Molecular and Morphological Characterization of *Colletotrichum* Species in the *Colletotrichum gloeosporioides* Complex Associated with Persimmon Anthracnose in South Korea

Oliul Hassan, Jong Yeob Jeon, Taehyun Chang,[†] Jun Sung Shin, and Nam Kwon Oh, School of Ecology and Environmental System, College of Ecology & Environmental Sciences, Kyungpook National University, Sangju, Gyeongsangbuk-do, 37224, Korea (Republic of); and Yong Se Lee, Division of Life and Environmental Sciences, College of Life and Environmental Sciences, Daegu University, Gyeongsan, Gyeongsangbuk-do, 38453, Korea (Republic of)

Abstract

Anthracnose is a major disease of persimmon in the pre- and postharvest phase. Several species of *Colletotrichum* (*Colletotrichum gloeosporioides*, *C. acutatum*, and *C. horii*) have been reported as causal agents of persimmon anthracnose in South Korea. In this study, a collection of 50 isolates associated with persimmon anthracnose were collected from Sangju ($n = 25$) and Cheongdo-gun ($n = 25$), South Korea. The morphological characteristics of all 50 *Colletotrichum* isolates were similar, and it was difficult to identify the isolates to the species level. A subsample of eight isolates was characterized phylogenetically to ascertain species. BLAST search and phylogenetic analysis of the internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase

(GAPDH), and actin (ACT) genes revealed two species: *C. horii* as well as a previously unreported persimmon anthracnose causal agent *C. siamense*. *C. siamense* isolates were confirmed again by phylogenetic analysis of the ITS, ACT, GAPDH, calmodulin, and Apn2-Mat1-2 intergenic spacer partial mating type genes. Koch's postulates for *C. horii* and *C. siamense* were fulfilled, confirming the pathogenicity of the two species in persimmon fruit. Morphological characteristics (colony morphology and size and shape of conidia and appressoria) from two representative isolates support results of the phylogenetic analysis and match those of previous descriptions of *C. horii* and *C. siamense*.

Persimmon (*Diospyros kaki* L. f.) is one of the popular fruit in the world. Persimmon fruit are believed to originate from Far East Asia (Badenes et al. 2003). Ancient records indicate that the domestication of *D. kaki* may have begun as early as 450 BCE (Luo and Wang 2008). The largest producers of persimmon fruit are China, Korea, Japan, Vietnam, Nepal, Brazil, Italy, Israel, and Spain. According to records from the Food and Agriculture Organization, China, Korea, and Japan produced 4,472,527 tons of persimmon fruit in 2014, which is approximately 95% of the total production worldwide. South Korea is the second most abundant producer of persimmon fruit in the world, providing approximately 10% of the world's fruit (FAO 2014).

In South Korea, as in other countries of the world, persimmon anthracnose is a serious disease, causing significant losses in yield and a reduction in fruit marketability. A single host plant can be infected by multiple *Colletotrichum* spp. (Huang et al. 2013; Y. C. Wang et al. 2016). *Colletotrichum* spp. responsible for persimmon anthracnose disease include *Colletotrichum gloeosporioides*, *C. acutatum*, and *C. horii* in Korea (Kwon and Kim 2011; Kwon et al. 2013); *C. gloeosporioides*, *C. horii*, and *C. karstii* in China (J. Wang et al. 2016; Xie et al. 2010; Zhang 2008); *C. acutatum* in the United States (Williamson and Sutton 2010); *C. gloeosporioides* in Spain (Palou et al. 2015); and *C. gloeosporioides* and *C. horii* in Brazil (May De Mio et al. 2015). *C. gloeosporioides* is a very common cause of anthracnose disease in all persimmon-growing countries. *C. gloeosporioides* is also responsible for anthracnose disease in others plant species, including peach, blueberry, strawberry, almond, avocado, apple, and citrus plants (Hu et al. 2015). *C. gloeosporioides* is a complex consisting of 22 species plus one subspecies and includes *C. asianum*, *C. cordylinicola*, *C. fructicola*, *C. gloeosporioides*, *C. horii*, *C. kahawae* subsp. *kahawae*, *C. musae*, *C. nupharicola*, *C. psidii*, *C. siamense*, *C. theobromicola*, *C. tropicale*,

C. xanthorrhoeae, *C. aenigma*, *C. aeshynomenes*, *C. alatae*, *C. alienum*, *C. aotearoa*, *C. clidemiae*, *C. kahawae* subsp. *ciggaro*, *C. salsolae*, and *C. ti*, plus nom. nov. *C. queenslandicum* (Weir et al. 2012). It is very difficult to separate these species (*C. gloeosporioides* is a species complex) based on a few distinguishing morphological characteristics. Colony colors and growth rate, conidia size, and shape of appressoria are some of the characteristics used to distinguish species of *Colletotrichum* (Hu et al. 2015; Hyde et al. 2009). However, these criteria alone are not sufficient to identify *Colletotrichum* spp. because of variations in the phenotypes of species under different environmental conditions (Hyde et al. 2009; Weir et al. 2012). Many *Colletotrichum* spp. were synonymized with the name of *C. gloeosporioides* because of the round ends and cylindrical conidia (Phoulivong et al. 2010). Later, several synonymized *C. gloeosporioides* species were identified as members of the *C. acutatum* and *C. boninense* species complexes (Damm et al. 2012a,b).

The available DNA (various genomic regions) sequences of *Colletotrichum* spp. have become increasingly important for identification. *Colletotrichum* spp. can be identified by comparing DNA sequences of isolates, which overcomes the limitations of morphological identification (Cai et al. 2009). Most *Colletotrichum* spp. were identified based on sequences of the nuclear ribosomal internal transcribed spacer (ITS) region or a combination of ITS and TUB2 sequences, which have been shown to be insufficient to fully differentiate *Colletotrichum* spp. (Diao et al. 2017; Liu et al. 2016). Multilocus molecular characteristics are also broadly applied for the identification of *Colletotrichum* spp.

Despite several reports of persimmon anthracnose caused by *Colletotrichum* spp. in South Korea, the *C. gloeosporioides* species complex has not yet been identified among fungal isolates from persimmon. Therefore, our research objectives were to characterize *C. gloeosporioides* isolates from the main persimmon-growing area in South Korea by molecular and morphological analyses. The pathogenicity of different *Colletotrichum* spp. on persimmon fruit was also assessed.

Materials and Methods

Collection of diseased persimmon fruit. Sampling sites for this study were 10 farmers' fields, with 5 in Sangju and 5 in Cheongdo-gun in South Korea. In total, 50 diseased persimmon fruit, with 5 fruit

[†]Corresponding author: T. Chang; E-mail: thchang@knu.ac.kr

O. Hassan and J. Y. Jeon contributed equally to this work.

Accepted for publication 11 December 2017.

showing anthracnose disease symptoms (Fig. 1A and B), were collected from each field and brought to the laboratory. Diseased fruit were cleaned under running tap water to remove foreign material and then stored in sterile polythene bags until further use.

Isolation of causal agents. The presumed causal agents of anthracnose disease were isolated from necrotic tissue of the diseased fruit. Before the isolation process, diseased fruit were surface disinfected in 95% ethanol and washed in distilled water. A small piece of necrotic tissue (1 cm²) was cut from the inside of a lesion on each fruit, surfaces were disinfected with 0.5% sodium hypochlorite solution for 2 min, and the pieces were washed thrice in sterile distilled water to remove the sterilizing agent, dried by blotting, and then placed on water agar containing streptomycin (0.05 g/liter). Plates were incubated at 25°C until fungal hypha emerged from the necrotic tissue. After 2 to 3 days of incubation, individual hyphal tips were transferred to fresh potato dextrose agar (PDA). For pure cultures, isolates were hyphal tipped twice, and 50 isolates were collected and grouped (four groups) based on morphological observations. Eight isolates, with an equal number representing each group, were selected for phylogenetic and morphological analyses. For long-term storage, three to four plugs (5 mm in diameter from the mycelium) of fungal colonies of each isolate were placed in a tube containing sterile aqueous solution of 20% glycerol and stored at -20°C.

Genomic DNA extraction, polymerase chain reaction, and sequencing. Total genomic DNA of all representative isolates selected for phylogenetic analysis was extracted using a HiGene Genomic DNA Prep Kit (Yuseong-Gu) following the manufacturer's instructions. Fungal colonies, 3 to 4 days old and grown at 25°C in the dark on PDA, were used to extract genomic DNA. Preliminary analysis of the isolates used partial gene sequences of three genomic loci:

ITS, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and actin (ACT) genes. Four isolates were confirmed to be *C. horii* and four isolates were preliminarily identified as *C. siamense*. Four *C. siamense* isolates were further analyzed using partial gene sequences of five genomic loci: ITS, ACT, GAPDH, Apn2-Mat1-2 intergenic spacer partial mating type (Mat1-2) (ApMat), and calmodulin (CAL) genes. The primer pairs ITS-1F/ITS4, GDF/GDR, ACT-512F/ACT-783R, CL1C/CL2C, and AMF1/AMR1 were used to amplify and sequence the ITS (ITS1-5.8S-ITS2) region, GAPDH, ACT, CAL, and ApMat genes, respectively, from the genomic DNA (Carbone and Kohn 1999; Silva et al. 2012; Templeton et al. 1992; Weir et al. 2012; White et al. 1990). All primers used in this study and their sequences are listed in Table 1. All polymerase chain reactions (PCR) were performed using a SimpliAmp thermal cycler (Thermo Fisher Scientific Inc.). The 25-μl PCR mixture contained 2.5 μl of 10× Ex Taq buffer, 0.5 μl of dNTP mix, 1 μl of forward primer (10 pmol), 1 μl of reverse primer (10 pmol), 1 μl of genomic DNA, 0.4 μl of Taq DNA polymerase, and 18.2 μl of sterilized water (BIOFACT). PCR for the ITS region was programmed with an initial denaturation at 95°C for 4 min; followed by 34 cycles of 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 45 s; and a final elongation at 72°C for 7 min. For genes, the PCR program was largely identical with that used for ITS amplification, except for annealing temperatures. The annealing temperatures were adjusted to 55, 58, 59, and 62°C for GAPDH, ACT, CAL, and ApMat, respectively. The PCR products were electrophoresed in 1% agarose gels at 130 V for 25 min in 1× Tris-borate-EDTA buffer to confirm the success of the reactions and then purified using a HiGene PCR Purification Kit (Yuseong-Gu). Both strands of the amplified DNA were sequenced directly with the help of Macrogen, Inc.

Fig. 1. Diseased persimmon fruit showing typical anthracnose disease symptoms. **A** and **B**, Symptoms caused by *Colletotrichum* spp. on fruit in infected fruit. **C**, Lesions generated by *Colletotrichum siamense* in detached persimmon fruit at 10 days after inoculation (wounding method). **D**, Lesions generated by *C. horii* in detached persimmon fruit at 10 days after inoculation (wounding method).

Phylogenetic analysis. The sequences of the ITS region and GAPDH and ACT genes of the present isolates were compared with *Colletotrichum* spp. sequences previously deposited in GenBank (<https://blast.ncbi.nlm.nih.gov/>). Multiple sequence alignments were performed using the Clustal Omega multiple sequence alignment program of MEGA, version 6.0 (Tamura et al. 2013). The ITS, GAPDH, and ACT sequences of 24 taxa in the *C. gloeosporioides* species complex (Weir et al. 2012) were retrieved from GenBank as reference sequences (Table 2). Neighbor-joining (NJ) and maximum-parsimony (MP) trees were constructed using MEGA, version 6.0 (Tamura et al. 2013), based on a combined dataset of the ITS, GAPDH, and ACT sequences. The relative stability of the branches was evaluated by bootstrapping with 1,000 replications. Concatenated sequences of ITS, ACT, GAPDH, CAL, and ApMat genes from four *C. siamense* complexes with selected reference type strains in the Musae clade of the *C. gloeosporioides* species complex (Table 2) were generated (Weir et al. 2012). NJ phylogenetic analyses were performed using MEGA, version 6.0 (Tamura et al. 2013).

Morphological examination. From each species, two representative isolates of each species (*C. siamense*, isolates ICK-23 and ICK-47; and *C. horii*, isolates ICK-103 and ICK-111) were selected for morphological characterization. To observe colony morphology and determine the mycelium growth rate, mycelial discs (5 mm in diameter) were removed from the actively growing edge of 4-day-old cultures, and each mycelial disc was placed onto a 90-mm PDA plate. The plates were incubated at 25°C in the dark. Colony characteristics (color, size, and texture) were noted after 5 days, and colony diameter was recorded daily, with three replicate cultures of each isolate and two measurements per replicate. The average growth rate was calculated by determining the millimeter change per day. To better understand conidial and appressoria morphologies, we followed the slide culture technique. Square blocks of the medium (10 by 10 mm) were cut from the PDA plate and aseptically transferred to the middle of a slide. Four sides of the agar square were inoculated with mycelial fragments of the same representative isolate using a sterile loop. Cover slips were placed on the inoculated agar samples, and then the slides were placed inside Petri dishes containing moist filter paper on a glass support. The slides were incubated at 25°C in the dark for 7 days. Conidia and appressoria formed across the underside of the cover slip, and their shapes and sizes were measured with a stage micrometer under an Olympus BX43 microscope (Olympus Corporation) at $\times 400$ magnification. For each isolate, at least 20 conidia and appressoria were measured. Analysis of variance ($P < 0.05$) and least significant different tests were conducted using the statistical software package JMP.

Pathogenicity test. Representative isolates of *C. siamense* and *C. horii* were tested for pathogenicity on mature and green persimmon fruit in the laboratory to confirm their ability to cause anthracnose disease. Persimmon fruit of similar stages and sizes were collected from persimmon orchards, thoroughly washed with tap water, and surface sterilized with 70% ethanol and 1% NaOCl. The disinfected fruit were then rinsed in sterile distilled water, and each fruit was placed in a small plastic saucer and allowed to dry before inoculation. Conidial

suspensions were prepared from 5- to 7-day-old cultures in distilled water. The concentrations of spore suspensions were adjusted to 10^5 conidia/ml using a hemocytometer. The sterilized fruit were inoculated with conidial suspension of each isolate by wounding methods. For the wounding method, persimmon fruit were wounded by pricking with a needle to about 5 mm in depth and placing a 10- μ l droplet of conidial suspension over the wound. Distilled water was used as a negative control. The plastic saucers with the inoculated fruit (positive and negative controls) were transferred in a sterile plastic box containing moist tissue paper, the lid was fixed, and the cultures were incubated at 25°C and high humidity until the disease developed. Lesion diameter (LD) was measured with a ruler and disease incidence (DI) was expressed as the percentage of infected fruit with the total number of fruit inoculated. Five replicate fruit were tested per isolate and the experiment was performed twice. LD and DI data of the two independent experiments were combined because variance was homogenous. Then, LD and DI of the two species were compared using Duncan's multiple-range test ($\alpha = 0.05$) with the help of JMP Pro 10.0.0 software. The causal agents were reisolated on PDA and identified based on morphological and molecular analyses, as described above.

Results

Molecular identification. All of the isolates were identified based on the obtained ITS, ACT, and GAPDH sequences (Table 3). A BLAST search resulted in sequences from two *Colletotrichum* spp. of >99% sequence similarity: *C. siamense* and *C. horii* (Table 3). The isolates ICK-3, ICK-22, ICK-23, and ICK-47 were identified as *C. siamense* and ICK-84, ICK-91, ICK-103, and ICK-111 as *C. horii*. Although the ITS sequences of ICK-3 and ICK-22 matched with undefined *Colletotrichum* spp. and ICK-47 matched with *C. gloeosporioides*. Their (ICK-3, ICK-22, ICK-23, and ICK-47) ACT and GAPDH gene sequence were most similar to those of *C. siamense* isolates (BML15, LQ22, Xt-18-1, and LC3049). Short descriptions of the isolates ICK-84, ICK-91, ICK-103, and ICK-111 as *C. horii* are available in our previous article (Jeon et al. 2017).

Phylogenetic analysis. A phylogenetic analysis was conducted using the NJ and MP methods (MEGA, version 6.0) to compare the ITS, ACT, and GAPDH nucleotide sequences from eight isolates obtained in this study and reference strains of the *C. gloeosporioides* complex (Table 2). The eight isolates clustered into two large groups in the MP phylogenetic tree (Fig. 2) with 99% bootstrap support. The isolates ICK-3, ICK-22, ICK-23, and ICK-47 formed a clade with *C. siamense* (reference strains ICMP 12567, ICMP 17795, ICMP 18118, and ICMP 18575) and reference strains of the Musae clade of the *C. gloeosporioides* complex. In this clade, only ICK-23 formed a distinct clade with strains ICMP 17795 and ICMP 18118 (*C. siamense*). The resulting topology of NJ analysis was largely identical to the topology of MP analysis (Fig. 3). For more clarification, a phylogenetic tree was constructed based on the combined nucleotide sequences of ITS, ACT, GAPDH, CAL, and ApMat from ICK-3, ICK-22, ICK-23, and ICK-47 and reference strains in the Musae clade of the *C. gloeosporioides* complex, including *C. siamense* (Fig. 4). In this tree, isolates ICK-3, ICK-22, ICK-23, and ICK-47

Table 1. Primers used in this study, with sequences and sources

Gene ^z	Primer name	Direction	Sequence (5'–3')	References
GAPDH	GDF	Forward	GCC GTC AAC GAC CCC TTC ATT GA	Templeton et al. 1992
	GDR	Reverse	GGG TGG AGT CGT ACT TGA GCA TGT	Templeton et al. 1992
ITS	ITS-1F	Forward	CTT GGT CAT TTA GAG GAA GTA A	White et al. 1990
	ITS-4	Reverse	TCC TCC GCT TAT TGA TAT GC	White et al. 1990
CAL	CL1C	Forward	GAA TTC AAG GAG GCC TTC TC	Weir et al. 2012
	CL2C	Reverse	CTT CTG CAT CAT GAG CTG GAC	Weir et al. 2012
Actin	ACT-512F	Forward	ATG TGC AAG GCC GGT TTC GC	Carbone and Kohn 1999
	ACT-783R	Reverse	TAC GAG TCC TTC TGG CCC AT	Carbone and Kohn 1999
ApMat	AM-F	Forward	TCATTCTACGTATGTGCCCG	Silva et al. 2012
	AM-R	Reverse	CCAGAAATACACCGAACTTGC	Silva et al. 2012

^z GAPDH = glyceraldehyde-3-phosphate dehydrogenase, ITS = internal transcribed spacer, CAL = calmodulin, and ApMat = Apn2-Mat1-2 intergenic spacer partial mating type.

Table 2. GenBank accession numbers of *Colletotrichum* isolates used in this study for molecular data analyses

Species, isolate ^z	Origin	Source	GenBank accession number ^y				
			GAPDH	ITS	ACT	CAL	ApMat
<i>Colletotrichum siamense</i>							
ICK-3	Korea	This study	LC260529	LC260488	LC260525	LC260532	LC307171
ICK-22	Korea	This study	LC260530	LC260489	LC260526	LC260533	LC307172
ICK-23	Korea	This study	LC260531	LC260490	LC260527	LC260534	LC307173
ICK-47	Korea	This study	LC208834	LC208833	LC260528	LC208835	LC307174
ICMP17795	United States	GenBank	JX010051	JX010162	JX009506	JX009703	...
ICMP 12567	Australia	GenBank	JX009940	JX010250	JX009541	JX009697	...
ICMP18118	Nigeria	GenBank	JX009941	JX010163	JX009505	JX009701	...
ICMP 18575	Thailand	GenBank	JX010059	JX010256	JX009455	JX009717	...
ICMP 18578*	Thailand	GenBank	JX009924	...	FJ907423	FJ917505	JQ899289
syn. <i>C. jasminisambac</i>							
ICMP19118	Vietnam	GenBank	HM131497	...	HM131507	JX009713	JQ807841
syn. <i>C. hymenocallidis</i>							
ICMP18642	China	GenBank	JX010019	...	GQ856775	JX009709	JQ899283
<i>C. horii</i>							
ICK-84	Korea	This study	LC186036	LC186040	LC186032	...	...
ICK-91	Korea	This study	LC186035	LC186039	LC186031	...	...
ICK-103	Korea	This study	LC186038	LC186042	LC186034	...	...
ICK-111	Korea	This study	LC186037	LC186041	LC186033	...	...
ICMP 12942	New Zealand	GenBank	GQ329685	GQ329687	JX009533	...	...
ICMP12951	New Zealand	GenBank	GQ329683	GQ329689	JX009466	...	...
ICMP 17968	China	GenBank	GQ329682	JX010212	JX009547	...	...
ICMP 17970	Japan	GenBank	GQ329686	JX010213	JX009467	...	...
<i>C. aenigma</i>							
ICMP 18686	Japan	GenBank	JX009913	JX010243	JX009519	JX009684	...
ICMP 18608*	Israel	GenBank	JX010044	...	JX009443	JX009683	KM360143
<i>C. aeschynomenes</i>							
ICMP 17673*	United States	GenBank	JX009930	JX010176	JX009483	JX009721	KM360145
<i>C. alatae</i>							
ICMP 17919*	India	GenBank	JX009990	JX010190	JX009471	JX009738	KC888932
<i>C. alienum</i>							
ICMP 18703	New Zealand	GenBank	JX010030	JX010252	JX009528	JX009656	...
ICMP 12071*	New Zealand	GenBank	JX010018	...	JX009580	JX009654	KM360144
<i>C. aotearoa</i>							
ICMP 18532	New Zealand	GenBank	JX009906	JX010220	JX009544	...	...
<i>C. asianum</i>							
ICMP 18696	Australia	GenBank	JX009915	JX010192	JX009576	JX009723	...
ICMP 18580*	Thailand	GenBank	JX009915	...	JX009576	FJ917506	FR718814

(Continued on next page)

^y GAPDH = glyceraldehyde-3-phosphate dehydrogenase, ITS = internal transcribed spacer, ACT = actin, CAL = calmodulin, and ApMat = Apn2-Mat1-2 intergenic spacer partial mating type.

^z Asterisks (*) indicate ex-holotype or ex-epitype cultures.

formed a distinct clade with *C. siamense* from other strains in the Musae clade with high bootstrap support (80%). The isolates ICK-84, ICK-91, ICK-103, and ICK-111 formed a distinct clade with *C. horii* (reference strains ICMP 12942, ICMP 12951, ICMP 17968, and ICMP 17970), with reference strains from both the Musae and Kahawae clades and with a posterior probability of 99% (Fig. 2).

Morphological characterization of the *Colletotrichum* spp. isolates obtained in the present study. Morphological characteristics of two *Colletotrichum* spp. are given below in detail.

Isolates ICK-23 and ICK-47 (C. siamense). Fast-growing colonies of *C. siamense* on PDA were white to off-white, with dense whitish mycelia. The reverse color was creamy yellow to dull yellow toward the center, with a yellowish conidial mass across the colony (Fig. 5A and B). The mycelium growth rate was 7.20 to 8.77 mm/day, with a mean of 8.35 mm/day at 25°C on PDA. Single-celled conidia were observed across the colony. The conidia were cylindric, straight, nonseptate, and fusiform, with obtuse ends and guttulate (Fig. 5C). The size of conidia ranged from 12.0 to 18.6 µm (mean 15 µm) long and 3.5 to 6.1 µm (mean 5 µm) wide (Fig. 5C). Brown-colored appressoria, observed in 2-day-old slide cultures, were globose, with a few clavate, but became complex with irregular lobes after 7 days of incubation. The sizes of the appressoria were 11.4 to 19.1 × 5.2 ×

9.1 µm (mean: 14.2 × 7.3 µm) (Fig. 5D and E). There were no clear differences in morphological characteristics between the *C. siamense* isolates tested in this study.

Isolate ICK-103 and ICK-111 (C. horii). Colonies of *C. horii* isolates were cottony and were initially white and then became grayish as the cultures aged on PDA, with dark concentric zonation and regular margins in reverse (Fig. 5A and B). The mycelium growth rate ranged 6.96 to 8.14 mm/day (mean: 7.51 mm) at 25°C on PDA. Few conidia (no conidial mass) were observed across the colony after 14 days of incubation at 25°C on PDA. Conidia were hyaline, one-celled, cylindrical, straight, a few slightly curved, obtuse apices, tapering toward the base and measuring up to 14.5 to 20.8 × 4.0 to 5.78 µm (mean: 17 × 4.5 µm) (Fig. 6C). Appressoria, arising from vegetative hyphae, were observed on the sterile covers slips. They were short, cylindric, light brown to brown, smooth, and mostly uniform in outline, with a few irregularly lobed (Fig. 6D and E). The sizes of the appressoria were 9.5 to 13.8 × 5.5 to 8.4 µm (mean: 11.1 × 7 µm) (Fig. 6 D and E). The morphological characteristics of both isolates tested in this study were similar.

Pathogenicity of *C. siamense* and *C. horii*. All tested *Colletotrichum* isolates caused anthracnose lesions on detached persimmon fruit inoculated by the wounding method (Fig. 1D and C). No lesions developed on fruit inoculated with water. *C. siamense* isolates

Table 2. (Continued from previous page)

Species, isolate ^z	Origin	Source	GenBank accession number ^y				
			GAPDH	ITS	ACT	CAL	ApMat
<i>C. boninense</i> ICMP 17904	Japan	GenBank	JX009905	JX010292	JX009827	...	...
<i>C. clidemiae</i> ICMP 18706	United States	GenBank	JX009909	JX010274	JX00947	...	...
<i>C. cordylinicola</i> ICMP 18579	Thailand	GenBank	JX009975	JX010226	HM470235	...	...
<i>C. fruticola</i> ICMP 12568	Australia	GenBank	JX009946	JX010166	JX009529	JX009680	...
ICMP 18581*	Thailand	GenBank	JX010033	...	FJ907426	FJ917508	JQ807838
<i>C. gloeosporioides</i> ICMP 18738	Australia	GenBank	JX009976	JX010151	JX009542	JX009730	...
ICMP 17821*	Italy	GenBank	JX010054	...	JX009558	JX009731	JQ807843
<i>C. hippeastri</i> ICMP 17920	The Netherlands	GenBank	JX009932	JX010293	JX009485	...	...
<i>C. kahawae</i> subsp. <i>ciggaro</i> ICMP 18728	Brazil	GenBank	JX010048	JX010239	JX009525	...	...
<i>C. musae</i> ICMP 12931	New Zealand	GenBank	JX009995	JX010140	JX009442	JX009688	...
ICMP 19119*	United States	GenBank	JX010050	...	JX009433	JX009742	KC888926
<i>C. nupharicola</i> ICMP 17938	United States	GenBank	JX009936	JX010189	JX009486	JX009661	...
ICMP 18658*	United States	GenBank	JX009972	...	JX009437	JX009663	JX145319
<i>C. psidii</i> ICMP 19120	Italy	GenBank	JX009967	JX010219	JX009515	...	...
<i>C. queenslandicum</i> ICMP 1780	Australia	GenBank	JX010010	JX010186	JX009504	JX009693	...
ICMP1778*	Australia	GenBank	JX009934	...	JX009447	JX009691	KC888928
<i>C. salsolae</i> ICMP 19051*	Hungary	GenBank	JX009916	JX010242	JX009562	JX009696	KC888925
<i>C. theobromicola</i> ICMP 18566	Australia	GenBank	JX009953	JX010282	JX009496	...	...
<i>C. ti</i> ICMP 4832	New Zealand	GenBank	JX009952	JX010269	JX009520	...	...
<i>C. tropicale</i> ICMP 18672	Japan	GenBank	JX010020	JX010275	JX009480	JX009722	...
ICMP 18653*	Panama	GenBank	JX010007	...	JX009489	JX009719	KC790728
<i>C. xanthorrhoeae</i> ICMP 17903	Australia	GenBank	JX009927	JX010261	JX009478	...	...

Table 3. Closest relatives of *Colletotrichum* spp. isolated from *Diospyros kaki* L. based on a BLAST search analyses of internal transcribed spacer (ITS), actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequences

Isolate	Closest organism in GenBank (accession number) based on		
	Resulting ITS sequences	Resulting actin sequences	Resulting GAPDH sequences
ICK 3	Uncultured <i>Colletotrichum</i> C203O7 (KF718216) Similarity: 100%	<i>C. siamense</i> BMLI15 (FJ907422) Similarity: 100%	<i>C. siamense</i> Xt-18-1 (KM053171) Similarity: 100%
ICK 22	Uncultured <i>Colletotrichum</i> C203O7 (KF718216) Similarity: 99%	<i>C. siamense</i> LQ22 (KX885157) Similarity: 100%	<i>C. siamense</i> LC3049 (KP703305) Similarity: 100%
ICK 23	<i>C. siamense</i> Cg363 (KX513879) Similarity: 100%	<i>C. siamense</i> LQ22 (KX885157) Similarity: 100%	<i>C. siamense</i> Xt-18-1(KM053171) Similarity: 100%
ICK 47	<i>C. gloeosporioides</i> 16-412(LC194223) Similarity: 99%	<i>C. siamense</i> LQ22 (KX885157) Similarity: 100%	<i>C. siamense</i> Xt-18-1(KM053171) Similarity: 100%
ICK 84	<i>C. horii</i> OGaC1 (KU239182) Similarity: 100%	<i>C. horii</i> CPC 20992 (KC566947) Similarity: 100%	<i>C. horii</i> C1069 (JX010001) Similarity: 100%
ICK 91	<i>C. horii</i> OGaC1 (KU239182) Similarity: 100%	<i>C. horii</i> CPC 20992 (KC566947) Similarity: 100%	<i>C. horii</i> C1069 (JX010001) Similarity: 100%
ICK 103	<i>C. horii</i> OGaC1 (KU239182) Similarity: 100%	<i>C. horii</i> CPC 20992 (KC566947) Similarity: 100%	<i>C. horii</i> C1069 (JX010001) Similarity: 100%
ICK 111	<i>C. horii</i> OGaC1 (KU239182) Similarity: 100%	<i>C. horii</i> CPC 20992 (KC566947) Similarity: 100%	<i>C. horii</i> C1069 (JX010001) Similarity: 100%

produced significantly larger lesions than those of *C. horii* isolates in inoculated fruit. LD of *C. siamense* were 17.26 to 22.85 mm, with mean of 19.80 ± 0.71 mm, whereas the LD of *C. horii* were 6.94 to 9.87 mm, with mean of 7.90 ± 0.52 mm. DI of both *Colletotrichum* spp. isolates were 100% (Table 4). Confirmation of pathogenicity was fulfilled by reisolating these species from the inoculated persimmon fruit. Conidia shape, size, and colony morphologies were identical to the originals, and the isolates were reidentified to the species level.

Discussion

Based on perceived scientific and economic importance, the genus *Colletotrichum* is considered the eighth most important group of

plant-pathogenic fungi in the world (Cannon et al. 2012; Dean et al. 2012). *C. gloeosporioides*, responsible for anthracnose on herbaceous and woody plants, is one of the most destructive species of *Colletotrichum* (over 700 species) (Dean et al. 2012). Persimmon anthracnose caused by *Colletotrichum* spp. has become increasingly common in South Korea. The main objectives of this study were to identify *Colletotrichum* isolates associated with persimmon anthracnose in key persimmon-growing areas of South Korea to the species level, based on morphology and a multigene phylogenetic analysis.

Colletotrichum spp. associated with persimmon anthracnose in Sangju and Cheongdo-gun, South Korea, were determined based on their phylogenetic affinities and pathogenicities. *C. siamense* and *C. horii*, which belong to the *C. gloeosporioides* species

Fig. 2. Phylogenetic analysis using the maximum-parsimony method to compare the nucleotide sequences of actin, glyceraldehyde-3-phosphate dehydrogenase, and internal transcribed spacer from the present isolates with those of other *Colletotrichum* spp. in the *Colletotrichum gloeosporioides* species complex retrieved from GenBank. Numbers above the branches represent bootstrap values.

Fig. 3. Phylogenetic analysis using the neighbor-joining method to compare the nucleotide sequences of actin, glyceraldehyde-3-phosphate dehydrogenase, and internal transcribed spacer from the present isolates with those of other *Colletotrichum* spp. in the *Colletotrichum gloeosporioides* species complex retrieved from GenBank. Numbers above the branches represent bootstrap values.

Fig. 4. Phylogenetic analysis using the neighbor-joining method to compare the nucleotide sequences of internal transcribed spacer, actin, glyceraldehyde-3-phosphate dehydrogenase, calmodulin, and Apn2-Mat1-2 intergenic spacer partial mating type from the present *Colletotrichum siamense* isolates with those of other *Colletotrichum* spp. within the Musae clade of the *C. gloeosporioides* species complex retrieved from GenBank. Numbers above the branches represent bootstrap values.

complex, were isolated and identified from Cheongdo-gun and Sangju, respectively. *C. siamense* was described in this study as a cause of anthracnose disease of persimmon in South Korea. *C. horii* was the only species from the *C. gloeosporioides* species complex

previously reported to cause anthracnose in persimmon in South Korea (Kwon and Kim 2011; Kwon et al. 2013).

Originally, *C. siamense* was isolated from coffee berries in Thailand (Prihastuti et al. 2009). *C. siamense* has also been reported as

Fig. 5. Culture and morphological characteristics of *Colletotrichum siamense* isolated from *Diospyros kaki*. Colony on potato dextrose agar after 7 days of incubation at 25°C **A**, from above; **B**, from below; **C**, conidia; **D**, conidia and appressoria; **E**, appressoria. Scale bars: C = 20 µm and D = 10 µm.

Fig. 6. Culture and morphological characteristics of *Colletotrichum horii* isolated from *Diospyros kaki*. Colony on potato dextrose agar after 7 days of incubation at 25°C **A**, from above; **B**, from below; **C**, conidia; **D**, conidia and appressoria; **E**, appressoria. Scale bars: C = 20 µm and D = 10 µm.

a disease-causing agent of *Persea americana*, *Pistacia vera*, *Hymenocallis americana*, *Dioscorea rotundata*, *Carica papaya*, *Cap-sicum annuum*, *Vitis vinifera*, *Fragaria* × *ananassa*, *Malus domestica*, and *Jasminum sambac* (Weir et al. 2012). *Colletotrichum horii* has been reported to cause minor signs of disease on inoculated fruit of *Cap-sicum annuum*, *Musa acuminata*, and *Cucurbita pepo* but it has not been shown to be responsible for disease in these hosts in the field (Weir et al. 2012). *Colletotrichum horii* is the species most frequently reported as a cause of persimmon anthracnose in the world (Jeon et al. 2017; Kwon and Kim 2011; Kwon et al. 2013; May De Mio et al. 2015; Weir and Johnston 2010; Xie et al. 2010; Zhang 2008).

C. siamense and *C. horii* have been isolated from astringent per-simmon (*Diospyros kaki*) in South Korea, although *C. horii* was previously isolated from sweet persimmon (Kwon and Kim 2011; Kwon et al. 2013). Phylogenetic analysis based on multigene sequences clearly differentiated between the two species. A phylogeny based on ITS, ACT, and GAPDH nucleotide sequences revealed that *C. horii* clustered in a distinct phylogenetic clade from other species of the *C. gloeosporioides* complex. ITS is considered to be the most informative gene marker to differentiate *C. horii* from all other *Colletotrichum* spp. (Weir et al. 2012). Morphological features of *C. horii* isolates in the present study were almost the same as those of *C. horii* isolates described by Weir and Johnston (2010) (Table 5).

ITS, ACT, and GAPDH nucleotide sequences from *C. siamense* were variable but the phylogenetic tree constructed from these genes revealed that all isolates clustered with *C. siamense* reference isolates, and that ICMP 12567, ICMP 17795, ICMP 18118, and ICMP 18575 clustered in the Musae clade of the *C. gloeosporioides* species complex. The combined gene analysis of ITS, ACT, and GAPDH did

not reliably separate *C. siamense* from the other *Colletotrichum* spp. in the Musae clade of the *C. gloeosporioides* complex. Identification of *C. siamense* was then confirmed by a combined ITS, ACT, GAPDH, CAL, and ApMat gene analysis, which best distinguished *C. siamense* species (Fig. 4). CAL is the most informative gene to differentiate *C. siamense* isolates from all other species, whereas the ApMat gene improves systematics of the *C. gloeosporioides* species complex (Silva et al. 2012; Weir et al. 2012).

The shape of the conidia and appressoria of the present isolates matched those of *C. siamense* described by Prihastuti et al. (2009) but they differed in size (Table 5). The conidia and appressoria of the present isolates were bigger than those of the reference *C. siamense* isolate. Morphological features may change in response to environment, growth media, and type of host (Hu et al. 2015; Hyde et al. 2009; Weir et al. 2012).

The identified *C. siamense* isolates and *C. horii* isolates showed distinct colony morphologies, with small variations in colony growth rates, conidia sizes and shape, and appressoria sizes and shape (Table 5). These variations were consistent with those of other *C. siamense* and *C. horii* (Weir et al. 2012).

The morphological characteristics of the present *C. siamense* and *C. horii* isolates overlapped with those of other species in the *C. gloeosporioides* complex (Weir et al. 2012). The analysis of the combined ITS, ACT, and GAPDH genes separated the above two taxa and supported the distinction between *C. horii* and other isolates in the *C. gloeosporioides* complex. An additional combined analysis of ITS, ACT, GAPDH, CAL, and ApMat genes supported the distinction of *C. siamense* from other isolates in the group. Recently, *C. siamense* has been recognized as a new species complex within the *C. gloeosporioides* complex, and this urgently needs phylogenetic reassessment (Doyle et al. 2013; Udayanga et al. 2013). Silva et al. (2012) reported that a new set of primers targeting the Apn2/MAT locus may be used to determine the phylogenetic relation of *C. siamense* with other members in the *C. gloeosporioides* complex. The problem in determining the placement of the tested *C. siamense* isolates was due to a lack of ApMat sequence data for novel species. These isolates may play a vital role in refining our understanding of the extent of cryptic diversification in *Colletotrichum* subcomplexes in the future.

All of the tested *Colletotrichum* isolates showed the same degree of virulence. Both groups of isolates (*C. horii* and *C. siamense*) were able to infect wounded persimmon fruit and produce black lesions. These black lesions are the visual sign of anthracnose disease. Though these experiments disregarded the importance of the epidermis as a resistance barrier to initial infection, the results proved that

Table 4. Lesion diameter and disease incidence of *Colletotrichum* spp. inoculated by wounding method in persimmon fruit^a

Species name	Lesion diameter on persimmon fruit (mm ± SE)	Disease incidence (%)
<i>Colletotrichum siamense</i> ICK-47	20.15 ± 1.40 a	100
<i>C. siamense</i> ICK-23	19.44 ± 0.67 a	100
<i>C. horii</i> ICK-103	7.64 ± 0.85 b	100
<i>C. horii</i> ICK-111	8.15 ± 0.75 b	100

^a SE = standard error. Treatment means followed by the same letter are not significantly different.

Table 5. Morphological characteristics of *Colletotrichum siamense* and *C. horii* species described in this study and closely related reference species

Characteristics	<i>C. siamense</i> (isolates from Thailand) ^y	<i>C. siamense</i> (present isolate)	<i>C. horii</i> (present isolate)	<i>C. horii</i> (isolate from Japan) ^z
Colony	At first white and becoming pale-brownish to pinkish, reverse pale yellowish to pinkish	White to off white, in reverse creamy yellow, dull yellow toward the center, yellowish conidial mass across the colony	White at first, became grayish over time, cottony, in reverse, darker concentric zone, margin of colony regular	Pale gray, cottony, conidia develop across the whole colony, in reverse, darker concentric bands, margin of colony regular
Conidia				
Shape	One-celled, smooth-walled, guttulate, hyaline, fusiform with obtuse to slightly rounded ends, sometimes oblong	Cylindric, straight nonseptate, fusiform with obtuse ends and guttulate	Cylindrical, straight, a few slightly curved, nonseptate, obtuse at both ends, a few tapering toward the base	Mostly cylindric, straight, ends broadly rounded, a few tapering toward the base
Size (μm)	7–18.3 × 3–4.3 (average: 10.18 × 3.46)	12.0–18.6 × 3.5–6.1 (average: 15 × 5)	14.5–20.8 × 4.0–5.78 (average: 17 × 4.5)	15–21 × 4–5.5 (average: 17.6 × 4.8)
Appressoria				
Shape	Brown, ovoid, sometimes clavate and often becoming complex with age	Brown, varied shapes, irregularly lobed, becoming complex with age	Short and cylindrical, light brown to brown, mostly uniform in outline, a few irregularly lobed	Short and cylindrical, usually uniform in outline, a few irregularly lobed
Size (μm)	4.7–8.3 × 3.5–5 (6.67 × 4.13)	11.4–19.1 × 5.2–9.1 (average: 14.2 × 7.3)	9.5–13.8 × 5.5–8.4 (average: 11.1 × 7)	9.5–13.5 × 6–8.5

^y Prihastuti et al. (2009).

^z Weir and Johnston (2010).

these two species of *Colletotrichum* were causal agents of anthracnose on persimmon. The enzyme cutinase produced by *C. truncatum* is very crucial to break down the structural defense of the host tissue (Auyong et al. 2015). Wounding in host tissue greatly enhance infectability of *Colletotrichum* spp. to cause disease (Pring et al. 1995).

In conclusion, here, we identified two species in the *C. gloeosporioides* complex that cause persimmon anthracnose in South Korea, based on morphological, molecular, and pathogenesis analyses. The identification of a new *Colletotrichum* spp. causing persimmon anthracnose reflects the importance of further research on *Colletotrichum* taxonomy to mitigate the risk to the South Korea persimmon fruit industry.

Literature Cited

- Auyong, A. S. M., Ford, R., and Taylor, P. W. J. 2015. The role of cutinase and its impact on pathogenicity of *Colletotrichum truncatum*. J. Plant Pathol. Microbiol. 6:259.
- Badenes, M., Garcés, A., Romero, C., Romero, M., Clavé, J., Rovira, M., and Llacer, G. 2003. Genetic diversity of introduced and local Spanish persimmon cultivars revealed by RAPD markers. Genet. Resour. Crop Evol. 50:579-585.
- Cai, L., Hyde, K. D., Taylor, P. W. J., Weir, B., Waller, J., Abang, M. M., Zhang, J. Z., Yang, Y. L., Phoulivong, S., and Liu, Z. Y. 2009. A polyphasic approach for studying *Colletotrichum*. Fungal Divers. 39:183-204.
- Cannon, P. F., Damm, U., Johnston, P. R., and Weir, B. S. 2012. *Colletotrichum*—Current status and future directions. Stud. Mycol. 73:181-213.
- Carbone, I., and Kohn, L. M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553-556.
- Damm, U., Cannon, P. F., Woudenberg, J. H. C., and Crous, P. W. 2012a. The *Colletotrichum acutatum* species complex. Stud. Mycol. 73:37-113.
- Damm, U., Cannon, P. F., Woudenberg, J. H. C., Johnston, P. R., Weir, B. S., Tan, Y. P., Shivas, R. G., and Crous, P. W. 2012b. The *Colletotrichum boninense* species complex. Stud. Mycol. 73:1-36.
- Dean, R., Van Kan, J. A., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J. J., Dickman, M., Kahmann, R., Ellis, J., and Foster, G. D. 2012. The top 10 fungal pathogens in molecular plant pathology. Mol. Plant Pathol. 13:414-430.
- Diao, Y.-Z., Zhang, C., Liu, F., Wang, W.-Z., Liu, L., Cai, L., and Liu, X.-L. 2017. *Colletotrichum* species causing anthracnose disease of chili in China. Persoonia 38:20-37.
- Doyle, V. P., Oudemans, P. V., Rehner, S. A., and Litt, A. 2013. Habitat and host indicate lineage identity in *Colletotrichum gloeosporioides* s.l. from wild and agricultural landscapes in North America. PLoS One 8:e62394.
- FAO. 2014. Crop production database FAOSTAT. Online publication. Food and Agriculture Organization of the United Nations (FAO). <http://faostat.fao.org/default.aspx>
- Hu, M. J., Grabke, A., and Schnabel, G. 2015. Investigation of the *Colletotrichum gloeosporioides* species complex causing peach anthracnose in South Carolina. Plant Dis. 99:797-805.
- Huang, F., Chen, G. Q., Hou, X., Fu, Y. S., Cai, L., Hyde, K. D., and Li, H. Y. 2013. *Colletotrichum* species associated with cultivated citrus in China. Fungal Divers. 61:61-74.
- Hyde, K. D., Cai, L., McKenzie, E. H. C., Yang, Y. L., Zhang, J. Z., and Prihastuti, H. 2009. *Colletotrichum*: A catalogue of confusion. Fungal Divers. 39:1-17.
- Jeon, J. Y., Hassan, O., Chang, T., Lee, D. W., Shin, J. S., and Oh, N. K. 2017. Anthracnose of Persimmon (*Diospyros kaki*) Caused by *Colletotrichum horii* in Sangju, Korea. Plant Dis. 101:1035.
- Kwon, J. H., and Kim, J. 2011. First report of fruit black spot of *Diospyros kaki* caused by *Colletotrichum acutatum* in Korea. Plant Pathol. J. 27:100.
- Kwon, J.-H., Kim, J., Choi, O., Gang, G.-H., Han, S., and Kwak, Y.-S. 2013. Anthracnose caused by *Colletotrichum horii* on sweet persimmon in Korea: Dissemination of conidia and disease development. J. Phytopathol. 161: 497-502.
- Liu, F., Tang, G., Zheng, X., Li, Y., Sun, X., Qi, X., You Zhou, Y., Xu, J., Chen, H., Chang, X., Zhang, S., and Gong, G. 2016. Molecular and phenotypic characterization of *Colletotrichum* species associated with anthracnose disease in peppers from Sichuan Province, China. Sci. Rep. 6: Article 32761.
- Luo, Z., and Wang, R. 2008. Persimmon in China: Domestication and traditional utilizations of genetic resources. Adv. Hortic. Sci. 22:239-243.
- May De Mio, L. L., Silva, F. A., Blood, R. Y., and Figueiredo, J. A. G. 2015. Twig blight and defoliation caused by *Colletotrichum horii* in persimmons in Brazil. Rev. Bras. Frutic. 37:256-260.
- Palou, L., Montesinos-Herrero, C., Tarazona, I., Besada, C., and Taberner, V. 2015. Incidence and etiology of postharvest fungal diseases of persimmon (*Diospyros kaki* Thunb. cv. Rojo Brillante) in Spain. Plant Dis. 99:1416-1425.
- Phoulivong, S., Cai, L., Chen, H., McKenzie, E. C., Abdelsalam, K., Chuakeitrote, E., and Hyde, K. 2010. *Colletotrichum gloeosporioides* is not a common pathogen on tropical fruits. Fungal Divers. 44:33-43.
- Prihastuti, H., Cai, L., Chen, H., McKenzie, E. H. C., and Hyde, K. D. 2009. Characterisation of *Colletotrichum* species associated with coffee berries in northern Thailand. Fungal Divers. 39:89-109.
- Pring, R. J., Nash, C., Zakaria, M., and Bailey, J. A. 1995. Infection process and host range of *Colletotrichum capsici*. Physiol. Mol. Plant Pathol. 46:137-152.
- Silva, D. N., Talhinas, P., Várzea, V., Cai, L., Paulo, O. S., and Batista, D. 2012. Application of the Apn2/MAT locus to improve the systematics of the *Colletotrichum gloeosporioides* complex: An example from coffee (*Coffea* spp.) hosts. Mycologia 104:396-409.
- Tamura, K., Stecher, G., Peterson, D., Filipinski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30: 2725-2729.
- Templeton, M. D., Rikkerink, E. H., Solon, S. L., and Crowhurst, R. N. 1992. Cloning and molecular characterization of the glyceraldehyde-3-phosphate dehydrogenase-encoding gene and cDNA from the plant pathogenic fungus *Glomerella cingulata*. Gene 122:225-230.
- Udayanga, D., Manamgoda, D. S., Liu, X., Chuakeitrote, E., and Hyde, K. D. 2013. What are the common anthracnose pathogens of tropical fruits? Fungal Divers. 61:165-179.
- Wang, J., Ai, C. X., Yu, X. M., An, M., Sun, S., and Gao, R. 2016. First report of *Colletotrichum karstii* causing anthracnose on persimmon leaves in China. Plant Dis. 100:532.
- Wang, Y. C., Hao, X. Y., Wang, L., Xiao, B., Wang, X. C., and Yang, Y. J. 2016. Diverse *Colletotrichum* species cause anthracnose of tea plants (*Camellia sinensis* (L.) O. Kuntze) in China. Sci. Rep. 6: Article 35287.
- Weir, B. S., and Johnston, P. R. 2010. *Characterisation* and neotypification of *Gloeosporium kaki* Hori as *Colletotrichum horii* nom. nov. Mycotaxon 111: 209-219.
- Weir, B. S., Johnston, P. R., and Damm, U. 2012. The *Colletotrichum gloeosporioides* species complex. Stud. Mycol. 73:115-180.
- White, T. J., Bruns, T., Lee, S. B., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to the Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
- Williamson, S. M., and Sutton, T. B. 2010. First report of anthracnose caused by *Colletotrichum acutatum* on persimmon fruit in the United States. Plant Dis. 94:634.
- Xie, L., Zhang, J., Cai, L., and Hyde, K. D. 2010. Biology of *Colletotrichum horii*, the causal agent of persimmon anthracnose. Mycology 1:242-253.
- Zhang, J.-Z. 2008. Anthracnose of persimmon caused by *Colletotrichum gloeosporioides* in China. Asian Australas. J. Plant Sci. Biotechnol. 2:50-54.