Diversity, Pathogenicity, and Fungicide Sensitivity of *Colletotrichum* Species Associated with Apple Anthracnose in South Korea

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Abstract

Apple fruits with anthracnose symptoms were collected from commercial apple orchards in different regions of the Republic of Korea, and isolations were made on potato dextrose agar to isolate the causal agents. The fungal isolates were identified based on their morphological characteristics, growth rates, and multigene sequences. Nine isolates were identified via phylogenetic analysis: three *Colletotrichum fructicola*, two *C. fioriniae*, one *C. gloeosporioides sensu stricto* (s.s.), two *C. nymphaeae*, and one *C. siamense* isolates. The pathogenicity of the *Colletotrichum* isolates was tested using detached apple fruits under laboratory conditions. This

study also reidentified six *Colletotrichum* isolates responsible for apple anthracnose, which were deposited in the Korean Agricultural Culture Collection. Among the six isolates, three were identified as *C. siamense* (deposited as *C. gloeosporioides* s.s.), and three were *C. nymphaeae* (deposited as *C. acutatum* s.s.). All the *Colletotrichum* species identified in this study were highly sensitive to tebuconazole in terms of inhibition of mycelial growth (EC₅₀ value of 0.12 to 2.1 µg/ml).

Keywords: *Colletotrichum* species, anthracnose, fungicide sensitivity

In addition to persimmon, pear, grape, and peach, apple is one of the major fruit crops commercially cultivated in South Korea (KREI 2018). On average, South Korea produces around 540,000 metric tons of apples a year. ‘Tsugaru’, ‘Hongro’, ‘Yangkwang’, ‘Gamhong’, and ‘Fuji’ are the most common apple cultivars grown in South Korea, with Fuji accounting for 70% of the total apple cultivation area (KREI 2018). Most apple growers choose Fuji because it has certain advantages over other cultivars, such as a longer storage life, and Korean consumers also prefer Fuji apples for their juicy flavor and crisp texture (Choi and Hinkle 2017).

The weather conditions and diseases caused by bacteria, viruses, and fungi are the main challenges for apple growers in South Korea. For example, in 2018, many orchards in Sangju experienced sunburn and smaller apple sizes due to long spells of high temperatures, which are known to reduce the size of apples (Basannagari and Kala 2013). Anthracnose, scab, apple blotch, white rot, *Alternaria* leaf spot, and bacterial shoot blight are the major apple diseases in South Korea (Cheon and Jeon 2015; Kim et al. 2018; KSPP 2009; Lee et al. 2017; Park et al. 2018). Among these diseases, anthracnose (also known as bitter rot) is an annual threat to the apple industry. Apple anthracnose is characterized by sunken lesions on apples and is responsible for their decay and reduction in quality. It is not possible to sell fruit affected by this disease on the market; thus, apple growers face severe economic losses if they cannot control it.

The fungus genus *Colletotrichum* consists of about 189 species, including 11 species complexes (Baroncelli et al. 2017). This pathogen is distributed across the world and is able to infect a wide range of hosts (Faedda et al. 2011; Fu et al. 2019; Guarnaccia et al. 2019). In particular, it is associated with apple anthracnose (Kim et al. 2018), with *Colletotrichum fructicola* and *C. siamense* reported to be the causal agents of apple anthracnose in South Korea (Kim et al. 2018; Park et al. 2018). Recently, *C. siamense*, *C. fructicola*, *C. fioriniae*, and *C. nymphaeae* have been reported

in *Malus domestica* in South Korea by Oo et al. (2018). In addition, a complex of *Colletotrichum* species such as *C. siamense*, *C. theobromicola*, *C. fructicola*, *C. fioriniae*, and *C. nymphaeae* has been reported to cause anthracnose in Kentucky orchards in the United States (Munir et al. 2016). *C. gloeosporioides sensu stricto* (s.s.), previously reported from apple in South Korea, was not identified by the study of Oo et al. (2018). Therefore, further research is needed on the etiology and epidemiology of apple anthracnose in South Korea. Oo et al. (2018) identified *Colletotrichum* species based on their morphological characteristics and nucleotide sequence data of internal transcribed spacer (ITS) regions and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Due to intraspecies overlap in terms of morphological characteristics, it has been difficult to distinguish closely related *Colletotrichum* species. ITS and GAPDH are not enough for distinguishing *Colletotrichum* species (Weir et al. 2012). Silva et al. (2012) suggested using the ApMat gene for the identification of *C. siamense*. Multilocus phylogenetic analysis has become a popular tool to delineate *Colletotrichum* species within a species complex (Damm et al. 2012; Weir et al. 2012). By applying this approach, several new species have been recorded recently. *C. bannanense*, *C. australisense*, and *C. cairnsense* have been recorded within the *C. acutatum* species complex, and *C. ledongense* has been identified in the *C. gloeosporioides* species complex (De Silva et al. 2017; Liu et al. 2018). Several articles focused on the diversity of *Colletotrichum* species associated to anthracnose of a single crop (Fu et al. 2019; Hassan et al. 2018, 2019a; Lubbe et al. 2004). The continuous recognition of novel species within particular species complexes, and several species being associated to a single crop in particular, make it more important for systematic studies to be conducted on the *Colletotrichum* species associated with apple anthracnose. In particular, the identification of *Colletotrichum* species is essential for the effective control of this disease. To enhance the effective management of anthracnose, determination of fungicide sensitivity of *Colletotrichum* spp. isolates is necessary. Sensitivity of various *Colletotrichum* spp. is not the same against a particular fungicide (Chen et al. 2016).

The objectives of this study were (i) to investigate the diversity of *Colletotrichum* species associated with apple anthracnose in Korea by studying their morphological characteristics and conducting multilocus phylogenetic analysis, (ii) to investigate the pathogenicity of different *Colletotrichum* species, and (iii) to examine the sensitivity of different *Colletotrichum* species isolated from apple to fungicides.

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Materials and Methods

Collection of fungal isolates. In 2018 and 2019, affected apples exhibiting typical anthracnose symptoms were collected from commercial Fuji, Tsugaru, and Hongro apple orchards in the Sangju, Gimcheon, and Gumi regions of South Korea. Fungal isolates were obtained from the diseased fruit using the tissue-isolation method. For this, the collected apples were washed thoroughly with tap water, and small pieces of the skin and pulp were cut from the outer margin of the lesions. To sterilize the surface, the skin and pulp were dipped in 0.1% NaOCl solution for 1 min, rinsed with sterilized distilled water three times, and finally air dried in a laminar flow hood. Disinfested fruit tissue was placed on a Petri plate of water agar and incubated at 25°C in the dark. After 2 days of incubation, the hyphal tips emerging from the skin and pulp were transferred to plates of potato dextrose agar (PDA, Difco, Becton Dickinson) and incubated in the dark at 25°C. Fungal isolates were preserved in 20% glycerol at -70°C in the plant tissue culture laboratory at Kyungpook National University for further use.

In addition, six *Colletotrichum* isolates responsible for apple anthracnose were taken from the Korean Agricultural Culture Collection (KACC), South Korea. Three of these isolates from the KACC have previously been identified as *C. gloeosporioides* s.s., and the other three as *C. acutatum* s.s. based on their ITS sequences and morphological characteristics.

DNA extraction, target gene amplification, and sequencing. The mycelium from a 4-day-old culture of each isolate was used for genomic DNA extraction. Around 10 g of the mycelium from each culture was lyophilized first, and the genomic DNA was then isolated using a HiGene Genomic DNA Prep Kit (Biofact, Yuseong-Gu, Daejeon, Korea) following the instructions of the

manufacturer. Variable regions of the genomic DNA were amplified and sequenced, including ITS and a partial sequence of beta-tubulin (TUB2), calmodulin (CAL), GAPDH, actin (ACT), chitin synthase 1 (CHS-1), and the Apn2–Mat1–2 intergenic spacer and the partial mating type (Mat1–2) gene (ApMat). Six loci (ITS, TUB2, GAPDH, CHS-1, CAL, and ApMat) and four loci (ITS, TUB2, GAPDH, and CHS-1) were selected for the *C. gloeosporioides* sensu lato (s.l.) and *C. acutatum* s.l. isolates, respectively. The primer sets ITS1 + ITS4 (White et al. 1990), Bt2a + Bt2b (Glass and Donaldson 1995), GDF + GDR (Templeton et al. 1992), CHS-79F + CHS-345R (Carbone and Kohn 1999), CL1C + CL2C (Weir et al. 2012), and AM-F and AM-R (Silva et al. 2012) were used for the amplification and sequencing of ITS, TUB2, GAPDH, CHS-1, CAL, and ApMat respectively (Table 1). The process and conditions for polymerase chain reaction (PCR) amplification were adopted from Hassan et al. (2018), with the purification and sequencing of the PCR products performed commercially at Macrogen (Seoul, Korea). The resulting sequences were deposited in NCBI GenBank; the accession numbers are listed in Table 2.

Phylogenetic analysis. The gene sequences of each ex-type *Colletotrichum* sp. used in the phylogenetic studies by Weir et al. (2012) and Damm et al. (2012) were downloaded from GenBank. Each gene sequence of the isolates from the present study and those from the ex-type *Colletotrichum* spp. were aligned using the MUSCLE multiple sequence alignment program in MEGA version 6.0 (Tamura et al. 2013), and all gaps were treated as missing data. The multiple sequence alignment for each gene was concatenated using Mesquite version 2.75 (Maddison and Maddison 2011). The concatenated sequences of different gene combinations were used to construct maximum likelihood and maximum parsimony phylogenetic trees using

Table 1. List of primers used for PCR amplification and sequencing

Gene	Primer name	Direction	Sequence (5'–3')	Annealing temperature (°C)	References
GAPDH	GDF	Forward	GCCGTCAACGACCCCTTCATTGA	60	Templeton et al. (1992)
	GDR	Reverse	GGGTGGAGTCGTACTTGAGCATGT	60	Templeton et al. (1992)
ITS	ITS1	Forward	TCCGTAGGTGAACCTGCGG	55	White et al. (1990)
	ITS4	Reverse	TCCTCCGCTTATTGATATGC	55	White et al. (1990)
CAL	CL1C	Forward	GAATTCAAGGAGGCCTTCTC	59	Weir et al. (2012)
	CL2C	Reverse	CTTCTGCATCATGAGCTGGAC	59	Weir et al. (2012)
ApMat	AM-F	Forward	TCATTCTACGTATGTGCCCG	62	Silva et al. (2012)
	AM-R	Reverse	CCAGAAATACACCGAACTTGC	62	Silva et al. (2012)
TUB2	Bt2a	Forward	GGTAACCAAATCGGTGCTGCTTTC	55	Glass and Donaldson (1995)
	Bt2b	Reverse	ACCCTCAGTGTAGTGACCTTGGC	55	Glass and Donaldson (1995)
CHS-1	CHS-79F	Forward	TGGGGCAAGGATGCTTGGAAGAAG	58	Carbone and Kohn (1999)
	CHS-345R	Reverse	TGGAAGAACCATCTGTGAGAGTTG	58	Carbone and Kohn (1999)

Table 2. GenBank accession numbers for the identified *Colletotrichum* isolates

Species	Isolate	GenBank accession number					
		ITS	GAPDH	TUB2	CHS-1	CAL	ApMat
<i>C. gloeosporioides</i>	ICKa85	LC499040	LC499057	LC499048	LC499071	LC499085	...
<i>C. siamense</i>	ICKa38	LC499039	LC499056	LC499047	LC499070	LC499084	LC499089
	KACC 42494	KA002861	LC499058	KA002890	LC499072	LC499086	LC499090
	KACC 42497	KA002863	LC499059	KA002888	LC499074	LC499087	LC499091
	KACC 42498	KA002864	L90C4960	KA002887	LC499075	LC499088	LC499092
<i>C. fruticola</i>	ICKa13	LC499038	LC499055	LC499046	LC499069	LC499083	...
	ICKa14	LC499037	LC499045	LC499054	LC499068	LC499082	...
	ICKa15	LC381301	LC499053	LC381302	LC381304	...	...
<i>C. nymphaeae</i>	ICKS18	LC499041	LC499061	LC499049	LC499075	...	...
	ICKS19	LC499042	LC499062	LC499050	LC499076	...	...
	KACC42403	KA002860	LC499065	KA002891	LC499079	...	...
	KACC40847	KA002638	LC499066	KA002682	LC499080	...	...
	KACC40848	KA002639	LC499067	KA002683	LC499081	...	...
<i>C. fioriniae</i>	ICKS30	LC499043	LC499063	LC499051	LC499077	...	...
	ICKS31	LC499044	LC499064	LC499052	LC499078	...	...

MEGA version 6.0 software (Tamura et al. 2013). A Bayesian inference consensus tree based on a 50% majority rule was constructed using MrBayes version 3.2 (Ronquist et al. 2012). A Markov chain Monte Carlo sampler was employed to sample trees and posterior probabilities of model parameters. Four Markov chains were run with the full data set for 1×10^6 generations, with a sampling frequency of once every 100 generations. The first 25% of the burn-in generations were discarded. The resulting 50% majority rule consensus tree was visualized using FigTree version 1.3.1 (Rambaut and Drummond 2009).

Morphological analysis. The colony color, mycelial growth rate, and morphology of each isolate (except the KACC isolates) on PDA were recorded over the course of 7 days of incubation at 25°C in the dark. The effects of temperature and the culture media on the mycelial growth of the isolates was also investigated. To assess the effect of temperature, a 5-mm mycelial plug from the 7-day-old culture of each isolate was placed on fresh PDA plates and incubated in the dark at 15, 20, 25, and 30°C. Similarly, a 5-mm mycelial plug from the 7-day-old culture of each isolate was placed on PDA, oatmeal agar (OA), malt agar (MA), and V8 and incubated at 25°C in the dark. Both experiments consisted of three replications and were repeated twice. The colony diameter was measured after 7 days of incubation. The shape and size of the conidia and appressoria were determined by mounting the conidial mass from each culture on a glass slide in distilled water. The sizes of 50 random conidia from each isolate were measured using an Olympus BX43 microscope with a magnification of 400×. To produce appressoria, a mounted conidial slide for each isolate was placed in a plastic box containing moistened tissue and incubated at 25°C in the dark. Appressoria were produced after 2 days of incubation. The sizes of 30 random appressoria from each isolate were measured in the same manner as for the conidia.

Pathogenicity testing. One representative isolate was chosen for pathogenicity testing from each recognized *Colletotrichum* sp. Healthy and unwounded mature apple fruits were collected from a commercial orchard, washed with tap water, and disinfected in 1% sodium hypochlorite for 3 min. The disinfected apples were washed with sterilized water three times, dried in a laminar flow hood, and then placed on a sterilized plastic saucer in a sterilized plastic box with wet tissue. A conidial suspension of *Colletotrichum* spp. (10^6

conidia/ml) was used to inoculate the disinfected fruit. This conidial suspension was prepared from a 7-day-old culture of each isolate on PDA. In each culture, 5 to 10 ml of sterile distilled water was added, and the mycelia were scraped from the plates and filtered through two layers of Kimtech wipes. To inoculate the apples, both wounding and nonwounding techniques were adopted. In the former, the epidermis of the apples was wounded with a sterile syringe needle, and a drop of 10 µl of the conidial suspension was placed over the wound (three wounds per fruit); in the latter, the conidial suspension was sprayed over the apples. Sterilized distilled water was used to treat the control fruit. The plastic boxes were sealed and maintained in an incubator at 25°C. After 5 days of incubation, the lesion size (diameter) and disease incidence in terms of percentage of lesion development per isolate were measured. For both inoculation methods, five apples were tested for each isolate, and the experiment was repeated twice.

Determination of sensitivity of *Colletotrichum* isolates to fungicides. Four fungicides, including thiophanate-methyl, tebuconazole, azoxystrobin, and carbendazim, were used for *Colletotrichum* species sensitivity analysis. For 25 different *Colletotrichum* species, 50% effective concentration (EC₅₀) values were determined using mycelial growth assays. Less than 1 week old and vigorously growing culture of each isolate on PDA was used for the experiment. Plugs of 5 mm in diameter, containing actively growing mycelium, were transferred to PDA amended with fungicides, and in addition nonamended PDA was used as a control. PDA was amended with the active ingredient of thiophanate-methyl at 0.05, 0.1, 0.5, 1, and 2 µg/ml; tebuconazole at 0.0083, 0.016, 0.083, 0.83, and 2 µg/ml; azoxystrobin at 0.05, 0.1, 0.5, 1, and 2 µg/ml; and carbendazim at 0.05, 0.1, 0.5, 1, and 2 µg/ml. All fungicides were added to PDA after autoclaving. A plug was placed in the center of the PDA plate. Petri plates were sealed with Parafilm and incubated at 25°C in the dark. Radial mycelium growth was measured after 3 days of incubation. The diameter of each colony was measured by subtracting the 5-mm plug from two perpendicular diameters and then averaging them. The relative mycelial growth percentage (RMG%) for each isolate at each test concentration was calculated by comparing the growth on amended media (GOA) compared with the growth on the nonamended control (GOC) as follows: $RMG\% = (GOA/GOC) \times 100$. The percentage inhibition of mycelium growth for each isolate at

Table 3. Preliminary identification of *Colletotrichum* isolates based on a BLASTn search using ITS sequences and conidial morphology

Isolates	Closest <i>Colletotrichum</i> spp. in GenBank based on resulting ITS sequences	Conidia	Remark
ICKS1	<i>C. gloeosporioides</i> (similarity: 100%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKS5	<i>C. gloeosporioides</i> (similarity: 99%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKS17	<i>C. siamense</i> (similarity: 99.2%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKa13	<i>C. fructicola</i> (similarity: 100%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKa14	<i>C. fructicola</i> (similarity: 100%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKa15	<i>C. fructicola</i> (similarity: 99.9%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKS18	<i>C. acutatum</i> (similarity: 99%)	Fusiform	<i>C. acutatum</i> s.l.
ICKS19	<i>C. acutatum</i> (similarity: 99%)	Fusiform	<i>C. acutatum</i> s.l.
ICKS20	<i>C. acutatum</i> (similarity: 100%)	Fusiform	<i>C. acutatum</i> s.l.
ICK21	<i>C. acutatum</i> (similarity: 99%)	Fusiform	<i>C. acutatum</i> s.l.
ICKS24	<i>C. gloeosporioides</i> (similarity: 99%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKa25	<i>C. fructicola</i> (similarity: 99.9%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKa28	<i>C. fructicola</i> (similarity: 99.27%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKS30	<i>C. acutatum</i> (similarity: 99.8%)	Fusiform	<i>C. acutatum</i> s.l.
ICKS31	<i>C. acutatum</i> (similarity: 100%)	Fusiform	<i>C. acutatum</i> s.l.
ICKa38	<i>C. siamense</i> (similarity: 99.2%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKS52	<i>C. fructicola</i> (similarity: 99.9%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKS54	<i>C. fructicola</i> (similarity: 99.9%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKS55	<i>C. acutatum</i> (similarity: 99%)	Fusiform	<i>C. acutatum</i> s.l.
ICKS57	<i>C. fructicola</i> (similarity: 99.9%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKS61	<i>C. acutatum</i> (similarity: 99.75%)	Fusiform	<i>C. acutatum</i> s.l.
ICKS63	<i>C. acutatum</i> (similarity: 99.45%)	Fusiform	<i>C. acutatum</i> s.l.
ICKS71	<i>C. acutatum</i> (similarity: 99.17%)	Fusiform	<i>C. acutatum</i> s.l.
ICKS85	<i>C. gloeosporioides</i> (similarity: 99.9%)	Cylindrical	<i>C. gloeosporioides</i> s.l.
ICKS92	<i>C. acutatum</i> (similarity: 99.17%)	Fusiform	<i>C. acutatum</i> s.l.

each test concentration (I) was also calculated as the difference between the radial growth of nonamended control (C) and the radial growth of each test concentration (T) as follows: $I (\%) = (1 - T/C) \times 100$. The EC_{50} values were calculated by linear regression of the probit-transformed relative inhibition on log10-transformed fungicide concentration using PROC PROBIT (SAS 9.2; SAS Institute, Cary, NC).

Statistical analysis. The average and standard deviation (SD) of the data were calculated using Microsoft Excel. The size of the conidia and appressoria and the diameter of the lesions are expressed as the average $\pm$ SD in this paper. EC_{50} values were subjected to one-way ANOVA and compared by using the least significant difference test in SAS (SAS 9.2).

Results

Preliminary identification of the fungal isolates. A total of 125 fungal isolates were collected from diseased apple fruits. The isolates were then grouped into five groups based on the similarity of morphology (colony color and conidial size and shape). A subsample of 25 isolates (five from each group) were selected for further study. Based on the morphological characteristics and ITS sequence data, 14 of these isolates were identified as *C. gloeosporioides* s.l., and 11 were found to be *C. acutatum* s.l. (Table 3). For morphological and molecular analysis, five isolates of the *C. gloeosporioides* species complex and four isolates of the *C. acutatum* species complex were selected randomly for identification at species level.

Phylogenetic analysis. Five combined gene alignment data matrixes (ITS, TUB2, GAPDH, CAL, and CHS-1) for 37 sequences were used to identify the *Colletotrichum* species within *C.*

gloeosporioides s.l. The combined matrixes of the aligned data consisted of 2,391 characters and gaps in the alignment. The identified isolates from the apples in the *C. gloeosporioides* complex are shown in Figure 1. The isolates in the *C. gloeosporioides* complex were clustered into three clades: one isolate clustered with *C. gloeosporioides* s.s., five (one from the present study and four from the KACC) with *C. siamense*, and three with *C. fruticicola*. The *C. siamense* isolate was further confirmed via phylogenetic analysis using the gene sequences of ApMat.

To identify the *Colletotrichum* species within *C. acutatum* s.l., four gene alignment data matrixes (ITS, TUB2, GAPDH, and CHS-1) for 44 sequences were used. The combined matrixes of the aligned data consisted of 1,576 characters and gaps in the alignment. The isolates identified in the *C. acutatum* complex are presented in Figure 2. The apple isolates in *C. acutatum* s.l. could be identified with confidence as *C. fioriniae* and *C. nymphaeae* because the two isolates clustered together with *C. fioriniae* ex-type isolates CBS 23549 and CBS 125396, and five isolates (two from the present study and three collected from KACC) clustered with the *C. nymphaeae* ex-type isolate CBS 100065.

Morphological characteristics. The 7-day-old colony of *C. gloeosporioides* on PDA was white with aerial mycelium and edges (Fig. 3). The conidia were cylindrical, aseptate, hyaline, rounded at both ends, and 13.0 to 18.3×4.8 to $7.1 \mu\text{m}$ (average $16.5 \pm 1.2 \times 6.2 \pm 0.6 \mu\text{m}$). The appressoria were globose to cylindrical, brown, and 8.2 to 12.07×5.9 to $9.8 \mu\text{m}$ (average $10.1 \pm 1.5 \times 8.0 \pm 1.1 \mu\text{m}$), with an entire margin (Table 4). The growth rate of isolate ICKa85 on PDA was 10.3 mm per day. Mycelial growth was fastest on MA, followed by PDA, V8, OA, and CA.

Fig. 1. Consensus tree based on the 50% majority rule obtained using Bayesian inference analysis of ITS, TUB2, GAPDH, CHS-1, and CAL sequence alignment. Bayesian posterior probability values ≥ 0.5 and bootstrap support values $\geq 50\%$ are presented at the nodes (posterior probability/maximum likelihood/maximum parsimony). *Colletotrichum boninense* was used as the outgroup. The sequences of reference *Colletotrichum* spp. within the *C. gloeosporioides* species complex were from Weir et al. (2012). The isolates obtained in this study are highlighted.

The 7-day-old colony of *C. siamense* on PDA was olivaceous white with whitish aerial mycelium and edges (Fig. 3). The conidia were cylindrical, aseptate, hyaline, rounded at both ends, and 12.5 to 19.5×4.9 to 7.2 μm (average $16.3 \pm 1.9 \times 6.5 \pm 0.9$ μm). The appressoria were globose to cylindrical, entire or lobed, 7.4 to 12.0×6.5 to 9.4 μm (average $10.3 \pm 1.6 \times 8.2 \pm 0.9$ μm), and brown (Table 4). The growth rate of isolate ICKa38 on PDA was 8.9 mm per day. Mycelial growth was fastest on MA followed by V8, PDA, OA, and CA.

The colony of *C. fruticola* after 7 days was light gray with whitish aerial mycelium and edges. The underside of the colony was gray with black zonation (Fig. 3). The conidia were cylindrical, aseptate, hyaline, rounded at both ends, and 14.6 to 24.4×5.4 to 7.5 μm (average $18.1 \pm 2.2 \times 6.4 \pm 0.6$ μm). The appressoria were ovoid, lobed, 8.6 to 17.8×5.6 to 14.33 μm (average $12.3 \pm 2.16 \times 8.6 \pm 1.8$ μm), and brown (Table 4). The growth rate of isolate ICKa15 on PDA was 12.10 mm per day, with mycelial growth fastest on PDA followed by MA, OA, V8, and CA.

After 7 days, the *C. fioriniae* colony on PDA was light gray with whitish aerial mycelium and edges. The underside of the colony was yellowish white with black zonation (Fig. 3). The conidia were fusiform to cylindrical, aseptate, hyaline, rounded at both ends, and 11.7 to 22.1×4.5 to 8.0 μm (average $17.1 \pm 2.3 \times 5.8 \pm 0.8$ μm); the appressoria were ovoid, lobed, 11.0 to 14.4×7.3 to 8.2 μm (average $12.5 \pm 1.1 \times 7.7 \pm 0.6$ μm), and brown (Table 4). The growth rate

of isolate ICKS30 on PDA was 6.40 mm per day. Mycelial growth was fastest on V8 followed by OA, PDA, MA, and CA.

C. nymphaeae on PDA was gray with a white margin after 7 days of incubation in the dark at 25°C (Fig. 3). The conidia were smooth-walled, aseptate, straight, cylindrical to cylindric-clavate, with one end rounded and one end rounded to acute, and 11.1 to 18.0×4.2 to 6.8 μm (average $13.8 \pm 1.4 \times 5.22 \pm 0.5$ μm). The appressoria were medium, brown, ovoid with smooth margins, and 7.0 to 11.2×5.7 to 8.1 μm (average $8.7 \pm 1.3 \times 7.0 \pm 0.8$ μm ; Table 4). The growth rate of isolate ICKS18 on PDA was 6.8 mm per day. Mycelial growth was fastest on OA followed by V8, PDA, MA, and CA.

The effect of temperature on mycelial growth of all isolates was almost similar. Active mycelium growth occurred at temperatures from 15 to 30°C . Radial mycelial growth gradually increased from 15 to 25°C and then decreased, indicating that maximum mycelial growth rate for all isolates was at 25°C .

Pathogenicity. Representative isolates of each *Colletotrichum* sp. were pathogenic on the detached apple fruits (Fig. 4). Inoculation with *C. fruticola*, *C. siamense*, *C. nymphaeae*, and *C. fioriniae* isolates led to the development of anthracnose symptoms on fruits using both the wounding and nonwounding approaches, whereas the *C. gloeosporioides* isolate was only capable of infecting wounded apples (Table 5). All of the *Colletotrichum* species exhibited higher virulence on the wounded apples compared with the nonwounded apples in terms of disease incidence and lesion size (Fig. 4;

Fig. 2. Consensus tree based on the 50% majority rule obtained using Bayesian inference analysis of ITS, TUB2, GAPDH, and CHS-1 sequence alignment. Bayesian posterior probability values ≥ 0.5 and bootstrap support values $\geq 50\%$ are presented at the nodes (posterior probability/maximum likelihood/maximum parsimony). The sequences of the reference *Colletotrichum* spp. within the *C. acutatum* species complex were from Damm et al. (2012). The isolates obtained in this study are highlighted.

Table 5). The fungal species were reisolated from the inoculated fruits and identified using the process described above to confirm Koch's postulates.

Sensitivity of *Colletotrichum* isolates to fungicides. Based on the EC₅₀ values of fungicides against mycelial growth of different *Colletotrichum* species, tebuconazole was more effective compared with other fungicides used in this study (Table 6). Based on the EC₅₀ values of different *Colletotrichum* species isolates, *C. fructicola* had significantly the highest EC₅₀ values against all fungicides except tebuconazole. EC₅₀ values (204.6 µg/ml) of mycelium growth of *C. fructicola* against thiophanate-methyl was higher than that of any other fungicide. The lowest EC₅₀ of *C. gloeosporioides* and *C. siamense* was against carbendazim, with values of 0.03 and

0.9 µg/ml, respectively. The lowest EC₅₀ of *C. fiorinia* was against tebuconazole and azoxystrobin, with values of 0.15 and 0.14 µg/ml, respectively; The lowest EC₅₀ of *C. nymphaeae* was against azoxystrobin with a value of 0.06 µg/ml (Table 6).

Discussion

Apple anthracnose is the most prevalent fruit disease in Gyeongbuk province in South Korea and the most challenging summer disease to control. Recently, many orchardists have reported that this disease could not be controlled even after employing the suggested spraying schedule. In this respect, a key finding of the present study is that different *Colletotrichum* spp. are responsible for apple anthracnose in Korea. Five phylogenetic species (*C. gloeosporioides*

Fig. 3. Morphological characteristics of the *Colletotrichum* isolates characterized in the present study: **A** to **E**, 7-day-old colony on PDA; **F** to **J**, conidia; and **K** to **O**, appressoria.

Table 4. Morphological characterization of representative *Colletotrichum* sp. isolates identified in this study

Characteristics	<i>C. gloeosporioides</i> (ICKa85)	<i>C. siamense</i> (ICKa38)	<i>C. fructicola</i> (ICKa15)	<i>C. fiorinia</i> (ICKS30)	<i>C. nymphaeae</i> (ICKS18)
Colonies, color on PDA	White with aerial mycelium and edges	Olivaceous white with whitish aerial mycelium and edges	Light gray with whitish aerial mycelium and edges, gray with black zonation in reverse	Yellowish white with black zonation	Gray at center, white at the margin, lack of aerial mycelium, reverse creamy white
Conidia, shape	Cylindrical, aseptate, hyaline, rounded at both ends	Cylindrical, aseptate, hyaline, rounded at both ends	Cylindrical, aseptate, hyaline, rounded at both ends	Fusiform to cylindrical, aseptate, hyaline, rounded at both ends	Smooth-walled, aseptate, straight, cylindrical to cylindric-clavate with one end round and one end rounded to acute
Conidia, size	13.0 to 18.3 × 4.8 to 7.1 µm (avg. 16.5 ± 1.2 × 6.2 ± 0.6 µm)	12.5 to 19.5 × 4.9 to 7.2 µm (avg. 16.3 ± 1.9 × 6.5 ± 0.9 µm)	14.6 to 24.4 × 5.4 to 7.5 µm (avg. 18.1 ± 2.2 × 6.4 ± 0.6 µm)	11.7 to 22.1 × 4.5 to 8.0 µm (avg. 17.1 ± 2.3 × 5.8 ± 0.8 µm)	11.1 to 18.0 × 4.2 to 6.8 µm (avg. 13.8 ± 1.4 × 5.22 ± 0.5 µm)
Appressoria, shape	Globose to cylindrical, entire margin	Globose to cylindrical, entire or lobed margin	Ovoid and lobed margin	Medium brown ovoid with lobed margin	Medium brown ovoid with smooth margin
Appressoria, size	8.2 to 12.07 × 5.9 to 9.8 µm (avg. 10.1 ± 1.5 × 8.0 ± 1.1 µm)	7.4 to 12.0 × 6.5 to 9.4 µm (avg. 10.3 ± 1.6 × 8.2 ± 0.9 µm)	8.6 to 17.8 × 5.6 to 14.33 µm (avg. 12.3 ± 2.16 × 8.6 ± 1.8 µm)	11.0 to 14.4 × 7.3 to 8.2 µm (avg. 12.5 ± 1.1 × 7.7 ± 0.6 µm)	7.0 to 11.2 × 5.7 to 8.1 µm (avg. 8.7 ± 1.3 × 7.0 ± 0.8 µm)

s.s., *C. fruticola*, *C. siamense*, *C. nymphaeae*, and *C. fiorinae*) belonging to the *C. gloeosporioides* and *C. acutatum* species complexes were identified in diseased apples using multigene phylogenetic and morphological analyses. This diverse range of causal agents may be a significant reason why numerous difficulties have been experienced in managing this disease.

A recent study based on a phylogenetic approach using ITS and GAPDH showed that *C. fruticola*, *C. siamense*, *C. nymphaeae*, and *C. fiorinae* were associated with apple anthracnose in South Korea (Oo et al. 2018). Along with four *Colletotrichum* species reported by Oo et al. (2018), this study identified *C. gloeosporioides* s.s. as a causal agent of apple anthracnose in South Korea.

Fig. 4. Anthracnose symptoms on naturally and artificially inoculated apples. **A**, Symptomatic fruit from an apple orchard. **B**, *Colletotrichum gloeosporioides* (ICKa85). **C**, *C. siamense* (ICKa38). **D**, *C. fruticola* (ICKa15). **E**, *C. fiorinae* (ICKS30). **F**, *C. nymphaeae* (ICKS18).

Table 5. Disease incidence and lesion diameter produced by different *Colletotrichum* isolates on apples^z

Species and isolate	Wounding method		Lesion diameter (nonwounding method)	
	Disease incidence (%)	Lesion diameter (cm)	Disease incidence (%)	Lesion diameter (cm)
<i>C. gloeosporioides</i> (ICKa85)	100	8.5 ± 1.25	0	–
<i>C. siamense</i> (ICKa38)	100	17.2 ± 1.83	10	5.2 ± 1.3
<i>C. fruticola</i> (ICKa15)	100	11.03 ± 45	13.3	4.5 ± 0.7
<i>C. fiorinae</i> (ICKS30)	100	14.52 ± 1.91	10	4.2 ± 0.1.2
<i>C. nymphaeae</i> (ICKS18)	100	9.34 ± 1.2	10	4.0 ± 0.5

^z Lesion diameter presented as the mean ± standard deviation.

Table 6. Mean half maximum effective concentration (EC₅₀) value of mycelial growth of *Colletotrichum* spp. to four fungicides^z

<i>Colletotrichum</i> spp.	EC ₅₀ (µg/ml)			
	Carbendazim	Thiophanate-methyl	Tebuconazole	Azoxystrobin
<i>C. fruticola</i>	150 ± 8.1 a	204.6 ± 10.2 a	0.8 ± 0.2 c	55.7 ± 2.3 a
<i>C. nymphaeae</i>	11.4 ± 1.3 b	14.02 ± 2.5 b	0.12 ± 0.02 d	0.06 ± 0.0 e
<i>C. fiorinae</i>	1.4 ± 0.2 c	5.9 ± 1.0 c	0.15 ± 0.06 d	0.14 ± 0.02 d
<i>C. siamense</i>	0.9 ± 0.2 d	1.38 ± 0.5 d	2.1 ± 0.4 a	11.6 ± 1.7 c
<i>C. gloeosporioides</i>	0.03 ± 0.01 e	0.51 ± 0.1 e	1.45 ± 0.1 b	36.3 ± 3.5 b

^z EC₅₀ values followed by different letters are significantly different among the species within each fungicide based on LSD test with *P* = 0.01.

C. gloeosporioides s.s. is a very common causal agent of *Citrus* anthracnose worldwide (Weir et al. 2012) and has been found in a number of other hosts, including *Broussonetia papyrifera*, *Ficus*, *Mangifera*, *Pueraria*, and *Vitis* (Weir et al. 2012; Yan et al. 2011). In Korea, it has been reported to be a causal agent of apple anthracnose (KSPP 2009). *C. gloeosporioides* s.s. has also been isolated from *Ficus carica*, *Liriodendron chinense*, *Prunus avium*, *Prunus salicina*, and *Diospyros kaki* in South Korea (Cheon et al. 2012; Choi et al. 2012, 2013; Hassan et al. 2019a; KSPP 2009; Lee and Kwak 2014). *C. gloeosporioides* s.s. is also often associated with the teleomorphic *Glomerella cingulata* (Weir et al. 2012). *C. siamense* is a biologically and geographically diverse species that has been recorded as a pathogen in multiple hosts (Cai et al. 2009; Weir et al. 2012). It was isolated and described by Prihastuti et al. (2009) as a causal agent for coffee berry disease. The present study identified *C. siamense* as a causal agent of apple anthracnose after it was previously reported to cause anthracnose in *D. kaki* and *P. salicina* in South Korea (Hassan et al. 2018, 2019a). Along with *C. siamense*, *C. fructicola* was also isolated and described by Prihastuti et al. (2009) for the first time as a causal agent for coffee berry anthracnose. This is a common pathogen, with a wide range of hosts, including mangos, grapes, pears, and cassava (Bragança et al. 2016; Jiang et al. 2014; Lima et al. 2013; Peng et al. 2013; Weir et al. 2012), possibly due to its parasitic and endophytic lifestyle. In our previous article, *C. fructicola* was identified as a causal agent of apple anthracnose (Kim et al. 2018), and it has also been isolated as a leaf endophyte in several plants (Lima et al. 2013; Weir et al. 2012). *C. gloeosporioides* s.s., *C. siamense*, and *C. fructicola* belong to the *Musae* clade of the *C. gloeosporioides* complex described by Weir et al. (2012). The morphological characteristics of these *Colletotrichum* species are almost identical; thus, separating them on the basis of their morphological features was very difficult. For example, cylindrical and round-ended conidia were common to all three species. The morphological characteristics of these isolates also overlapped with other taxa within *C. gloeosporioides* s.l., with the colony appearance, conidial measurements, and growth rates of the *Colletotrichum* spp. within the *C. gloeosporioides* complex almost identical to or within the range of the type specimens (De Silva et al. 2017; Weir et al. 2012). Given this high degree of morphological similarity, multigene phylogenetic analysis can be used to disentangle *Colletotrichum* spp. (Cai et al. 2009; Weir et al. 2012). Phylogenetic analysis using ITS, TUB2, GAPDH, CAL, and CHS-1 sequences clearly separated the three species, which were located within a distinct phylogenetic clade in *C. gloeosporioides* s.l., with a high posterior probability (Fig. 1). The *C. siamense* isolates were further verified via phylogenetic analysis using ApMat sequence data, with ApMat possibly a strongly informative gene for the identification of *C. siamense* (Silva et al. 2012; Weir et al. 2012).

The isolates that we identified as *C. fioriniae* and *C. nymphaeae* belong to the *C. acutatum* species complex. *C. fioriniae* has been reported to be endophytic in some cases and entomopathogenic in others. It has also been reported as a causal agent of anthracnose in a wide range of crops and wild plants worldwide, including apple (Baroncelli et al. 2014; Damm et al. 2012; Faedda et al. 2011; Guarnaccia et al. 2019; Lubbe et al. 2004). In Korea, *C. fioriniae* has been isolated as a pathogen from different crops including *Lycium chinense*, *P. salicina*, *Prunus persica*, and *Solanum melongena* (Hassan et al. 2019a; Lee et al. 2018; Oo and Oh 2017; Xu et al. 2018). To the best of our knowledge, the present study is the second to report apple anthracnose caused by *C. fioriniae* in Korea. *C. nymphaeae* is a very common anthracnose pathogen in strawberry crops within *C. acutatum* s.l. (Damm et al. 2012; Jayawardena et al. 2016). It has been isolated as a causal agent of strawberry anthracnose in Bulgaria, Canada, China, France, Israel, Italy, Kenya, Netherlands, South Africa, Spain, Switzerland, the United Kingdom, and the United States (Damm et al. 2012; Jayawardena et al. 2016). *C. nymphaeae* is also associated with anthracnose in many other hosts, such as *Eriobotrya japonica*, *Nuphar luteum*, and *M. domestica* (Damm et al. 2012; Velho et al. 2014; Wu et al. 2018). Very recently, it has been isolated from Japanese plum and persimmon in Korea

(Hassan et al. 2019a, 2019b). To the best of our knowledge, the present study represents the second time *C. nymphaeae* has been identified as a causative agent of apple anthracnose.

The *C. fioriniae* isolates were easily differentiated from those of *C. nymphaeae* based on their fusiform conidia (Fig. 3). Molecular analysis using ITS, TUB2, GAPDH, and CHS-1 sequence data also clearly separated these two species. The colony color and conidial shape of *C. nymphaeae* were not identical with *C. gloeosporioides* s.l. isolates (Fig. 3), although it is known that the separation of *C. nymphaeae* from *C. gloeosporioides* based on morphological characteristics is very difficult (Johnson et al. 1997). However, multigene sequence analysis, especially that using TUB2, is unequivocal in distinguishing *C. nymphaeae* from other species (Damm et al. 2012).

Pathogenicity assays were employed in the present study to determine the aggressiveness of the different species identified within *C. gloeosporioides* s.l. and *C. acutatum* s.l. Aggressiveness was estimated based on lesion size and disease incidence. In terms of disease incidence, all the isolates exhibited comparable aggressiveness except *C. gloeosporioides* s.s., which did not infect unwounded apples. All the isolates showed variable aggressiveness in terms of disease incidence (Table 5).

Another important outcome of this study was the reassessment of six isolates collected from the KACC in South Korea. Three isolates (KACC42494, KACC42497, and KACC42498) deposited in the KACC as *C. gloeosporioides* s.s. were identified as *C. siamense* in the present study, and the isolates KACC40847, KACC40848, and KACC42403, deposited in the KACC as *C. acutatum* s.s., were identified as *C. nymphaeae*. This illustrates the importance of verifying the *Colletotrichum* strains deposited in the KACC.

Many apple growers in South Korea rely on application of fungicides with different modes of action. This study determines the selectivity of different *Colletotrichum* species to fungicides (thiophanate-methyl, tebuconazole, azoxystrobin, and carbendazim) having different modes of action. EC₅₀ values estimated in this study for *Colletotrichum* species against tebuconazole were comparatively low, suggesting this fungicide may provide good control efficacy in field conditions. To choose effective fungicides, more field study is required in the future. *C. fructicola* showed more tolerance to fungicides (except tebuconazole) compared with other *Colletotrichum* species. The EC₅₀ value of specific *Colletotrichum* species varied greatly among the fungicides, which deserves to be further investigated to improve the efficacy of the management strategies of apple bitter rot. In conclusion, the present study provides a better insight into the diversity of *Colletotrichum* species in the incidence of apple anthracnose in commercial Korean apple orchards in Gyeongbuk province. *C. gloeosporioides* s.s., *C. fructicola*, *C. siamense*, *C. nymphaeae*, and *C. fioriniae* were identified as causal agents of apple anthracnose based on colony and conidial morphological characteristics, molecular features, and pathogenicity testing. It also provides useful information for the effective control of apple anthracnose in South Korea.

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