

Identification and Characterization of *Colletotrichum* Species Associated with Bitter Rot Disease of Apple in South Korea

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Bitter rot caused by *Colletotrichum* species is a common fruit rotting disease of apple and one of the economically important disease in worldwide. In 2015 and 2016, distinct symptoms of bitter rot disease were observed in apple orchards in five regions of South Korea. In the present study, infected apples from these regions were utilized to obtain eighteen isolates of *Colletotrichum* spp. These isolates were identified and characterized according to their morphological characteristics and nucleotide sequence data of internal transcribed spacer regions and glyceraldehyde-3-phosphate-dehydrogenase. Molecular analyses suggested that the isolates of *Colletotrichum* causing the bitter rot disease in South Korea belong to 4 species: *C. siamense*; *C. fructicola*; *C. fioriniae* and *C. nymphaeae*. *C. siamense* and *C. fructicola* belonged to Musae Clade of *C. gloeosporioides* complex species while *C. fioriniae* and *C. nymphaeae* belonged to the Clade 3 and Clade 2 of *C. acutatum* complex species, respectively. Additionally, we also found that the isolates of *C. gloeosporioides* species-complex were more aggressive than those in the *C. acutatum* species complex via pathogenicity tests. Taken together, our results suggest that accurate identification of *Colletotrichum* spp. within each species complex is required for management of bitter rot disease on apple fruit in

South Korea.

Keywords : apple, bitter rot, *Colletotrichum*, pathogenicity

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The apple tree (*Malus pumila*, commonly known as *Malus domestica*) is a deciduous tree belonging to the family Rosaceae, and apples are the third most traded fruit internationally followed by bananas and grapes (Lynch, 2010). Apple cultivation originated in Central Asia and with time has spread worldwide. Apple is best known for their sweet taste and is extremely rich in important dietary fibers, antioxidants, and flavonoids. The antioxidants and flavonoids in apples help in reducing the risk of developing hypertension, heart disease, cancer and diabetes (Boyer and Liu, 2004). In South Korea, apple is grown in a large amount with the expected cultivation area of 33,600 ha in 2017/2018 and has played a critical role in Korean economic development (Choi and Hinkle, 2017). The major varieties of apples grown in Korea are Tsugaru (Aori) (harvested in August), Hongro (harvested in September), Fuji (harvested from October to November), Yang Kwang (harvested from September to October), and Kam Hong (harvested in October) (Choi and Hinkle, 2017). However, numerous destructive pathogens infect apple trees and cause significant yield loss in apple production worldwide (Munir et al., 2016; Sutton, 1990).

Among these diseases, bitter rot caused by *Colletotrichum* species is one of the important apple fruit diseases in worldwide and causes significant yield losses to the apple producers (Munir et al., 2016; Uhm, 2010). The symptom of apple bitter rot initially appears as a small circular lesion on fruits that are light to dark brown in color. On mature fruits, these areas may be surrounded by a red halo (Beever et al., 1995; Struble et al., 1950). Under favorable condi-

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tions, these lesions subsequently enlarge rapidly and cover the entire fruit surface. As the spots enlarge, the fruit rot symptoms can vary depending on the fungal isolate and the type of spore that initiates infection. Spots initiated by isolates that only produce conidia are sunken and circular with concentric rings (Beever et al., 1995; Struble et al., 1950). Within the rings, copious amounts of conidia are produced in fruiting bodies called acervuli. Under moist, humid conditions, the spore masses appear creamy and are salmon to pink in color. Spots initiated by isolates that produce conidia and ascospores are usually not sunken and are darker brown in color than spots produced by conidial isolates. Within the spots, structures that contain ascospores (perithecia) form in black clumps over the surface (Beever et al., 1995; Struble et al., 1950). As spots enlarge, the rot progresses inward towards the core. After making a cross-section of the infected fruit a V-shaped, brown watery lesion can be observed (Velho, 2014). This is a key diagnostic feature of bitter rot, which distinguishes it from other fruit rots disease. Moreover, the bitter rot pathogens overwinter as perithecia and acervuli in mummified apples, and as mycelia in colonized dead wood, unpruned branches and cuttings that are left on the ground (Sutton, 1990). Without proper disease management, bitter rot can destroy an entire fruit crop in just a few weeks during periods of warm, wet weather (Sutton, 1990).

Genus *Colletotrichum* is one of the most important pathogenic fungi causing economically significant diseases on the fruits and leaves of a wide range of subtropical, tropical, and temperate crops, fruits, and ornamental plants (Cannon et al., 2012; Sutton, 1992). Moreover, genus *Colletotrichum* is a cosmopolitan fungal genus and comprises 11 major clades (Jayawardena, 2016). Some *Colletotrichum* species have wide host ranges, while others can infect only a single host species (Damm et al., 2012; Weir et al., 2012). Diverse species of *Colletotrichum* can cause bitter rot diseases in apples. A previous report demonstrated that two species of *C. gloeosporioides* and *C. acutatum* are the most common species causing bitter rot disease on apple fruit in worldwide (González et al., 2006). In South Korea, *C. gloeosporioides* and *C. acutatum* were also recorded as causal pathogens for bitter rot on apples (KSPP, 2009; Lee et al., 2007). Additionally, Park et al. (2018) reported two new species of *C. siamense* and *C. fructicola* as causal agents of bitter rot disease of apple in South Korea. However, their identification method using morphological characteristics may not provide reliable characterization because there are no definite morphotaxonomic characters on these species (Bernstein et al., 1995; Cai et al., 2009). Therefore, the objective of this study was to identify *Colle-*

totrichum species associated with bitter rot disease of apple in South Korea and to characterize and compare the pathogenicity of the isolated species.

Materials and Methods

Sampling and isolation of the isolated fungus. In 2015 and 2016, symptomatic fruits were collected from some private orchards in different locations of South Korea. Samples of fruits affected by typical disease symptoms were washed with water and dried in a laminar air flow chamber. After surface sterilization, infected fruits tissues were cut into 5 mm pieces and disinfected by dipping in 1% Sodium hypochlorite (NaClO) for 3 minutes, rinsed 3 times with sterile distilled water, and dried on sterilized tissue paper (Nam et al., 2013). After drying, the samples were cultured on 90- mm Petri dishes containing blotter paper and incubated at room temperature ($25 \pm 2^\circ\text{C}$) for 12 photoperiods. After 2-3 days, spore layers were picked with a smear loop or autoclaved toothpick and mixed with 1ml of sterilized distilled water supplement with Streptomycin (300 ppm for 1 l) to prevent the bacteria contamination. After mixing with this solution and spore, the mixture was then spread on Water Agar culture medium (containing Streptomycin 300 ppm for 1 l) for 2-3 days at room temperature ($25 \pm 2^\circ\text{C}$) (Prihastuti et al., 2009). Two or 3 days after isolation, a single isolated spore of emerging fungi was picked from the sporulating colonies and transferred to new potato dextrose agar (PDA; Difco, Sparks, MD, USA) plates to obtain the pure culture and were further grown for DNA extraction, pathogenicity tests and morphological characterization. The culture plates were checked every day with naked eyes for measuring hyphal growth.

Morphological characterization. To examine the morphological characteristics, fungal species were cultured on PDA and V8 juice media at $25 \pm 2^\circ\text{C}$ in the darkness for 7 days. Small discs (5 mm diameter) of mycelium plugs were aseptically punched from actively sporulating areas of 4 - 5 days old culture and transferred to the center of V8 juice and PDA media plate and then incubated at room temperature ($25 \pm 2^\circ\text{C}$). Three cultures of every isolates were investigated. After 7 days, colony color, size, texture, and zonation of every culture were recorded from V8 juice and PDA media. Colony colors were named according to the mycological color chart (Rayner, 1970). Colony diameter (mm) was measured and calculated based on the average of the perpendicular diameter and the data were examined to compute the mycelial growth rate (mmd^{-1}). The sporulating pattern was analyzed under a Olympus BX40 dissecting

microscope (Olympus optical Co., Ltd, Japan). Conidia of every isolate were characterized using conidia from 10 days old colonies on PDA media, mounted in a drop of lactophenol and examined using Olympus BX50 Fluorescence Microscope with digital camera (Olympus optical Co., Ltd, Japan), at least 24 conidia were measured for each isolate (Cappuccino and Sherman, 2001).

Appressoria were produced by a slide culture technique (Sutton, 1980). For that, small square of PDA media was cut into $10 \times 10 \text{ mm}^2$ pieces and settled on vacant Petri dish. A small spore fragment taken from a sporulating fungal culture was distributed on the surface of this small square piece of PDA media and promptly concealed with a cover slip. After 14 days, the cover slip which formed the appressoria across the underside of the cover slip and media was detached and placed on a drop of Lacto phenol on a glass slide. Approximately 20-30 appressoria were microscopically monitored for each isolate.

DNA extraction, PCR amplification, and sequencing.

All the selected fungal isolates were grown on V8 juice media for 7 days and genomic DNA was extracted using a protocol as described in Cenis (1992) with minor modifications. The mycelia mat of each isolate were scraped with glass slides and transferred to 1.5 ml Eppendorf tubes. Three hundred microliters of extraction buffer (250 mM NaCl, 200 mM Tris HCl pH 8.5, 0.5% SDS, 25 mM EDTA) was added to fungi filled E-tubes and ground with an autoclavable plastic pestle (8.5 cm). Afterwards, 150 μl of 3 M $\text{C}_2\text{H}_3\text{NaO}_2$ (Sodium acetate, pH 5.2) was added and tubes were placed at -20°C for about 10 min. Tubes were then centrifuged in a microfuge at 13,000 rpm for 5-10 minutes and the supernatants were transferred to other tubes. Thereafter, an equal volume of isopropanol (isopropyl alcohol) was added and kept for at least 5 min at room temperature. The tubes were placed on ice for 10 minutes and then precipitated DNA was pelleted by centrifugation at 13,000 rpm for 20 minutes. After a wash with 500 μl of 70% ethanol for 3 minutes centrifugation, the pellet was vacuum dried for 5 minutes and dissolved in 50 μl of DW (sterilized distilled water) and stored at -20°C until further use. A sample of each DNA isolate was confirmed by visualizing under ultraviolet illumination with gel electrophoreses and DNA sizes were compared with standard marker.

To confirm the morphological characterization, genomic region were amplified using; an internal transcribed spacer (ITS) rDNA region using universal primers ITS1 and ITS4 (White et al., 1990) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene using primers GDF and GDR (Templeton et al., 1992) respectively. PCR amplification

was accomplished using 20 μl reaction volume containing 1 μl of each primer, 1 μl of Genomic DNA (100 ng) and sterilized distilled water. Negative controls containing the same reagents but without DNA template were also involved in all PCR reactions. The amplicons were analyzed by running the samples on a 1% ethidium bromide (EtBr)-stained agarose gels in TAE buffer and the DNA bands were visualized in a UV transilluminator. The PCR products were then purified and directly sequenced with the same primers by a commercial sequencing service provider (Macrogen, Daejeon, South Korea). For all these isolates, sequencing was carried out in both forward and reverse direction to avoid errors. Homology searches for resulting sequences were performed by comparing with those deposited in GenBank Nucleotide database hosted by the NCBI (National Center for Biotechnology Information) using BLAST (Boratyn et al., 2013) and obtaining forward and reverse sequences which were aligned with T-coffee Multiple Sequence Alignment program. All the sequences generated in this study were deposited in GenBank and assigned accession numbers.

Phylogenetic analysis. The phylogenetic analysis was accomplished with multilocus alignment with two combined genes (ITS and GAPDH) using MEGA 7.0 software (Kumar et al., 2016). Sequence similarity searches were performed using the nucleotides BLAST program (<http://blast.nih.gov/Blast.cgi>) and sequence alignment was manually done with closely related reference sequences of other isolated *Colletotrichum* species available in NCBI database using ClustalW2 software (<http://www.ebi.ac.uk/Tools/phylogeny/clustalw2-phylogeny/>). The reliability of the evolutionary relations in this tree was evaluated with 1,000 bootstrap replications to determine the percentage for each clade. The sequence of *C. orbiculare* was used as an outgroup in all analyses.

Pathogenicity test. All 18 resultant isolates were tested on two apple cultivars; Tsugaru (Aori) cultivar and Fuji cultivar. For preparation of spore suspensions, the selected isolates were incubated on V8 juice agar media at $25 \pm 2^\circ\text{C}$ in the dark for 7 days, as V8 juice media enables more rapid growth and conidia production than PDA media. To obtain the inoculum, the culture was flooded with 10 ml of sterile distilled water and the surface was slightly scraped by microscope glass slide. The suspension was then filtered through two layers of muslin cloth and the conidia concentration was examined and adjusted to 10^6 conidia/ml using a hemocytometer. For fungal pathogenicity tests, the 18 isolated fungi were inoculated on healthy fruits by wound/

drop method at the middle of each fruit and 2 apple cultivars (Aori and Fuji) were used.

For each isolate, 3 healthy fruits were rinsed with tap water, sterilized with 1% sodium hypochlorite (NaClO) solution for 3 minutes and rinsed twice with distilled water. After surface sterilization, the fruits were dried with sterilized tissue paper and placed on a plastic box (28 × 20 × 9 cm; length × width × height) with 3 fruits per box. Then, the apple fruits were pinpricked to 1 mm depth at the middle of each fruit and 10 µl spore suspension (1 × 10⁶ conidia/ml) was pipetted on this wounded spot. All the control fruits were treated with 10 µl of sterilized distilled water. To develop the disease, inoculated fruits were secured in plastic containers lined with wet paper towels and incubated for 14 days at 25°C in constant light. Three fruits were selected per isolate in each cultivar and the experiment was performed twice. Containers were opened once a day to check the development lesions and lesion diameters were measured. Fourteen days after inoculation (DAI), the fruits were cut through the centers of lesions to observe the

V-shape symptoms and measure of lesion depth. Isolates were considered pathogenic when the disease lesion area progressed over a 5 mm diameter.

To achieve the Koch's postulates, the developing symptoms on the inoculated fruits were compared with original symptoms which were observed in the field and the fungi were re-isolated from inoculated fruits following the above method.

Results

Isolates obtained from infected fruits. A total of 18 *Colletotrichum* isolates were obtained from typical symptomatic fruits acquired from different apple orchards in South Korea (Table 1). From 7 day old colonies of these 18 isolates, *Colletotrichum* spp. were segregated into 4 morphological subtypes (Type 1, 2, 3 and 4) according to colony characteristics and conidial shape; type 1, 2, 3 and 4, respectively comprised of 3, 2, 8, and 5 isolates. These 4 types shared resemblance to the morphological character-

Table 1. Sources of isolates used in this study and GenBank accession number of *Colletotrichum* spp. isolated from apple fruits

Isolates	Morphotype	Host	Species	Location	GenBank Accession Number	
					ITS	GAPDH
CNU160021	Type 1	<i>Malus domestica</i>	<i>C. fioriniae</i>	Gyeongbuk Andong, Korea	MG751877	MG751880
CNU160022	Type 1	<i>Malus domestica</i>	<i>C. fioriniae</i>	Gyeongbuk Cheongsong, Korea	MG751878	MG751881
CNU160023	Type 1	<i>Malus domestica</i>	<i>C. fioriniae</i>	Gyeongbuk Andong, Korea	MG751879	MG751882
CNU160031	Type 2	<i>Malus domestica</i>	<i>C. nymphaeae</i>	Andong, Korea	MG751883	MG751885
CNU160032	Type 2	<i>Malus domestica</i>	<i>C. nymphaeae</i>	Andong, Korea	MG751884	MG751886
CNU160011	Type 3	<i>Malus domestica</i>	<i>C. siamense</i>	Andong, Korea	MG751897	MG751905
CNU160012	Type 3	<i>Malus domestica</i>	<i>C. siamense</i>	Andong, Korea	MG751898	MG751906
CNU160013	Type 3	<i>Malus domestica</i>	<i>C. siamense</i>	Yesan, Korea	MG751899	MG751907
CNU160014	Type 3	<i>Malus domestica</i>	<i>C. siamense</i>	Andong, Korea	MG751900	MG751908
CNU160015	Type 3	<i>Malus domestica</i>	<i>C. siamense</i>	Mungyeong, Korea	MG751901	MG751909
CNU160016	Type 3	<i>Malus domestica</i>	<i>C. siamense</i>	Yesan, Korea	MG751902	MG751910
CNU160017	Type 3	<i>Malus domestica</i>	<i>C. siamense</i>	Andong, Korea	MG751903	MG751911
CNU160018	Type 3	<i>Malus domestica</i>	<i>C. siamense</i>	Gyeongbuk, Korea	MG751904	MG751912
CNU160001	Type 4	<i>Malus domestica</i>	<i>C. fruticicola</i>	Andong, Korea	MG751887	MG751892
CNU160002	Type 4	<i>Malus domestica</i>	<i>C. fruticicola</i>	Jeonbuk, Korea	MG751888	MG751893
CNU160003	Type 4	<i>Malus domestica</i>	<i>C. fruticicola</i>	Andong, Korea	MG751889	MG751894
CNU160004	Type 4	<i>Malus domestica</i>	<i>C. fruticicola</i>	Andong, Korea	MG751890	MG751895
CNU160005	Type 4	<i>Malus domestica</i>	<i>C. fruticicola</i>	Gyeongbuk Cheongsong	MG751891	MG751896
CBS125396	-	<i>Malus domestica</i>	<i>C. fioriniae</i>	USA	JQ948299	JQ948629
CBS100064	-	<i>Anamone</i> sp.	<i>C. nymphaeae</i>	Netherlands	JQ948224	JQ948554
CBS125379	-	<i>Hymenocallis americana</i>	<i>C. siamense</i>	China	JX010258	JX010060
CBS125395	-	<i>Theobroma cacao</i>	<i>C. fruticicola</i>	Panama	JX010172	JX009992
CBS126521	-	<i>Anamone</i> sp.	<i>C. acutatum</i>	Netherlands	JQ948366	JQ948697
CBS112999	-	<i>Citrus sinensis</i>	<i>C. gloeosporioies</i>	Italy	JX010152	JX010056
CBS570.79	-	<i>Cucumis sativus</i>	<i>C. orbiculare</i>	UK	KF178466	KF178490

Table 2. Description of morphological characters of isolated *Colletotrichum* species based on colony character, conidial shape, and appressoria

Morphotype	Colony on PDA media		Colony on V8 Juice media		Conidia			Appressoria	
	Characters	Growth rate (mm/wk)	Characters	Growth rate (mm/wk)	Length (µm)	Width (µm)	Shape	Length (µm)	Width (µm)
Type 1	Slightly yellow pigment in culture	29-31	Pale wine pigment in culture	40-45	9.5-17	4-5.5	Fusiform with pointed ends	6-12	4.5-7
Type 2	white mycelium with light orange conidial masses	32-35	Pale grey mycelium	41-44	10-18	4-6	Fusiform with pointed ends	5-13	3.5-7.5
Type 3	Light olivaceous grey mycelia	45-48	Dark grey mycelium	Above 60	10-15.5	4-6.2	Cylindrical with rounded ends	6-12	4-7
Type 4	Cottony, grey with abundant mycelia	46-48	Milky fluffy mycelium	Above 60	10.5-16	3.5-5	Cylindrical with rounded ends	5.5-11	3.5-7.5

istics of 2 *Colletotrichum* species that have been frequently correlated with the bitter rot disease of apple, *C. acutatum* and *C. gloeosporioides*.

Morphological characteristics. Measurement and appearance of colony characteristics on PDA and V8 juice were described in Table 2. Isolates belonging to Type 1 subtype produced pale yellow colored pigmentation with orange clusters of conidial masses which were submerged in mycelia on PDA. Colonies on V8 juices were smoky grey on the front side and purplish grey on the reverse side. Conidia were $9.5-17 \times 4-5.5 \mu\text{m}$ and fusiform in shape with both ends pointed or slightly acute. Appressoria were clavate to irregular in outline, brown colored, and $6-12 \times 4.5-7 \mu\text{m}$. The conidial shapes of the isolates in Type 1 resembled those of *C. acutatum*.

Type 2 isolates formed white to grey-colored mycelia with orange-colored conidial masses on PDA media. Colonies on V8 juices appeared white to pale grey-colored on the top and pale orange on the underside. Conidia were $10-18 \times 4-6 \mu\text{m}$, mostly fusiform in shape, but some were obclavate (both ends pointed or one acute \pm rounded). Appressoria were single, medium brown, clavate or irregular in outline, and $5-13 \times 3.5-7.5 \mu\text{m}$. Type 2 conidia shape resembled those of *C. acutatum*.

Type 3 isolates formed olive grey to iron grey mycelia in the center of PDA plates. Colonies on V8 juices had light grey mycelium on front view and pale orange aerial mycelium on back view. Conidia were mostly cylindrical in shape with rounded ends, up to $10-15.5 \mu\text{m} \times 4-6.2 \mu\text{m}$. Appressoria were ovoid to slightly irregular in shape, dark brown in color, and ranged from $6-12 \mu\text{m} \times 4-7 \mu\text{m}$. The features of the isolates in Type 3 were similar to those of *C.*

gloeosporioides.

Isolates of Type 4 formed cottony grey-colored colonies with abundant mycelia on PDA media. Colonies on V8 juices had a white to milky-fluffy mycelium on top view and the underside was pale yellow. Conidia in all isolates were $10.5-16 \times 3.5-5 \mu\text{m}$ and had cylindrical-shape with slightly rounded (sometimes oblong) ends. Appressoria were clavate, brown to dark brown in color, ovoid and slightly irregular in shape, with dimension of $5.5-11 \times 3.5-7.5 \mu\text{m}$. Based on these morphological features, the isolates in Types 4 resembled the description of *C. gloeosporioides*. The description of morphological characteristics for the *Colletotrichum* species in Type 1-4 is shown in (Fig. 1A, 1B).

Molecular characteristics and Phylogenetic analysis.

The ITS and GAPDH regions of all isolated fungi were amplified and sequenced. The PCR products obtained from ITS and GAPDH gene sequence of isolated fungi were approximately 550 and 250 base pairs (bp) long respectively and homology searches with each individual sequence suggested that all tested isolates corresponded to the genus *Colletotrichum*. The sequences of all isolates within the same type were 100% identical to each other with no evident differences.

Based on the sequence comparison of all the obtained isolates with published sequences in GenBank, isolates in Type 1 matched well with sequences belonging to *C. fioriniae*, the isolates in Type 2 shared homology to *C. nymphaeae*, all isolates in Type 3 had sequence identity to *C. siamense* and the sequences from isolates in Type 4 corresponded to the sequences from *C. fructicola*.

Molecular phylogenetic analyses based on final sequence

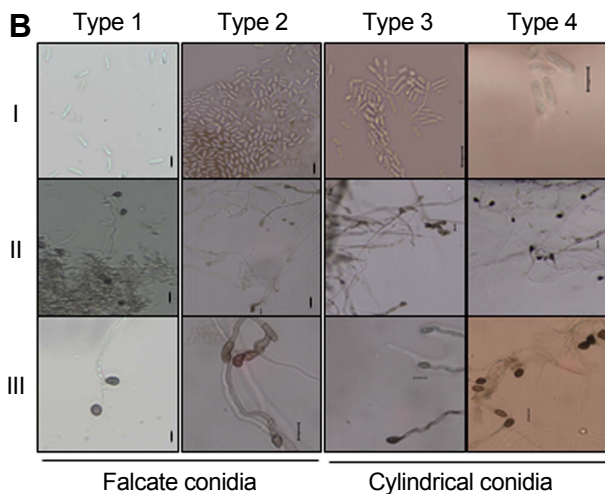
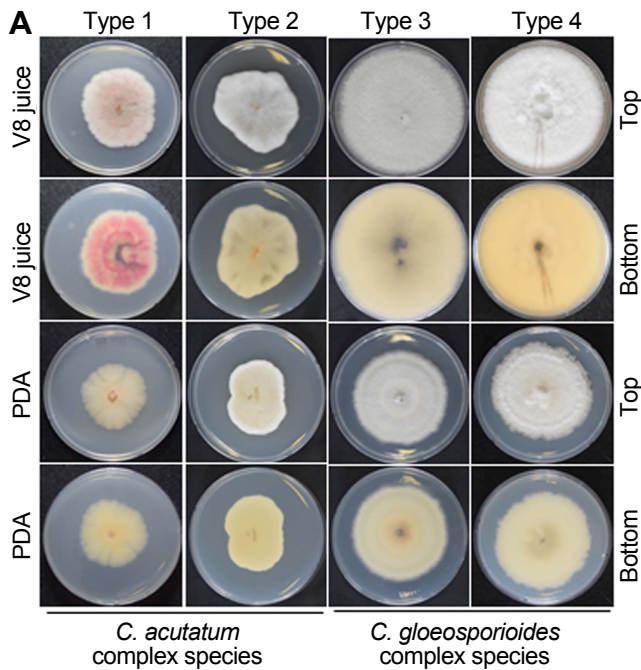


Fig. 1. (A) Colony morphology of 4 selected *Colletotrichum* species on PDA and V8 juice media. (B) Morphological characteristics of conidia and appressoria of *Colletotrichum* species in Type 1-4. I, Conidia; II-III, Appressoria. Bars = 10 μ m.

alignment of a combined data set of ITS and GAPDH regions in a separate analysis classified the *Colletotrichum* isolates into two major clades, corresponding to the *C. acutatum* species complex and *C. gloeosporioides* species complex, with bootstrap support of 100% (Fig. 4). The *C. acutatum* species complex clade included two separate sub-clades: *C. fioriniae* and *C. nymphaeae*. The *C. gloeosporioides* species complex clade comprised of two sub-clades: of *C. siamense* and *C. fructicola*. Notably, this is the first time that these 4 species have been identified as the causal

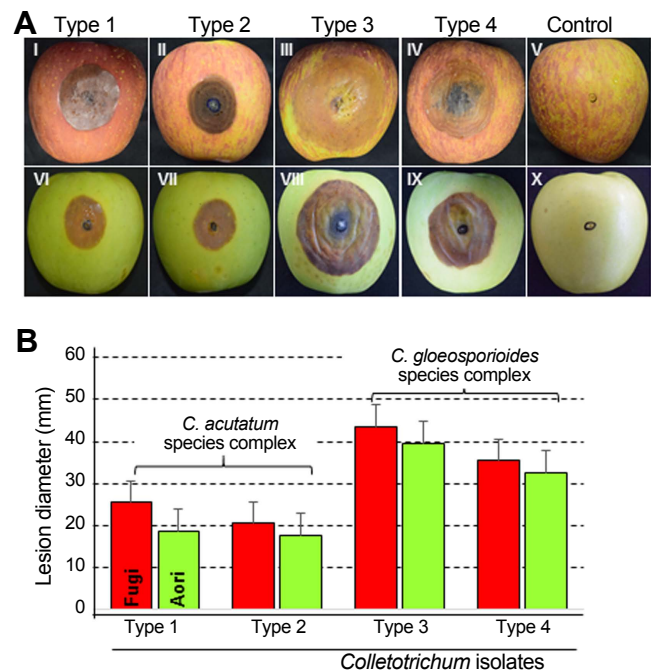


Fig. 2. (A) Symptoms on apple fruits after wound/drop inoculation with *Colletotrichum* spp. Symptoms produced by (I, IV) Type 1, *C. fioriniae*; (II, VII) Type 2, *C. nymphaeae*; (III, VIII) Type 3, *C. siamense*; (IV, IX) Type 4, *C. fructicola*; (V, X) control fruits. Fruits (I-V) on Fuji cultivar and (VI-X) on Aori cultivar. Lesions were 14 days after inoculation (DAI). (B) Comparison of lesion length among four types of *Colletotrichum* species on Fuji and Aori cultivars.

agents of bitter rot disease on apple in South Korea.

The representative ITS and GAPDH genetic sequences obtained from the isolates were deposited in GenBank with accession numbers given in Table 1. The final sequence alignment of combined datasets of ITS and GAPDH containing 25 taxa (including current 18 isolates and 7 reference sequences from GenBank) were used to compare the tree output. All *Colletotrichum* isolates resulted from the current study. The species used in this study are presented in Table 1.

Pathogenicity test. All 18 isolates of *Colletotrichum* species were pathogenic and produced the typical bitter rot symptoms on inoculated-apple fruits belonging to Fuji and Aori cultivars (Fig. 2A). These symptoms consisted of sunken lesions with orange to pink-colored acervuli arranged concentrically. V-shaped necrotic tissue zones were also observed when the fruits were cut through the centers of lesions (Fig. 3). The Fuji cultivar developed the lesion symptom earlier than that of Aori cultivar. All the isolates belonging to the *C. gloeosporioides* species com-



Fig. 3. V-shaped necrotic tissues by cause of wound/drop inoculation of (A, E) Type 1, *C. fioriniae*; (B, F) Type 2, *C. nymphaeae*; (C, G) Type 3, *C. siamense*; (D, H) Type 4, *C. fructicola* on (A, B, C, D) Fuji cultivar and (E, F, G, H) Aori cultivar. Necrotic tissues symptoms were 14 days after inoculation (DAI).

Table 3. Pathogenicity and fruits lesion diameter of apple (Fuji and Aori cultivars) fruits caused by four *Colletotrichum* species 14 days after inoculation with wound/drop method^a

Species	Morphotype	Fuji cultivar		Aori cultivar	
		Lesion size (mm)	Depth (mm)	Lesion size (mm)	Depth (mm)
<i>C. fioriniae</i>	Type 1	25.5 ± 1.3	17.5 ± 1.6	18.5 ± 1.6	15.5 ± 1.7
<i>C. nymphaeae</i>	Type 2	20.5 ± 1.2	16.5 ± 1.5	17.5 ± 1.8	14.0 ± 1.9
<i>C. siamense</i>	Type 3	43.5 ± 1.6	30.5 ± 1.9	39.5 ± 1.9	25.5 ± 2.0
<i>C. fructicola</i>	Type 4	35.5 ± 1.7	25.5 ± 1.7	32.5 ± 1.8	25.0 ± 1.9

^aAll experiments were repeated three times.

plex, formed bigger and deeper lesions than the isolates belonging to the *C. acutatum* species complex isolates (Fig. 2B). Fruit lesion diameters of the 18 isolates are described in Table 3. Among all 18 isolates, Type 3 isolates (*C. siamense*) showed the widest and deepest lesions (43.5 mm in diameter and 30.5 mm in depth on Fuji cultivar; 39.5 mm in diameter and 25.5 mm in depth on Aori cultivar) two week after inoculation (Fig. 2). After *C. siamense*, Type 4 isolates (*C. fructicola*) produced the second largest lesion among these 4 species (35.5 mm in diameter and 25.5 mm in depth on Fuji cultivar; 32.5 mm in diameter and 25.0 mm in depth on Aori cultivar). In the *C. acutatum* species complex (*C. nymphaeae* and *C. fioriniae*), the lesion produced by the Type 1 isolates (*C. fioriniae*) was a slightly larger (25.5 mm in diameter and 17.5 mm in depth on Fuji cultivar; 18.5 mm in diameter and 15.5 mm in depth on Aori cultivar) than that produced by Type 2 isolates (*C. nymphaeae*) (20.5 mm in diameter and 16.5 mm in depth on Fuji cultivar; 16.5 mm in diameter and 14.0 in depth on Aori cultivar). However, the depth of the lesion caused by these two species was not significantly different. No symptoms were observed on control fruits treated with sterile

distilled water (Fig. 2A). Consistent results were obtained in the replicated experiments.

Discussion

The current study is focused on the *Colletotrichum* species isolates obtained from apple fruits afflicted with bitter rot disease in South Korea. Based on the morphological and molecular characteristics (including ITS and GAPDH gene sequences) the resulting 18 isolates were divided in 4 groups: Type 1 (3 isolates), Type 2 (2 isolates), Type 3 (8 isolates) and Type 4 (5 isolates) as described in Table 1. Thus, the present study showed that the isolates of *Colletotrichum* spp. causing the bitter rot disease in Korea belong to 4 species: *C. fioriniae* (Type 1); *C. nymphaeae* (Type 2); *C. siamense* (Type 3); *C. fructicola* (Type 4) where *C. fioriniae* and *C. nymphaeae* belong to Clade 3 and Clade 2 respectively of *C. acutatum* species complex and *C. siamense* and *C. fructicola* belong to Musae Clade of *C. gloeosporioides* complex species. Interestingly, to the best of our knowledge, this is the first report that implicates these 4 species in the pathogenesis of bitter rot disease in

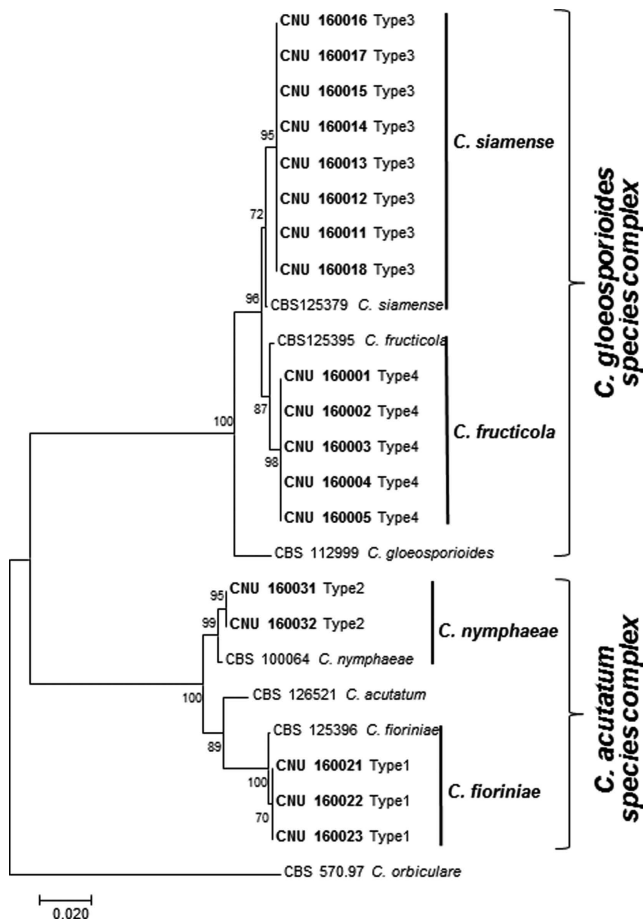


Fig. 4. Maximum parsimony analysis showing phylogenetic relationships among 18 isolates of *Colletotrichum* from apple and other related *Colletotrichum* spp. based on combined dataset of internal transcribed spacer (ITS) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene sequences. Numbers beside each branch represent bootstrap values obtained after a bootstrap test with 1,000 replications. Present isolates are shown in boldface. Bar indicates the number of nucleotide substitutions. The tree is rooted with *Colletotrichum orbiculare*.

South Korea. Therefore, the population *Colletotrichum* species associated with Apple bitter rot disease in South Korea maybe more complex than was previously recognized.

According to the morphological result, our result also showed that the isolates of *C. gloeosporioides* complex species grew faster than that of *C. acutatum* complex species isolates on PDA and V8 juices media. Generally, spore shape has traditionally been used for differentiating *C. acutatum* from *C. gloeosporioides*. However, identifying species within the *Colletotrichum* genus using morphological characteristics is challenging because of increasing complexity and morphological similarity within different members of *Colletotrichum* species (Park et al., 2018). The morphological characteristics are ambiguous, and indefi-

nite morphotaxonomic characters tend to vary depending on the experimental method used and conditions at the time of experiment (Oo et al., 2016). Thus, the novelty of this study is the combination of morphological characteristics and molecular traits to distinguish different species of *Colletotrichum* and confirm the identity of extracted isolates. The present study showed that morphological characteristics alone could not distinguish the species within a species complex as these features could discriminate only the *C. acutatum* species from *C. gloeosporioides* species. Identification of *Colletotrichum* species using species-specific primers was found to be useful as it gave reliable and replicable results. Even though the ITS sequences have been widely used in identification of *Colletotrichum* species, they could not clearly differentiate the species within species complex. GAPDH sequences, on the contrary, gave the accurate differentiation of *Colletotrichum* species within *C. acutatum* and *C. gloeosporioides* complex species. The multi-sequence alignment using ITS sequence data did not show the conflict in tree topology suggesting that the tree sequence datasets of ITS and GAPDH could be combined for further analysis. Therefore, the results derived from combined molecular approaches provided more reliable identification method to distinguish *Colletotrichum* species.

Pathogenicity tests showed that all the tested isolates from four *Colletotrichum* species were pathogenic to wounded apple fruits and lesion sizes did not differ in the two tested apple cultivars, Aori and Fuji cultivars (Fig. 2A). However, pathogenic comparison between these 4 species showed that the current isolates belonging to the *C. gloeosporioides* species complex (Type 3 and Type 4 isolates; *C. siamense* and *C. fructicola*) produced larger lesions than isolates belonging to the *C. acutatum* species complex (Type 1 and Type 2 isolates; *C. fioriniae* and *C. nymphaeae*). Additionally, *C. siamense* in the *C. gloeosporioides* complex species produced the biggest, widest lesions and was found to be the most aggressive among these 4 species. Even though the current 18 isolates are not enough to represent all the species causing bitter rot disease in Korea, there is a high probability that isolates (*C. siamense* and *C. fructicola*) belonging to *C. gloeosporioides* complex species is the dominant pathogen in Korea as compared to the other isolates (*C. fioriniae* and *C. nymphaeae*) in *C. acutatum* complex species (Fig. 4). Therefore, the current result is consistent with a previous report (Munir et al., 2016).

In conclusion, our results indicate that accurate identification of pathogens within a species complex of genus *Colletotrichum* is required for management of bitter rot disease on apple fruit. Moreover, these results emphasized

molecular phylogenetic analysis and pathogenicity tests along with the morphological identification are better tools for the correct identification and characterization of the *Colletotrichum* species.

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