,000 replications. Marks AJ and AY are DNA sequence accession numbers from NCBI GenBank.

Pathogenicity. Pathogenicity of *C. acutatum* and *C. gloeosporioides* isolates to leaves of grape cultivars was examined. Symptoms first appeared as blackish brown spots two days after inoculation. The spots were expanded more rapidly by the wound inoculation than by the unwound inoculation. Among the *C. acutatum* isolates tested, C0569 and C0570 of group A2 and C0599 of group A3 were strongly pathogenic to cultivars of Campbell Early and Tamnara but weakly pathogenic to MBA (Table 2). C0617 of group A3 was weakly pathogenic to all the varieties tested. No symptom was produced on leaves of unwounded grape and control. Among *C. gloeosporioides* isolates tested, C0564 showed strongly pathogenic to all the varieties tested and unwounded leaves of Tamnara. C0614 of *C. gloeosporioides* was strongly pathogenic to cultivar Campbell Early but weakly pathogenic to cultivars MBA and Tamnara.

Discussion

C. gloeosporioides is known to be a pathogen of grape ripe rot in Korea (Cho and Shin, 2004). However, present study showed that *C. acutatum* as well as *C. gloeosporioides* is a causal fungus of grape ripe rot and widely distributed in the country. The symptoms on grape caused by the two species were not distinguished each other.

C. acutatum has been differentiated from *C. gloeosporioides* based on phenotypic traits such as mycelial growth rate, characters of conidia and appressoria, colony appearance and production of setae (Dyko and Mordue, 1979; Sutton, 1998). It was reported that *C. acutatum* grew slower than *C. gloeosporioides* (Simmonds, 1965; Talhinhas et al., 2002). Korean isolates of the two species usually showed the same tendency in growth rate as above descriptions but some isolates were undistinguishable in growth rates between the two species. Therefore, growth rate could be used as one of characters for differentiation of the two species but not be absolute. Colony color of the two species in culture was very variable. *C. acutatum* isolates could be divided into a chromogenic or non-chromogenic form. This was in agreement with previous reports that colony in *C.*

Table 2. Pathogenicity of *C. acutatum* and *C. gloeosporioides* isolates on grape leaves by artificial inoculation

Colletotrichum species	Isolate	Lesion diameter (mm)[a] on leaves of grape cultivars					
		Campbell Early		MBA		Tamnara	
		Wounded	Unwounded	Wounded	Unwounded	Wounded	Unwounded
C. acutatum	C0569	17.5±3.8	–[b]	3.3±1.0	–	23.3±2.2	–
C. acutatum	C0570	14±6.1		3.3±0.5	–	20.8±3.8	–
C. acutatum	C0599	20.5±1.0	–	8.8±3.4	–	14.3±2.9	–
C. acutatum	C0617	7.5±3.5	–	5.8±2.9	–	5.9±4.9	–
C. gloeosporioides	C0564	15.7±1.5	7.0±1.2	14.5±3.8	–	17.8±3.0	19.5±5.0
C. gloeosporioides	C0614	19.5±3.8	–	5.8±1.7	–	3.3±4.6	–
Control		–	–	–	–	–	–

[a]Lesion diameter was measured five days after inoculation. The data represent means±standard deviations of three replicates. [b]–, no symptom.

acutatum populations had different colors (Lardner et al., 1999; Baxter et al., 1992).

Unlike *C. gloeosporioides* conidia with cylindrical form, conidial shape of *C. acutatum* is fusiform or acuminate at least on one end (Simmonds, 1965; Dyko and Mordue, 1979; Sutton, 1998). Most Korean isolates of the fungus showed similar conidial shape and size to the description by Gunnell and Gubler (1992) but fell outside that of Simmonds (1965). Some Korean isolates of *C. acutatum* such as C0602 sometimes showed intermediate conidial form of the two species and resulted in difficult to identify the species. Shape and size of appressoria has been used for taxonomy of the genus *Colletotrichum* (Sutton, 1998). Korean isolates of *C. acutatum* showed slightly smaller appressoria than *C. gloeosporioides*, corresponding to description of Sutton (1998). The shape of appressoria of *C. acutatum* isolates showed always ovate, whereas that of *C. gloeosporioides* isolates were variable. Therefore, appressoria was one of the features to differentiate the two species but difficult to set up a standard key for identification of the species.

Molecular tools were evaluated for the ability to differentiate *C. acutatum* and *C. gloeosporioides*, and to clarify genetic variation within isolates of the two species. RAPD-PCR analysis using primer URP-2R could separate *C. acutatum* and *C. gloeosporioides*, and divide into two clusters for isolates of the two species, respectively. This result was largely consistent with that of phylogenetic analysis of ITS and β-tubulin-2 sequences. This indicates that RAPD-PCR for *C. acutatum* and *C. gloeosporioides* isolates had a resolution comparable to ITS and β-tubulin-2 sequences, and provided information enough to represent intra-specific variation of the two species. However, ITS and β-tubulin-2 sequences have an advantage that RAPD-PCR clusters can be assigned to known groups of *Colletotrichum* species using comparative analysis with sequences from GenBank database. A phylogenetic tree of ITS sequences showed that Korean isolates of *C. acutatum* were clustered into groups of A2 and A3 among the eight global groups of Sreenivasaprasad and Talhinhas (2005). Sequences of β-tubulin-2 also produced a phylogenetic tree similar to those of ITS but Korean isolates within the A2 and A3 groups were further divided into subgroups different from published sequences of GenBank database. Therefore, the sequences of β-tubulin-2 had a better resolution than ITS sequences, and could reflect geographic origins. Korean isolates of group A2 did not produce pigment in culture but pink or red pigment for those of group A3. This did not fit with Sreenivasaprasad and Talhinhas (2005), who reported pink and red pigmentation in culture for group A3 and A5 isolates, respectively. This showed that colors of pigment in culture might not be always correlated with specific molecular groups of *C. acutatum*. The global groups of *C. acutatum* isolates from grape were A2, A3, A4, A5 and A9 (Sreenivasaprasad and Talhinhas, 2005; Whitelaw-Weckert et al., 2007). In Korea, it was reported that only group A2 and A3 of *C. acutatum* was found on apple and pepper (Kim et al., 2006; Lee et al., 2007), suggesting that *C. acutatum* isolates distributed in Korea represent a low level of genetic diversity.

ITS tree for *C. gloeosporioides* isolates produced two groups as in RAPD-PCR except isolate C0568. However, β-tubulin-2 tree for the majority of Korean isolates produced one group except for independent positioning of isolates C0614 and C0622. These results showed that ITS sequences of *C. gloeosporioides* unlike *C. acutatum* had a better resolution for genetic diversity than β-tubulin-2 sequences. However, further study is needed to clarify whether one of these has better resolution.

Pathogenicity test for *C. acutatum* and *C. gloeosporioides* isolates revealed that *C. acutatum* isolates were as virulent as *C. gloeosporioides* isolates on the leaves of grape. Both *C. acutatum* and *C. gloeosporioides* were therefore confirmed as pathogens responsible for grape ripe rot in Korea. However, there was difference in aggressiveness among isolates of the two species and disease resistance among grape cultivars. Therefore, it is desired that several isolates of *C. acutatum* and *C. gloeosporioides* should be used to test resistance of grape cultivars to the pathogens.

References

von Arx, J. A. 1957. Die Arten der Gattung *Colletotrichum* Cda. *Phytopathogische Zeitschrift* 29:413-468.

Baxter, A. P., van der Westhuizen, G. C. A. and Eicker, A. 1983. Morphology and taxonomy of South African isolates of *Colletotrichum*. *S. Afr. J. Bot.* 2:259-289.

Cho, W. D. and Shin, H. D. eds. 2004. *List of plant diseases in Korea*. Fourth edition. The Korean Society of Plant Pathology. Suwon, Korea. 779 pp. (In Korean).

Daykin, M. E. and Milholland, R. D. 1984. Ripe rot of muscadine grape caused by. *Colletotrichum gloeosporioides* and its control. *Phytopathology* 74:710-714.

Dyko, B. J. and Mordue, J. E. M. 1979. *Colletotrichum acutatum*. *CMI Descriptions of Pathogenic Fungi and Bacteria*. No. 630. C.M. I., Kew, U.K.

Glass, N. L. and Donaldson, G. 1995. Development of primer sets designed for use with PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61:1323-1330.

Gunnell, P. S. and Gubler, W. D. 1992. Taxonomy and morphology of *Colletotrichum* species pathogenic to strawberry. *Mycologia* 84:157-165.

Johnston, P. R. and Jones, D. 1997. Relationships among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. *Mycologia* 89:420-430.

Johnston, P. R. 2000. The importance of phylogeny in understanding host relationships within *Colletotrichum*. *In*: *Colletotrichum. Host specificity, pathology and host-pathogen interaction*. Prusky, D., Freeman, S. and Dickman eds. APS press, St. Paul, MN, USA.

Kang, H. W., Park, D. S., Go, S. J. and Eun, M. Y. 2003. Fingerprinting of diverse genomes using PCR with universal rice primers generated from repetitive sequence of Korean weedy rice. *Mol. Cells* 13:1-7.

Kim, D. H., Jeon, Y. A., Go, S. J., Lee, J. K. and Hong, S. B. 2006. Reidentification of *Colletotrichum gloeosporioides* and *C. acutatum* isolates stored in Korea Agricultural Cultural Collection (KACC). *Res. Plant Dis.* 12:168-177. (In Korean).

Kim, J. T., Park, S. Y., Choi, W. C., Lee, Y. H. and Kim, H. T. 2008. Characterization of *Colletotrichum* isolates causing anthracnose of pepper in Korea. *Plant Pathol. J.* 24:17-23.

Kim, S. H., Choi, S. Y., Lim, Y. S., Yoon, J. T. and Choi, B. S. 2001. Etiological characteristics and chemical control of ripe rot in grape cultivar Campbell Early. *Res. Plant. Dis.* 7:140-144 (In Korean).

Kim, W. G. and Hong, S. K. 2008. Occurrence of anthracnose on peach tree caused by *Colletotrichum* species. *Plant Pathol. J.* 24:80-83.

Kim, W. G., Hong, S. K. and Park, Y. S. 2007. Occurrence of anthracnose on fruits of Asian pear tree caused by *Colletotrichum acutatum*. *Mycobiology* 35:238-240.

Lardner, R., Johnston, P. R., Plummer, K. M. and Pearson, M. N. 1999. Morphological and molecular analysis of *Colletotrichum acutatum sensu lato*. *Mycol. Res.* 103:275-285.

Lee, D. H., Kim, D. H., Jeon, Y. A., Uhm, J. Y. and Hong, S. B. 2007. Molecular and cultural characterization of *Colletotrichum* spp. causing bitter rot of apples in Korea. *Plant Pathol. J.* 23:37-44.

Martinez-Culebras, P. V., Querol, A., Suarez-Fernandez, M. B., Garcia-Lopez, M. D. and Barrio, E. 2003. Phylogenetic relationships among *Colletotrichum* pathogens of strawberry and design of PCR primers for their identification. *J. Phytopathology.* 151:135-143.

Melksham, K. J., Weckert, M. A. and Steel, C. C. 2002. An unusual bunch rot of grapes in sub-tropical regions of Australia caused by *Colletotrichum acutatum*. *Australas. Plant Pathol.* 31:193-194.

Mordue, J. E. M. 1971. *Colletotrichum gloeosporioides. CMI Descriptions of Pathogenic Fungi and Bacteria.* No. 315. C.M.I. Kew, U.K.

Rohlf, E. J. 1993. NTSYS-pc: numerical taxonomy and multivariate analysis system, version 1.80. Applied Biostatistics Inc., Setauket, New York.

Simmonds, J. H. 1965. A study of the *Colletotrichum* causing ripe fruit rots in Queensland. *Queensl. J. Agr. Anim. Sci.* 22:437-459.

Southworth, E. A. 1891. Ripe rot of grapes and apples. *J. Mycol.* 6:64-173.

Sreenivasaprasad, S. and Talhinhas, P. 2005. Genotypic and phenotypic diversity in. *Colletotrichum acutatum*, a cosmopolitan pathogen causing anthracnose on a wide range of hosts. *Mol. Plant Pathol.* 6:361-378.

Sutton, B. C. 1998. *The Coelomycetes*. Fungi imperfecti with pycnidia, acervuli and stromata. CMI, U.K. 696 pp.

Talhinhas, P. Sreenivasaprasad, S., Neves-Martins, J. and Oliveira, H. 2002. Genetic and morphological haracterization of *Colletotrichum acutatum* causing anthracnose of lupins. *Phytopathology* 92:986-996.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596-1599.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nuc. Acids Res.* 22:4673-4680.

Whitelaw-Weckert, M. A., Curtin, S., Huang, R., Steel, C. C., Blanchard, C. L. and Roffey, P. 2007. Phylogenetic relationships and Pathogenicity of grape isolates of *Colletotrichum acutatum* in sub-tropical Austrialia. *Australas. Plant Pathol.* 56:448-463.

Yamamoto, J., Sato, T. and Tomioka, K. 1999. Occurrence of ripe rot of grapes (*Vitis vinifera* L.) caused by *Colletotrichum acutatum* Simmonds ex Simmonds. *Ann. Phytopathol. Soc. Jpn.* 65:83-86.