Taxonomic Re-evaluation of *Colletotrichum gloeosporioides* Isolated from Strawberry in Korea

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For the past two decades, the causal agent of anthracnose occurring on strawberry in Korea was considered *Colletotrichum gloeosporioides*. However, the recent molecular analysis has shown that the genus *Colletotrichum* has undergone many taxonomic changes with introduction of several new species. As a result, it revealed that *C. gloeosporioides* indeed consisted of more than 20 distinct species. Therefore, the Korean pathogen isolated from strawberry should be reclassified. The shape and size of the conidia of the pathogen were not distinctly different from those of *C. gloeosporioides* and *C. fructicola*, but it differed in shape of the appressoria. A combined sequence analysis of partial actin, glyceraldehydes-3-phosphate dehydrogenase genes, and the internal transcribed spacer regions showed that the strawberry isolates formed a monophyletic group with authentic strains of *C. fructicola*. On the basis of these results, the anthracnose fungi of the domestic strawberry in Korea were identified as *C. fructicola* and distinguished from *C. gloeosporioides*.

Keywords : anthracnose, *Colletotrichum fructicola*, strawberry

Anthracnose crown rot, to which all currently grown strawberry (*Fragaria × ananassa* Duch.) cultivars are susceptible, is a major disease in over 30% of nurseries and after transplanting stage in Korea (Nam et al., 2009). The causal pathogen *Colletotrichum gloeosporioides* was first reported by Kim et al. (1992).

C. gloeosporioides that occurs in many crops is a species aggregate containing a number of polymorphic subgroups which show varying degrees of pathogenicity, host-specificity and genetic homogeneity (Hyde et al., 2009a). However, the recent epitypification of *C. gloeosporioides* has enabled accurate classification by comparative analysis

with pathogens isolated from the various plants (Cannon et al., 2008). The sequence analysis of the type strains was an important element in the research of identification and phylogenetic relationship of *Colletotrichum* (Cai et al., 2009; Phoulivong et al., 2010). Anthracnose pathogens, previously reported to be *Colletotrichum* sp., that occur on coffee in Thailand were shown as three new species of *C. asianum*, *C. fructicola*, and *C. siamense* based on the multi-gene sequence analysis and morphological characteristics (Prihastuti et al., 2009). In the past few years, anthracnose pathogens from tropical fruits, which were identified as *C. gloeosporioides* based on their morphological characteristics, are subdivided into *C. asianum*, *C. fructicola*, *C. horii*, *C. kahawae*, and *C. gloeosporioides* by comparing of the nucleotide sequences with that of the *C. gloeosporioides* epitype (Phoulivong et al., 2010). As a result, many species of *Colletotrichum*, including *C. gloeosporioides*, have been defined, mainly based on the results of molecular phylogenetic analysis (Damm et al., 2010). Although the internal transcribed spacer (ITS) sequence do not separate *C. gloeosporioides* complex, some single genes or combinations of genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glutamine synthetase, can be used to reliably distinguish most taxa (Weir et al., 2012).

Therefore, *C. gloeosporioides*, previously reported pathogen on strawberries in Korea, is necessary to re-evaluate, taking into account the changed species boundary and the recent phylogenetic results of the genus *Colletotrichum*.

Isolation and culture of strains. Eleven strains isolated from strawberry plants from 2005 to 2011 (Table 1). Diseased petiole, roots, and crown tissues were surface sterilized by dipping in 2% NaOCl for 2 min, rinsed thrice with sterile water, and dried on sterile tissue paper. Samples were then placed on water agar and incubated at 27°C. The growing edges of any fungal hyphae developing from the tissue were then transferred aseptically to potato dextrose agar (PDA; Difco, Detroit, MI, USA). Single spore isolations were also carried out using the procedure described

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Table 1. Sources and pathogenicity test of *Colletotrichum* isolates used in this study

Isolates	Isolated year	Cultivar	Plant part	Origin	Pathogenicity test (DI) ^a	Sequence analysis ^b	
						ACT	GAPDH
CGF050604	2005	Johong	Crown	Busan	1.3 ± 0.7	K1	K1
CGF060617	2006	Redpearl	Crown	Gangneung, Gangwon	1.0 ± 0.7	K1	K1
CGF071204	2007	Seolhyang	Root	Nonsan, Chungnam	3.0 ± 0.6	K2	K2
CGF080805	2008	Kumhyang	Petiole	Nonsan, Chungnam	1.8 ± 0.7	K2	K2
CGF080806	2008	Seolhyang	Petiole	Nonsan, Chungnam	1.7 ± 0.8	K2	K2
CGF080901	2008	Seolhyang	Crown	Buyeo, Chungnam	2.5 ± 0.7	K2	K2
CGF081103	2008	Seolhyang	Crown	Nonsan, Chungnam	1.5 ± 0.5	K2	K2
CGF090501	2009	Seolhyang	Crown	Gyeryong, Chungnam	0.5 ± 0.2	K2	K2
CGF090701	2009	Seolhyang	Crown	Nonsan, Chungnam	2.3 ± 0.6	K2	K2
CGF100713	2010	Sinseolmae	Crown	Eumseong, Chungbuk	3.2 ± 0.4	K2	K2
CGF110101	2011	Seolhyang	Crown	Iksan, Jeonbuk	3.0 ± 0.4	K2	K2

^aDI (Disease index): 0, healthy; 1, < 50% petioles affected; 2, > 50% petioles affected; 3, wilted; 4, necrosis formed on the entire plants.

^bHaplotypes of the Korean isolates.

by Prihastuti et al. (2009). Pure cultures were stored at 4°C on PDA slants.

Pathogenicity test. Each isolated pathogen was prepared at 1×10^5 conidia/mL and sprayed 1 mL per plant on the Seolhyang cultivar of strawberry plants. Six plants per an isolate were used for test. The inoculated plants were incubated in a dew plastic house at 27°C and 100% relative humidity (RH) for 2 days and then moved to a plastic house held between 24°C and 30°C. After 60 days, disease index on each plant was rated on a scale of 0–4: 0, healthy; 1, < 50% of petioles affected; 2, > 50% of petioles affected; 3, wilted; 4, necrosis formed on the entire plant (Nam et al., 2006). Disease index data from isolates were subjected to analysis of means and standard error. All data analysis was performed using the Costat program (CoHort software, Berkeley, CA). All isolates caused anthracnose symptoms

on the Seolhyang strawberry cultivar (Table 1). Disease index of the isolates was the highest (DI > 3) for CGF071204, CGF100713, and CGF110101. Smith and Black (1990) showed that pathogenicity of *C. gloeosporioides*, *C. acutatum*, and *C. fragariae* can be differentiated by factors such as strawberry tissue and cultivars. *C. gloeosporioides* and *C. acutatum* were reported to have a wide host range, while *C. fragariae* were restricted to strawberry plants (Freeman et al., 1998; Hyde et al., 2009b). *Colletotrichum* isolated from strawberry in Korea and Japan was pathogenic to strawberry and apple fruit (Nam et al., 1998), and avocado, broad bean, common sowthistle, cyclamen, pea, and strawberry under non-wound inoculation condition (Okayama and Tsujimoto, 1994), respectively.

Morphological and cultural characteristics. Isolates were measured for colony characteristics, and shape and size of

Table 2. Morphological characteristics of *Colletotrichum* species from strawberry

Species	Colony characters	Conidia			Appressoria			Growth rate (mm/day)
		Length (μm)	Width (μm)	Shape	Length (μm)	Width (μm)	Shape	
<i>Colletotrichum</i> sp. (CGF050604, 060617, 071204, 080806, 090701)	White, becoming grey to dark grey at the center	14.6±0.47, n = 10	3.99±0.11, n = 10	Cylindrical	9.93±0.32, n = 10	4.90±0.10, n = 10	Ovoid, sometimes clavate	9.98±1.8, n = 8
<i>C. gloeosporioides</i> (Prihastuti et al., 2009)	Grey, becoming dark grey to black	8-11	3-4.5	Cylindrical	4.5-10	4-7.5	Circular to slightly irregular	N/A
<i>C. fructicola</i> (Prihastuti et al., 2009)	Cottony, dense pale grey aerial mycelium, grey to dark grey colony, fast growing	11.37±0.96 (9.7-14)	3.54±0.35 (3-4.3)	Cylindrical	7.54±1.55 (4.3-11.7)	4.35±0.85 (3-7.3)	Ovoid, sometimes clavate	10.72±0.53 (9.67-11.5)
<i>C. fragariae</i> (Gunnell and Gubler, 1992)	Pale mouse grey to dark mouse grey	14-22.5	3.5-5	Cylindrical	N/A	N/A	N/A	13-14

the conidia after 7 days of incubation on PDA media. The colony diameters were measured daily for 7 days, and the growth rate for these 7 days was calculated as the average of mean daily growth (mm per day). The colors of the conidial masses and zonation were recorded at 7 days. Appressoria were produced using a slide culture technique, in which a conidial suspension was placed in a hole on the glass slide and covered with a cover slip. The slide was placed in an empty Petri dish with wet filter paper and incubated at 28 °C for 2–4 days. Colonies of the isolates on

PDA first appeared as white, then becoming grey to dark grey at the center over time, and growth rate was shown 9.98 ± 1.8 mm per day (Table 2). The shape and size of the conidia were cylindrical and $14.6 \pm 0.47 \times 3.99 \pm 0.11$ μ m, respectively. The shape and size of the appressoria were ovoid, sometimes clavate and $9.93 \pm 0.32 \times 4.90 \pm 0.10$ μ m, respectively (Table 2). Prihastuti et al. (2009) reported that the colony characteristics and conidial shape and size of *C. fructicola* were similar to those of *C. gloeosporioides*, but that ovoid, sometimes clavate appressoria in *C. fructicola*

Table 3. Reference sources of *Colletotrichum gloeosporioides* complex isolates used in this study

Species	Source	GenBank accession number		
		ITS	GAPDH	ACT
<i>C. aenigma</i>	ICMP 18608 ^{Ta}	JX010244	JX010044	JX009443
<i>C. aeshynomenes</i>	ICMP 17673 ^T	JX010176	JX009930	JX009483
<i>C. alatae</i>	ICMP 17919 ^T	JX010190	JX009990	JX009471
<i>C. alienum</i>	ICMP 12071 ^T	JX010251	JX010028	JX009572
<i>C. aotearoa</i>	ICMP 18537 ^T	JX010205	JX010005	JX009564
<i>C. asianum</i>	ICMP 18580 ^T	FJ972612	JX010053	JX009584
<i>C. boninense</i>	ICMP 17904 ^T	JX010292	JX009905	JX009583
<i>C. clidemiae</i>	ICMP 18658 ^T	JX010265	JX009989	JX009537
<i>C. cordylinicola</i>	ICMP 18579 ^T	JX010226	JX009975	HM470235
<i>C. fructicola</i>	ICMP 18581 ^T	JX010165	JX010033	FJ907426
<i>C. fructicola</i> (syn. <i>C. ignotum</i>)	ICMP 18646 ^(T)	JX010173	JX010032	JX009581
<i>C. fructicola</i> (syn. <i>Glomerella cingulata</i> var. <i>minor</i>)	ICMP 17921 ^(T)	JX010181	JX009923	JX009495
<i>C. gloeosporioides</i>	ICMP 17821 ^T	JX010152	JX010056	JX009531
<i>C. gloeosporioides</i> (syn. <i>Gloeosporium pedemontanum</i>)	ICMP 19121 ^(T)	JX010148	JX010054	JX009558
<i>C. horii</i>	ICMP 10492 ^T	GQ329690	GQ329681	JX009438
<i>C. kahawae</i> subsp. <i>ciggaro</i>	ICMP 18539 ^T	JX010230	JX009966	JX009523
<i>C. kahawae</i> subsp. <i>ciggaro</i> (syn. <i>Glomerella cingulata</i> var. <i>migrans</i>)	ICMP 17922 ^(T)	JX010238	JX010042	JX009450
<i>C. kahawae</i> subsp. <i>ciggaro</i> (syn. <i>Glomerella rufomaculans</i> var. <i>vaccinii</i>)	ICMP 19122 ^(T)	JX010228	JX009950	JX009536
<i>C. kahawae</i> subsp. <i>kahawae</i>	ICMP 17816 ^T	JX010231	JX010012	JX009452
<i>C. musae</i>	ICMP 19119 ^T	JX010146	JX010050	JX009433
<i>C. nupharicola</i>	ICMP 18187 ^T	JX010187	JX009972	JX009437
<i>C. psidii</i>	ICMP 19120 ^T	JX010219	JX009967	JX009515
<i>C. queenslandicum</i>	ICMP 1778 ^T	JX010276	JX009934	JX009447
<i>C. salsolae</i>	ICMP 19051 ^T	JX010242	JX009916	JX009562
<i>C. siamense</i>	ICMP 18578 ^T	JX010171	JX009924	FJ907423
<i>C. siamense</i> (syn. <i>C. hymenocallidis</i>)	ICMP 18642 ^(T)	JX010278	JX010019	GQ856775
<i>C. siamense</i> (syn. <i>C. jasmini-sambac</i>)	ICMP 19118 ^(T)	HM131511	HM131497	HM131507
<i>C. theobromicola</i>	ICMP 18649 ^T	JX010294	JX010006	JX009444
<i>C. theobromicola</i> (syn. <i>C. fragariae</i>)	ICMP 17927 ^(T)	JX010286	JX010024	JX009516
<i>C. theobromicola</i> (syn. <i>C. gloeosporioides</i> f. <i>stylosanthi</i>)	ICMP 17957 ^(T)	JX010289	JX009962	JX009575
<i>C. ti</i>	ICMP 4832 ^T	JX010269	JX009952	JX009520
<i>C. tropicale</i>	ICMP 18653 ^T	JX010264	JX010007	JX009489
<i>C. xanthorrhoeae</i>	ICMP 17903 ^T	JX010261	JX009927	JX009478
<i>Glomerella cingulata</i> f. sp. <i>camelliae</i>	ICMP 10643	JX010224	JX009908	JX009540

^aT = ex-type or authentic, (T) = ex-type or authentic of synonymised taxon.

were different from circular to slightly irregular one in the latter species. Size of conidia and appressoria of the strawberry isolate differed from *C. fragariae* by Gunnell and Gubler (1992). Therefore, *Colletotrichum* isolates from strawberries in Korea were similar to *C. gloeosporioides* and *C. fructicola* in shape and size of conidia, but the shape of the appressoria was close to *C. fructicola* rather than *C. gloeosporioides*.

Phylogenetic analysis. The sequences of eleven strains from strawberry and the ex-type or authentic strains of anthracnose available from GenBank (Table 3) were used in this study. Genomic DNA was extracted by using the method of Park et al. (2005). For the amplification of the ITS, partial actin (ACT), and GAPDH genes, 3 different primer sets were used: ITS5 and ITS4 (White et al., 1990); ACT512F and ACT783R (Prihastuti et al., 2009); and GDF1 and GDR1 (Peres et al., 2008); respectively. The amplification was performed with Maxime polymerase chain reaction (PCR) PreMix (I-Taq, iNtRoN BioTechnology, Korea) in a final volume of 20 µl containing 10 pmol of each primer set and PCR conditions were performed as described by Prihastuti et al. (2009). PCR products were electrophoresed through a 1% agarose gel stained with ethidium bromide and purified using a PCR quick-spin PCR product purification kit (iNtRoN BioTechnology, Korea) according to the manufacturer's instructions. The nucleotide sequences were determined by Bioneer Corporation (Chungwon, Korea). The sequences were proofread, edited, and merged onto comparable sequences using the PHYDIT program version 3.2 (Chun, 1995). To determine the phylogenetic positions of the *Colletotrichum* isolates, the alignments of the 3 gene sequence of 11 Korean isolates and 34 reference sequences retrieved from GenBank were performed with Clustal × 1.81 (Thompson et al., 1997). Nucleotide sequences were aligned manually where necessary. A maximum likelihood tree was inferred in MEGA v. 5.05, using TN93+I that was selected as the most suitable nucleotide mode by Modeltest (Tamura et al., 2011). Bootstrap analysis was performed with 1,000 replications for branch stability. Sequences of ITS, ACT, and GAPDH regions of three isolates CGF080901, CGF090501, and CGF100713 were deposited in GenBank (JX125050,

JX125051, and JX125052; KC283023, KC283024, and KC283025; KC283017, KC283018, and KC283019, respectively).

PCR amplification of the ITS region generated 466 bp fragments for all isolates from strawberry plants. Sequence analysis of ITS region showed no sequence difference among all isolates and authentic of *C. fructicola* (ICMP 18581, ICMP 18646, and ICMP 17921) (data not shown).

PCR amplification of the ACT gene generated 217–226 bp fragments for all isolates from strawberry plants. Korean isolates present two haplotypes (K1 and K2) (Table 1). The ACT sequence of K1 (CGF050604 and CGF060617) differed from those of other nine Korean isolates (K2) by 3nt polymorphic characters at 14, 96, and 129 position and 7nt deletion at 23–29 position (Table 4). ACT sequence of K1 is identical to that of *C. fructicola* strain ICMP 17921 (syn. *Glomerella cingulate* var. *minor*), whereas K2 showed a sequence similarity of 99.4% with *C. fructicola* ICMP 18646 (syn. *C. ignotum*).

PCR amplification of the GAPDH genes generated 232 bp fragments for all isolates. K1 differed from those of other Korean isolates (K2) by 3nt polymorphic characters at 47, 57 and 117 position (Table 4). GAPDH sequence of K1 showed a sequence similarity of 99.5% with that of *C. fructicola* strain ICMP 17921, whereas K2 showed a sequence similarity of 99.1% with *C. fructicola* ICMP 18581 and *C. fructicola* ICMP 18646.

The combined analysis showed that the 11 strains isolated from Korea formed a monophyletic group with authentic of *C. fructicola* (ICMP 18581, ICMP 18646, and ICMP 17921) and clearly distinct from other *C. gloeosporioides* complex (Fig. 1). All isolates were divided into two subgroups: one subgroup (K1) contained two Korean isolates and *C. fructicola* strain ICMP 17921 with a bootstrap value of 77%. The other subgroup (K2) contained 9 Korean isolates and *C. fructicola* ICMP 18581 and *C. fructicola* ICMP 18646 with a bootstrap value of 63%.

Since *C. fructicola* was first isolated from the berries of *Coffea arabica* in the Chiang Mai region of Thailand (Prihastuti et al., 2009), it has been found in tropical fruits such as chili, papaya, and longan (Phoulivong et al., 2010). Recently, *G. cingulata* var. *minor* and *C. ignotum* placed in synonymy with *C. fructicola* (Weir et al., 2012). K1 was

Table 4. Polymorphisms in ACT and GAPDH for two haplotypes (K1 and K2) of Korean isolates

Haplotype	Nucleotide position												
	ACT										GAPDH		
	14	23	24	25	26	27	28	29	96	129	47	57	117
K1	T	-	-	-	-	-	-	-	T	T	G	A	C
K2	C	C	C	G	C	A	G	A	C	G	C	G	T

Fig. 1. The maximum-likelihood tree inferred from a combined dataset of ACT, GAPDH and ITS sequences showing phylogenetic relationships among *Colletotrichum* from strawberry plants in Korea and representative species of *C. gloeosporioides* complex. Numbers above each branch represent bootstrap values obtained after a bootstrap test with 1,000 replications. Bar indicates the number of nucleotide substitutions.

more closely related to *C. fructicola* strain ICMP 17921 (syn. *G. cingulata* var. *minor*) from *Ficus* than K2, whereas K2 grouped to *C. fructicola* ICMP 18581 and *C. fructicola* ICMP 18646 (syn. *C. ignotum*).

The *Colletotrichum* species isolated from strawberries worldwide are as follows: *C. gloeosporioides* (Gunnell and Gubler, 1992), *C. fragariae* (Smith et al., 1990), *C. acutatum* (Smith et al., 1986), *C. dematium* (Beraha and Wright, 1973), and *Gloeosporium* spp. (Wright et al., 1960), but as *C. gloeosporioides* (perfect stage: *G. cingulata*) in Korea (Kim et al., 1992; Nam et al., 1998). Three anthracnose stains (KACC 40695, KACC 40696, and KACC 40812) from strawberry plants stored in Korean Agricultural Culture Collection (KACC) were identified as *C. gloeosporioides* based on sequence analysis of ITS and partial β -tubulin gene and cultural characteristics (Kim et al., 2006). In ITS analysis, KACC 40695 and KACC 40812 formed the mono-

phyletic group with K2 and showed 100% sequence similarity among K2, while KACC 40696 was closely related to *C. aenigma* strain ICMP 18608, *C. alienum* strain ICMP 12071, and *C. siamense* ICMP 18578 (syn. *C. hymenocallidis*) and differed from ex-types of *C. fructicola* (data not shown). As we performed comparative analysis with three KACC strains using ITS sequence in the present study, phylogenetic analysis with combined genes will be needed to establish for accurate identification of the three stains. The *C. fragariae*, originally reported as a disease of strawberry in Florida, USA (Brooks, 1931), placed in synonymy with *C. theobromicola* (Weir et al., 2012). All isolates from Korea were clearly distinguished from *C. theobromicola* (syn. *C. fragariae*) based on morphological and molecular phylogenetic analysis.

In the present study, anthracnose fungi that occur in the domestic strawberry were identified as *C. fructicola* and

distinguished from *C. gloeosporioides* based on their morphological and molecular phylogenetic features.

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