Investigation of the *Colletotrichum gloeosporioides* Species Complex Causing Peach Anthracnose in South Carolina

Meng-Jun Hu, Anja Grabke, and Guido Schnabel, School of Agricultural, Forest and Environmental Sciences, Clemson University, Clemson, SC 29634

Abstract

Hu, M.-J., Grabke, A., and Schnabel, G. 2015. Investigation of the *Colletotrichum gloeosporioides* species complex causing anthracnose fruit rot of peach in South Carolina. Plant Dis. 99:797-805.

Colletotrichum gloeosporioides, *C. acutatum*, and *C. truncatum* are causal agents of anthracnose disease of peach in South Carolina, but more recent investigations show that *C. gloeosporioides* is a species complex that has not yet been investigated among peach isolates. A total of 28 *Colletotrichum* spp. isolates associated with peach fruit anthracnose were collected in 2012 from Chesnee (10 isolates), McBee (10 isolates), Monetta (2 isolates), and Ridge Spring (6 isolates), South Carolina. Morphological characteristics indicated that all 28 isolates belonged to the *C. gloeosporioides* species complex. Phylogenetic analysis of the combined calmodulin (*CAL*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and β -tubulin (*TUB2*) gene sequences identified two species, *C. siamense* and *C. fructicola*.

Cultural characteristics such as colony growth rate, shape and size of conidia, and appressoria from representative isolates of the two species largely matched previous descriptions for *C. siamense* and *C. fructicola*. Koch's postulates for *C. siamense* and *C. fructicola* were fulfilled, confirming pathogenicity of the two species on peach. A new, two-step multiplex PCR assay was developed to facilitate differentiation of the four known *Colletotrichum* spp. causing anthracnose of peach in South Carolina, including *C. acutatum*, *C. truncatum*, *C. siamense*, and *C. fructicola*. The first step distinguished *C. acutatum* from *C. truncatum* and the two members of the *C. gloeosporioides* species complex. The second assay distinguished *C. siamense* from *C. fructicola* isolates.

The filamentous fungal plant pathogen *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (teleomorph = *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk) causes anthracnose disease on various fruits including peach (Bernstein et al. 1995; Kim and Hong 2008; Ramsey et al. 1951), blueberry (Hartung et al. 1981; Smith et al. 1996), strawberry (Denoyes and Baudry 1995; Freeman et al. 2002; Smith and Black 1990), almond (Ogawa and English 1991), avocado (Stanley et al. 1996), apple (Bernstein et al. 1995; Shi et al. 1996), papaya (Gamagae et al. 2003), and citrus (Alahakoon and Brown 1994; Liyanage et al. 1992). The disease can develop on stems or twigs, leaves, and fruit of host plants. On peach, the pathogen often occurs in areas and during seasons with high rainfall and warm temperatures (Ogawa et al. 1995). Symptoms include sunken lesions with circular rings where conidia of the fungus are produced in acervuli. Anthracnose disease can occasionally cause yield loss in commercial peach orchards of the southeastern United States, especially during wet and warm summers.

Prior to the availability of DNA sequence data, taxonomic concepts were based on host specificity and morphological features such as colony growth rate, color of cultures, conidial size and shape, and shape of appressoria. However, many of the morphological features can change with repeated subculturing or vary under different growing conditions. Therefore, it is not sufficient to identify fungal species only based on morphological features (Weir et al. 2012). Penzig (1882) first proposed the name *Colletotrichum gloeosporioides* based on *Vermicularia gloeosporioides*, which was collected from citrus in Italy. Von Arx (1957) defined *C. gloeosporioides* as the anamorph of all fungi with the *G. cingulata* teleomorph and artificially expanded the spore size range for *C. gloeosporioides* to overcome the instability of spore morphology from different hosts or measured under different cultural conditions. As a consequence, large numbers of *Colletotrichum* strains

with cylindrical conidia from various studies were synonymized with the name *C. gloeosporioides* (Phoulivong et al. 2010). Some of the synonymy was subsequently found to be incorrect. For example, *C. acutatum* (Simmonds 1965) and *C. boninense* (Moriwaki et al. 2003) were segregated from *C. gloeosporioides* defined by von Arx (1957). In addition, several species regarded as synonyms of the *C. gloeosporioides* sensu von Arx (1957) were later identified to be members of the *C. acutatum* or the *C. boninense* species complex (Damm et al. 2012a, b).

Cannon et al. (2008) used a *C. gloeosporioides* isolate from an Italian orange as epitype. Living strains and sequence data are available from the epitype so that *Colletotrichum* isolates can be compared by DNA sequence analyses to identify species, overcoming the inadequacies of traditional morphological identification (Cai et al. 2009; Hyde et al. 2009a). More than 86% of the ITS sequences from isolates designated as *C. gloeosporioides* in GenBank were not conspecific with the *C. gloeosporioides* epitype (Cai et al. 2009; Hyde et al. 2009b). Therefore, multi gene loci, which have more informative sites compared with the ribosomal ITS regions, along with morphological analyses were used to distinguish *Colletotrichum* species (Cai et al. 2009; Prihastuti et al. 2009; Yang et al. 2009). Recently, Weir et al. (2012) systematically described the *C. gloeosporioides* species complex based on phylogenetic analyses of up to eight genes. He reported 22 species plus one subspecies within the *C. gloeosporioides* species complex. All taxa accepted within this clade were morphologically similar to the broadly defined *C. gloeosporioides*, as it has been applied in the literature for the past 50 years (Weir et al. 2012).

Bernstein et al. (1995) identified *C. gloeosporioides* and *C. acutatum* as causal agents of peach anthracnose. Recently, a third species, *C. truncatum*, was reported to cause anthracnose on peach in South Carolina (Grabke et al. 2014). Anthracnose symptoms were observed frequently on peach fruit grown in Aiken, Chesterfield, Saluda, and Spartanburg counties in South Carolina during a disease survey in August of 2012. Because the *C. gloeosporioides* species complex has not been investigated in depth for isolates causing peach anthracnose, there was potential for improving taxonomic delineation of existing species. The objectives of this study were to morphologically and phylogenetically characterize *C. gloeosporioides* isolates from key peach production areas in South Carolina and to develop

Corresponding author: G. Schnabel; Email: SCHNABE@clemson.edu

Accepted for publication 3 January 2015.

a rapid, PCR-based method that differentiates the identified *Colletotrichum* species.

Materials and Methods

Fungal isolates and culture conditions. Peach fruit showing symptoms of anthracnose disease were collected from commercial peach farms in South Carolina during summer 2012. Twenty-eight isolates were collected from different locations in South Carolina, which included Chesnee (10 isolates), McBee (10 isolates), Monetta (2 isolates), and Ridge Spring (6 isolates). The locations represent three key production areas in South Carolina, including the “Ridge” (Monetta and Ridge Springs), the “Piedmont” (Chesnee), and the “North of Midlands” (McBee). To isolate *Colletotrichum* spp. from these peaches, fruits were surface-sterilized in 10% bleach for 2 min, and then rinsed with sterile distilled water. Lesions were cut in half, and necrotic tissue from the inside of the fruit was placed on acidified potato dextrose agar (APDA). Plates were incubated at 22°C with a 12-h cycle of fluorescent light until sporulating colonies of *Colletotrichum* spp. had grown. For single-spore isolation, conidia were scraped off the plate using a sterile toothpick, and suspended in 1 ml sterile distilled water. Fifty microliters of the conidial suspension was spread on 1.5% (w/v) water agar plates and incubated at 22°C overnight. One germinated conidium was transferred onto potato dextrose agar (PDA) plates (Difco Laboratories, Sparks, NJ), and plates were incubated at 22°C with a 12-h cycle of fluorescent light to induce sporulation. Each isolate was stored as dried mycelium on filter paper (Whatman Inc., Maidstone, UK) at –20°C as previously described (Zhu et al. 2012). In addition, an isolate of *C. acutatum* (Schnabel et al. 2006) and an isolate of *C. truncatum* (Grabke et al. 2014), both from peach, were used to validate the identification method developed in this study.

DNA extractions. Isolates selected for phylogenetic analysis were grown on PDA at 25°C for 4 days in the dark. Single agar plugs containing actively growing mycelium were taken from the periphery of the advancing colonies and transferred to 250-ml flasks containing 40 ml of potato dextrose broth (PDB). Flasks were shaken at 110 rpm for 4 days at 25°C. Developing mycelium was removed from the broth, rinsed under sterile deionized water, and genomic DNA was subsequently extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions.

PCR amplification and sequencing. Gene nuclear sequences were obtained from the calmodulin (*CAL*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and β -tubulin (*TUB2*) genes, respectively. The *CAL* gene region was amplified from genomic DNA with primer pairs CL1C/CL2C (Weir et al. 2012). The standard *GAPDH* (Templeton et al. 1992) and *TUB2* (O’Donnell and Cigelnik 1997) primers yielded poor or nonspecific amplification. Therefore,

new primers were designed. Forward primer (GDF1) was designed for *GAPDH* amplification and reverse primer (TubR1) was designed for *TUB2* amplification based on the *C. graminicola* M1.001 (also known as M2) genome sequence and based on the *TUB2* sequence of a *C. gloeosporioides* isolate from GenBank (U14138), respectively (Table 1).

All amplifications were performed using the T100 Bio-Rad thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA) in a total volume of 25 μ l. The PCR mixtures contained 1 $\times$ PCR buffer, 20 ng template DNA, 0.6 μ M of each primer, 200 μ M of each dNTP, and 1 unit of Taq DNA polymerase (New England Biolabs, Ipswich, MA). The amplification parameters for the *CAL* gene consisted of an initial denaturation at 94°C for 3 min followed by 34 cycles of 94°C for 40 s, 57°C for 40 s, and 72°C for 1 min, and a final elongation step of 72°C for 5 min. The parameters for amplification of the *GAPDH* and *TUB2* genes were largely as described above, except that the annealing temperature were reduced to 55°C, and extension times were 30 s and 2 min for the *GAPDH* and *TUB2* amplifications, respectively. Unincorporated primers and dNTPs remaining in the PCR products were removed using ExoSAP-IT reagent according to the manufacturer’s instruction. Sequencing was conducted at Clemson University Genomics Institute (CUGI; Clemson, SC).

Phylogenetic analysis. Phylogenetic analysis was conducted to characterize and identify *Colletotrichum* species of peach. Weir et al. (2012) determined that *C. siamense* and *C. fruticola* are separate species. We performed an analysis for a combined data matrix of the three coding loci (*CAL*, *GAPDH*, and *TUB2*). Multiple sequence alignments were conducted using DNASTAR (DNASTAR Inc., Nevada City, CA) and CLUSTALX v. 2.1 (Larkin et al. 2007; Thompson et al. 1997). *CAL*, *GAPDH*, and *TUB2* nucleotide sequences of *Colletotrichum* species within the *Musae* clade (Weir et al. 2012) were obtained from GenBank as reference sequences, including those for *C. siamense*, *C. fruticola*, *C. aenigma*, *C. aescrynomenes*, *C. alienum*, *C. asianum*, *C. gloeosporioides*, *C. musae*, *C. nupharicola*, *C. queenslandicum*, *C. salsolae*, and *C. tropicale* (Table 2). Maximum parsimony (MP) method and maximum likelihood (ML) methods were used to carry out phylogenetic reconstruction respectively using MEGA version 6.0 (Tamura et al. 2013). The MP tree was inferred using the subtree-pruning-regrafting (SPR) search option and 1,000 random sequence additions (level = 1). For the ML tree, the following settings were used: the Tamura 3-parameter model, gamma distributed (5 categories), and heuristic method using SPR-extensive. The bootstrap consensus tree inferred from 1,000 replicates was used to represent the evolutionary history of the taxa analyzed (Felsenstein 1985).

Morphological and cultural characterization. Starter cultures were prepared by plating each isolate onto PDA at 25°C. Five-millimeter-diameter plugs were aseptically removed from the periphery of actively growing, 4-day-old cultures using a sterile cork borer.

Table 1. Primers used for PCR amplification and sequencing

Primer name	Primer sequence (5'→3')	Description
CL1C ^a	GAATTC AAGGAGGCCTTCTC	1) To amplify and sequence <i>CAL</i> in <i>Colletotrichum</i> spp 2) Species-specific primer for <i>C. fruticola</i> from <i>CAL</i>
CL2C ^a	CTTCTGCATCATGAGCTGGAC	To amplify <i>CAL</i>
GDF1	ATGGCTCCCATCAAGGTCG	1) To amplify and sequence <i>GAPDH</i> in <i>Colletotrichum</i> spp 2) Specific primer for <i>Colletotrichum</i> spp. from <i>GAPDH</i>
GDR ^b	GGGTGGAGTCGTA CTGAGCATGT	To amplify <i>GAPDH</i>
T1 ^c	AACATGCGTGAGATTGTAAGT	To amplify and sequence <i>TUB2</i> in <i>Colletotrichum</i> spp.
TubR1	TTCTGGACGTTGCGCATCTG	To amplify <i>TUB2</i> in <i>Colletotrichum</i> spp.
CALF1	ACAAGGACGGCGATGGTCAGT	Common forward primer for <i>C. siamense</i> , <i>C. fruticola</i> and <i>C. acutatum</i> from <i>CAL</i>
Cg-R	AACTCGTTGTCTACACCGCC	Specific primer for <i>C. gloeosporioides</i> species complex
Ca-R1	GTCAAAGACCTGTCTCTGAT	Species-specific primer for <i>C. acutatum</i>
C-GAPDH-R	TACTTGAGCATGTAGGCCTG	Specific primer for <i>Colletotrichum</i> spp. from <i>GAPDH</i>
CALR1	CCTACTCAGAGTCAACATCA	Species-specific primer for <i>C. fruticola</i> from <i>CAL</i>
G3F1	AGGTCGAGATCGTTGCCGTC	Same as C-GAPDH-R

^a Weir et al. 2012.

^b Templeton et al. 1992.

^c O’Donnell and Cigelnik 1997.

Each plug was placed onto a petri dish (90 mm diameter) filled with 20 ml of PDA and grown at 25°C in the dark. Three replicate cultures of each isolate were investigated. The colony diameter was measured daily for at least 5 days and the growth rate was calculated in millimeter per day. After 6 days of incubation, conidia were harvested in 200 µl of sterile water. The length and width of at least 20 conidia from each isolate were measured with a stage micrometer using an OLYMPUS BX41TF microscope (Olympus Optical Co., LTD., Japan) at 400× magnification. For appressorial formation, two drops of a conidial suspension (20 µl; 10⁵ conidia/ml) were placed on a microscope slide and then placed inside petri dishes containing moist filter paper. The slides were incubated at 25°C in darkness for 2 days. Appressoria were formed from germ tubes and their sizes and shapes were measured as described above. Mean and standard deviation values were calculated for mycelial growth rate, the size of conidia, and appressoria.

Pathogenicity assay on detached fruit. Ripe but still firm peach fruit were washed with soap and water, surface sterilized for 2 min in 10% bleach, and rinsed with sterile distilled water. Wetted paper towels were placed on the bottom of a carbon fiber tray containing 18 perforated pockets. Fruit were placed in a randomized block design on 4 cm i.d. plastic rings located in the pockets of the tray and air dried overnight. For inoculation, a 10-µl droplet of conidial suspension prepared in sterile distilled water (1 to 2 × 10⁴ spores/ml) was placed on three equidistant points, each about 2 cm apart, on each peach fruit. Control fruit received sterile water without conidia. The trays were covered with domes, wrapped in a clear plastic bag for 2 days to keep the relative humidity high, and incubated at room temperature for 6 days. Disease incidence (DI) was expressed as the percentage of infected spots compared with the total number of spots inoculated. Lesion diameters (LDs) were measured with a ruler. Three isolates of each species were used for inoculation and six fruit were used for each isolate. The experiment was performed twice. Bartlett's test for equal variances was performed to test whether the LD data of the two independent experiments could be combined.

T-test and Fisher's exact test were used to determine whether the LD and DI of the two species were statistically different from each other, respectively. Analysis of variance and significant difference procedures were conducted using JMP Pro 10.0.0 software (SAS Institute Inc., Cary, NC).

Development of a multiplex PCR method to distinguish the *Colletotrichum* species. A two-step PCR assay was developed to distinguish among *Colletotrichum* species from peach. In the first PCR, five primers were combined to amplify fragments from both *CAL* and *GAPDH* genes. Primer pair GDF1 / C-GAPDH-R amplified a fragment from *GAPDH* from each of the four species as a positive control. Primer pair CALF1 / Cg-R amplified a fragment of *CAL* only from the *C. gloeosporioides* species complex and primer pair CALF1 / Ca-R1 amplified a fragment of *CAL* only from *C. acutatum*. The second PCR distinguished the two species in the *C. gloeosporioides* species complex. This PCR cocktail contained four primers, including primer pair G3F1 / C-GAPDH-R that amplified a *GAPDH* gene fragment from both species as a positive control. Primer pair CL1C / CALR1 amplified a fragment from the *CAL* gene from *C. fruticola* isolates but not from *C. siamense* isolates. *GAPDH* gene primers for species delineation were designed based on the aligned sequences from the *C. gloeosporioides* species complex (*C. siamense* and *C. fruticola*) isolates, *C. acutatum* (JQ948686), and *C. truncatum* (KC109617). *CAL* gene primers were designed based on the aligned sequences from *C. siamense*, *C. fruticola*, and *C. acutatum* isolates from our collection with the exception of the CL1C primer (Weir et al. 2012). PCR reactions were carried out in a volume of 25 µl containing 1 × PCR buffer, 200 µM of each dNTP, 20 ng template DNA, 0.25 µM of each primer, and 1 unit of Taq DNA polymerase (New England Biolabs, Ipswich, MA). For the first PCR, the amplification program consisted of an initial denaturation at 94°C for 4 min followed by 35 cycles of 40 s at 94°C, 40 s at 56°C, and 1 min at 72°C, and a final extension step at 72°C for 5 min. For the second PCR, amplification parameters were identical to the first PCR, except that the annealing temperature was increased to 58°C. Products were

Table 2. GenBank accession numbers of *Colletotrichum* isolates used in the phylogenetic analyses

Species	Culture	Source	GenBank accession number		
			<i>CAL</i>	<i>GAPDH</i>	<i>TUB2</i>
<i>C. siamense</i>	OD12-1	This study	KJ769234	KJ769240	KM245089
	EY12-1	This study	KJ769236	KJ769246	KM245086
	EY12-2	This study			KM245087
	EY12-3	This study		KJ769245	
	EY12-4	This study		KJ769242	
	EY12-7	This study	KJ769235	KJ769244	
	EY12-8	This study			KM245088
	RR12-4	This study		KJ769241	KM245090
	RR12-6	This study		KJ769243	KM245091
	ICMP 17795	GenBank	JX009703	JX010051	JX010393
	ICMP 18121	GenBank	JX009715	JX009942	JX010402
	ICMP 18642	GenBank	JX009709	JX010019	JX010410
<i>C. fruticola</i>	SE12-1	This study	KJ769237	KJ769248	
	RR12-1	This study	KJ769238		
	RR12-3	This study	KJ769239	KJ769247	KM245092
	RR12-5	This study			KM245093
	ICMP 18645	GenBank	JX009666	JX009992	JX010408
	ICMP 18646	GenBank	JX009674	JX010032	JX010409
	ICMP 18727	GenBank	JX009682	JX010035	JX010394
	ICMP 18608	GenBank	JX009683	JX010044	JX010389
<i>C. aenigma</i>	ICMP 18608	GenBank	JX009721	JX009930	JX010392
<i>C. aeshynomenes</i>	ICMP 17673	GenBank	JX009721	JX010028	JX010411
<i>C. alienum</i>	ICMP 12071	GenBank	JX009654	JX010028	JX010411
<i>C. asianum</i>	ICMP 18696	GenBank	JX009723	JX009915	JX010384
<i>C. gloeosporioides</i>	ICMP 17821	GenBank	JX009731	JX010056	JX010445
<i>C. musae</i>	ICMP 17817	GenBank	JX009689	JX010015	JX010395
<i>C. nupharicola</i>	ICMP 18187	GenBank	JX009663	JX009972	JX010398
<i>C. queenslandicum</i>	ICMP 1778	GenBank	JX009691	JX009934	JX010414
<i>C. salsolae</i>	ICMP 19051	GenBank	JX009696	JX009916	JX010403
<i>C. tropicale</i>	ICMP 18653	GenBank	JX009719	JX010007	JX010407

resolved on 1.5% agarose gel in 1 × TBE buffer for 1 h at 100v. Gels were stained with GelRed (Phenix Research, Candler, NC) and visualized using a Kodak EDAS 290 Electrophoresis Documentation and Analysis System (Kodak, Rochester, NY).

Results

Phylogenetic analysis of *CAL*, *GAPDH*, and *TUB2* sequences. Phylogenetic analysis was conducted using the combined sequences of the *CAL*, *GAPDH*, and *TUB2* genes from the 28 isolates of this study and reference strains (Table 2). There were 1,664 nucleotide positions included in the final dataset. In the MP phylogenetic tree (Fig. 1), the 28 isolates from South Carolina clustered into two large groups with high bootstrap support (99%). All isolates coded OD12, EY12, and isolates RR12-2, RR12-4, and RR12-6 formed a clade with *C. siamense* reference strains ICMP 17795, ICMP 18121, and ICMP 18642. This clade was most closely related to a clade formed by two reference isolates of the *C. aeshynomenes* and *C. tropicale* species. All isolates coded SE12, and isolates RR12-1 and RR12-5 clustered with *C. fruticola* reference strains ICMP 18646 and ICMP 18727, whereas isolate RR12-3 clustered with another *C. fruticola* reference strain ICMP 18645 (Fig. 1). The ML analysis of the combined DNA sequences generated a topological structure that was largely identical to the structure obtained using MP analysis (Fig. 2). *CAL*, *GAPDH*, and *TUB2* nucleotide sequences were deposited in GenBank (Table 2).

Colony morphology and mycelial growth rate. Distinct colony morphology on PDA medium was observed in *C. siamense* and *C. fruticola* after 6 days of incubation (Fig. 3A and B). Colonies produced by isolates from *C. siamense* varied in color from white or gray to pale brown with dense whitish-gray aerial mycelium and a few bright orange conidial masses near the center (Fig. 3A). Colonies produced by *C. fruticola* isolates were more uniform and were white or gray at first, and later became dark green with dense pale gray aerial mycelium (Fig. 3B).

The mean and range of colony growth rate on PDA medium were similar for *C. siamense* and *C. fruticola* (Table 3). The mean colony growth rates of *C. siamense* isolates were 12.9 ± 1.9 mm per day, ranging from 9.7 to 17 mm per day. The mean colony growth rate of *C. fruticola* was 13.1 ± 1.7 mm per day, ranging from 9.8 to 16.5 mm per day (Table 3).

Characterization of conidia and appressoria. There was little difference between the *C. siamense* and *C. fruticola* isolates in regard to the size and shape of conidia. Conidial shape of these two species were both cylindrical with obtuse to rounded ends. The mean length and width of conidia was $16.0 \pm 2.1 \times 4.9 \pm 0.2$ μ m, ranging from 8.4 to 20.5 $\times$ 4.8 to 6.0 μ m for *C. siamense*, whereas the mean length and width was $16.8 \pm 3.3 \times 5.0 \pm 0.5$ μ m, ranging from 8.4 to 26.5 $\times$ 4.8 to 6.0 μ m for *C. fruticola* (Table 3). Appressorial shapes produced by *C. siamense* were ovate,

Fig. 1. Phylogeny of the *Colletotrichum* isolates from peach and species within the *Musae* clade of the *Colletotrichum gloeosporioides* complex. Maximum parsimony (MP) tree inferred from the data set containing the combined DNA sequences of *CAL*, *GAPDH*, and *TUB2*. The numbers labeled at each node indicate the bootstrap (BS) percentage ($n = 1,000$). The consistency index, retention index, and composite index for parsimony-informative sites were 0.679245, 0.915842, and 0.622081, respectively, and the composite index for all sites was 0.790787. There were a total of 1,664 positions in the final dataset.

whereas the shape varied from clavate to ovate in *C. fruticola* isolates. The average appressorial size of *C. fruticola* isolates was larger than that for *C. siamense* isolates (Table 3). The mean length and width of appressoria was $7.3 \pm 1.0 \times 4.8 \pm 0.8 \mu\text{m}$, ranging from 5.1 to 9.6×3.1 to $6.7 \mu\text{m}$ for *C. siamense*, whereas the mean length and width was $8.2 \pm 1.1 \times 5.1 \pm 0.6 \mu\text{m}$, ranging from 6.0 to 10.8×3.6 to $6.0 \mu\text{m}$ for *C. fruticola*.

Pathogenicity of *C. siamense* and *C. fruticola*. Both *C. siamense* and *C. fruticola* tested were pathogenic and sporulated on peach fruit. Koch's postulates were fulfilled by reisolating the fungus from symptomatic fruit and reidentifying the pathogen to the species level (data not shown). LD data of each isolate of the two independent experiments was merged, because variances between experiments were equal ($P > 0.05$). The average LD of *C. fruticola* isolates was significantly larger than that of *C. siamense* isolates ($P < 0.05$). The mean LD for *C. fruticola* was $1.57 \pm 0.34 \text{ cm}$, and ranged from 0.60 to 2.15 cm, whereas the mean LD for *C. siamense* was $1.40 \pm 0.30 \text{ cm}$, ranging from 0.70 to 1.85 cm. There was no significant difference between the DI values of *C. siamense* isolates and *C. fruticola* isolates ($P > 0.05$). The average DI value of *C. siamense* isolates was 92.6% and ranged from 91.7 to 94.4%, whereas the average value for *C. fruticola* isolates was 95.4% ranging from 88.9 to 100% (Table 4). *C. siamense* and *C. fruticola* isolates produced largely identical symptoms, except that the lesions on fruit inoculated

with *C. siamense* developed a denser, whiter mycelium than those of *C. fruticola* isolates (Fig. 4A and B).

Development of a molecular tool to distinguish *Colletotrichum* species affecting peach. A two-step multiplex PCR method was developed to differentiate South Carolina *Colletotrichum* species on peach. The first PCR amplified a 359-bp fragment from the *GAPDH* gene with primer pair GDF1 / C-GAPDH-R from all *Colletotrichum* species. The cocktail also contained forward primer CALF1 and reverse primers Cg-R and Ca-R1, which produced amplicons 649 or 491 bp in length from the *C. gloeosporioides* species complex (*C. siamense* and *C. fruticola*) or *C. acutatum*, respectively (Fig. 5A). For the second PCR, primer pair G3F1 / C-GAPDH-R generated a 280-bp fragment from the *GAPDH* gene from both *C. siamense* and *C. fruticola* and primer pair CL1C / CALR1 generated a 550-bp fragment from the *CAL* gene from *C. fruticola*, but not from *C. siamense* (Fig. 5B). All isolates used in this study for the phylogenetic analysis and pathogenicity tests were subjected to this test and produced the expected amplicon sizes with this new multiplex PCR tool.

Discussion

The main objective of this study was to identify and characterize the *Colletotrichum* species associated with anthracnose of peach in South Carolina based on DNA sequence data and morphological characters. All isolates collected for this study were

Fig. 2. Maximum likelihood (ML) tree inferred from the data set containing the combined DNA sequences of *CAL*, *GAPDH*, and *TUB2*. The tree with the highest log likelihood (−3,934.3901) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ method. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.1303)). There were a total of 1,664 positions in the final dataset.

micromorphologically typical of *C. gloeosporioides* according to von Arx (1970) and Sutton (1992). However, previous identification methods were based on morphology alone, which cannot unequivocally place an isolate in this species complex. For example, species in *C. gloeosporioides* species complex (Cannon et al. 2012; Weir et al. 2012) are micromorphologically similar to *C. cliviae* (Yang et al. 2009) and members of the *C. boninense* species complex (Damm et al. 2012b). Weir et al. (2012) defined the limit of the *Colletotrichum* species complex genetically on the basis of up to eight gene sequences, and delimited 22 species within the species complex that formed a strongly supported clade based on ITS sequences (Cannon et al. 2012). It clearly indicated 22 species plus one subspecies within the *C. gloeosporioides* species complex. But further analyses determined that not all eight gene sequences are necessary to reliably

distinguish *Colletotrichum* species; *GAPDH* and *TUB2* gene sequences distinguished species reliably for most taxa within this species complex (Weir et al. 2012). In addition, *CAL* gene sequences distinguished between *C. siamense* and *C. fructicola* better than sequences of ITS, *GAPDH*, *TUB2* (β -tubulin 2), *ACT* (actin), *CHS-1* (chitin synthase), *GS* (glutamine synthetase), or *SOD* (manganese superoxide dismutase) (Weir et al. 2012). In this study, the combined sequence data of *GAPDH*, *TUB2*, and *CAL* clearly identified *C. gloeosporioides* isolates from South Carolina to be either *C. siamense* or *C. fructicola* when compared with sequences available in GenBank.

This is the first time *C. siamense* and *C. fructicola* were isolated from peach. Both species were originally isolated from coffee berries in northern Thailand (Prihastuti et al. 2009). According

Fig. 3. Characteristics of single spore isolates of *Colletotrichum* species grown on potato dextrose agar medium at 25°C in darkness for 6 days. **A**, Colonies of *Colletotrichum siamense* (isolates EY12-9, RR12-4, and EY12-1 from left to right). **B**, Colonies of *C. fructicola* (isolates SE12-1, RR12-1, and RR12-3 from left to right).

Table 3. Morphological characterization of *Colletotrichum siamense* and *C. fructicola*

Species (isolate)	Colony characteristics	Mycelial growth rate (mm per day)	Conidial shape and size (μm)	Appressorial shape and size (μm)
<i>C. siamense</i> (RR12-6, EY12-9, OD12-2, OD12-9, EY12-4, RR12-4, EY12-5, EY12-1, RR12-2)	White, becoming pale brown or greenish gray; reverse pale yellowish or greenish	9.7 to 17 Average = 12.9 ± 1.9 $n = 43$	Cylindrical, with obtuse to rounded ends 8.4 to 20.5×4.8 to 6.0 Average = $16.0 \pm 2.1 \times 4.9 \pm 0.2$ $n = 205$	Ovate, medium brown 5.1 to 9.6×3.1 to 6.7 Average = $7.3 \pm 1.0 \times 4.8 \pm 0.8$ $n = 53$
<i>C. fructicola</i> (RR12-5, SE12-1, SE12-2, RR12-1)	White, gray to dark gray with age; reverse white at margin, greenish black	9.8 to 16.5 Average = 13.1 ± 1.7 $n = 20$	Cylindrical, with obtuse to rounded ends 8.4 to 26.5×4.8 to 6.0 Average = $16.8 \pm 3.3 \times 5.0 \pm 0.5$ $n = 90$	Clavate to ovate, medium brown 6.0 to 10.8×3.6 to 6.0 Average = $8.2 \pm 1.1 \times 5.1 \pm 0.6$ $n = 24$

to Weir et al. (2012), these two species are biologically and geographically diverse. Besides infecting *Coffea*, *C. siamense* has been found on many hosts including *Persea americana*, *Pistacia vera*, *Hymenocallis americana*, *Dioscorea rotundata*, *Carica papaya*, *Capsicum annuum*, *Vitis vinifera*, *Fragaria* × *ananassa*, *Malus domestica*, and *Jasminum sambac*, whereas *C. fructicola* was isolated from *Pyrus pyrifolia*, *Limonium*, *Malus domestica*, *Fragaria* × *ananassa*, *Persea americana*, *Ficus*, *Dioscorea*, *Theobroma*, and *Tetragastris* (Weir et al. 2012). *C. siamense* and *C. fructicola* have been isolated from strawberry and *C. siamense* from

blueberry from the southeastern United States, which along with this study supports their role as fruit pathogens in the southeast (Hu et al. 2015).

CAL, *GAPDH*, and *TUB2* nucleotide sequences from *C. siamense* isolates were variable but phylogenetic analysis revealed all isolates from our collection clustered around *C. siamense* reference isolates ICMP 17795, ICMP 18642, and ICMP 18121. The genetic variability may in part be explained by the geographic isolation of some of the distinct genotypes (e.g., OD isolates were collected 160 km apart from other isolates). Rojas et al. (2010) noted the occurrence of two distinct haplotype subgroups (A4-3 and A5-4) within their species concept of *C. ignotum* (*C. fructicola*), which was confirmed by Weir et al. (2012). Together, the haplotype subgroups were monophyletic within the *Musae* clade of the *C. gloeosporioides* species complex. Both clades include isolates from a wide range of hosts from many countries, and both are similar in cultural appearance and morphology (Weir et al. 2012). Two isolates from group I (ICMP 18646 and ICMP 18727) and one isolate from group II (ICMP 18645; named “group I” and “group II” for the two subgroups in this study) of *C. fructicola* were selected as references for phylogenetic analysis. Results of this study showed that isolates from both groups existed on peaches from South Carolina and both occurred at the same location (Ridge Spring; Figs. 1 and 2). In comparison with the previous study (Weir et al. 2012), our MP and ML analyses indicated group II isolates of *C. fructicola* were more closely related to the species of *C. tropicale* and *C. aeshynomenes* than to group I isolates of *C. fructicola* (Figs. 1 and 2). The difference between the studies may be a result of different nucleotide sequences used in each phylogenetic analysis.

Table 4. Lesion diameter and disease incidence of *Colletotrichum* isolates on peach fruit^a

Isolates	Lesion diameter (cm)		Disease incidence (%)	
	Mean	SD	Mean	Mean of three isolates
<i>C. siamense</i>				
EY12-3	1.49	0.28	94.4	92.6
EY12-7	1.26	0.31	91.7	
RR12-2	1.46	0.28	91.7	
<i>C. fructicola</i>				
RR12-1	1.60	0.41	97.2	95.4
RR12-3	1.48	0.40	88.9	
SE12-1	1.62	0.20	100	

^a The mean and standard deviation values indicate the average values of two independent experiments.

Fig. 4. Symptoms of infection by *Colletotrichum siamense* and *C. fructicola* on peach fruit (cv. Red Globe) after 6 days of incubation at room temperature. **A**, Lesions resulting from infection by *C. siamense* (isolates EY12-7, EY12-2, and RR12-2 from left to right). **B**, Lesions resulting from infection by *C. fructicola* (isolates RR12-3, SE12-1, and RR12-1 from left to right).

In addition to the phylogenetic analysis, morphological features including cultural characteristics, colony growth rate, shape and size of conidia, Appressoria, and detached fruit assays were investigated in this study (Table 3). Cultural characteristics of *C. siamense* and *C. fructicola* isolates were in agreement with previous studies (Prihastuti et al. 2009; Yang et al. 2009). Colonies of *C. siamense* were either white with some gray or white with some orange, while *C. fructicola* colonies had dark gray centers and white peripheral mycelium. Moreover, the differences of conidia size between our *C. siamense* and *C. fructicola* isolates were consistent with the variation of conidia size for *C. siamense* and *C. fructicola* (Weir et al. 2012). For example, conidial size variation of these two species was similar within the *C. gloeosporioides* species complex. Mean length of *C. siamense* conidia was slightly shorter than that of *C. fructicola*, whereas the mean width of *C. siamense* conidia was nearly identical to that of *C. fructicola* (Weir et al. 2012). In addition, the colony growth rate and appressorial size of *C. fructicola* were higher and larger, respectively, compared with those of *C. siamense* when grown on PDA at 28°C in the dark, which was consistent with data published previously (Prihastuti et al. 2009). However, there were differences in size of conidia and appressoria between the studies. For example, the mean conidia length of our *C. siamense* isolates was 16.0 µm, whereas Prihastuti et al. (2009) reported a mean length of 10.17 µm. This may be a result of a different cultural condition or because isolates were from different hosts. Yang et al. (2009) used the same *C. siamense* isolates of the Prihastuti et al. (2009) study and determined conidial length to be 15.3 µm.

Four *Colletotrichum* species are now known to cause anthracnose fruit rot of peach in South Carolina. They are *C. siamense*, *C. fructicola* (both described in this study), *C. acutatum*, and *C. truncatum* (Grabke et al. 2014; Schnabel et al. 2006). The prevalence of anthracnose fruit rot is still very low in South Carolina according to a survey conducted at recent production meetings in Gaffney and Edgefield, SC. Previous studies had shown that *C. acutatum* and species within the *C. gloeosporioides* species complex are the most common species and that they can easily be confused based on morphological parameters (Damm et al. 2012a; Simmonds 1965). Therefore, a new PCR-based method was developed in this study to differentiate *Colletotrichum* spp. infecting peach. The method is based on a two-step PCR assay that distinguished the four species and included an internal PCR control in both steps to verify suitable PCR amplification material and conditions. The *Colletotrichum* isolates included in

this study were successfully identified as *C. siamense*, *C. fructicola*, or *C. acutatum* using our new PCR-based assay. Additional *C. acutatum* isolates in our collection that were characterized in a previous study (Schnabel et al. 2006) were also accurately identified (data not shown). We do not know the composition of species within the *C. gloeosporioides* complex from other peach production areas and therefore this PCR assay may have to be validated with isolates from other geographic areas. Also, this identification method may not be applicable to distinguish *C. gloeosporioides* and *C. acutatum* isolates from other hosts on a species level since both species represent species complexes that include more than 20 different species each (Damm et al. 2012a; Weir et al. 2012).

C. gloeosporioides and *C. acutatum* are common causal agents of anthracnose on different fruits (Adaskaveg and Hartin 1997; Bernstein et al. 1995; Kim and Hong 2008; Phoulivong et al. 2010). Bernstein et al. (1995) identified most of the isolates collected from peach in South Carolina to be *C. acutatum*; only one isolate of 19 was *C. gloeosporioides*. This is in sharp contrast to the collection examined in this study, as we only found two isolates of *C. acutatum* in the locations sampled (data not shown). This may have been due to our small sample size. However, a more likely explanation is based on inherent differences in fungicide sensitivity of *C. acutatum* compared with *C. gloeosporioides*. Bernstein et al. (1995) found that *C. acutatum* strains were more resistant to benzimidazole (also known as MBC) fungicides. Relative growth of *C. acutatum* isolates ranged between 17 and 47% on the discriminatory dose of 2 µg/ml benomyl, whereas none of the *C. gloeosporioides* isolates from peach grew at that dose. MBC fungicides were used more commonly at and prior to the Bernstein study for control of different fruit diseases compared with recent years, which may have given *C. acutatum* a selective advantage. However, MBC fungicides were gradually replaced by new site-specific fungicides for disease management over the past two to three decades due to benzimidazole resistance concerns in the major target pathogen *Monilinia fructicola* (Zehr et al. 1991).

In conclusion, this study establishes the existence of two species within *C. gloeosporioides* species complex causing anthracnose fruit rot of peach in South Carolina as documented by pathogenicity studies. Together with *C. acutatum* and *C. truncatum*, a total of four species are now known to cause peach anthracnose in the southeast. A molecular assay designed based on *GADPH* and *CAL* nucleotide sequences data was developed and used successfully to distinguish the four species.

Fig. 5. Multiplex PCR results for *Colletotrichum* species from peach. GeneRuler 100 bp DNA ladder (Thermo Scientific) is shown in lane M. In the first PCR (**A**), primer pair GDF1/C-GAPDH-R produced a 359-bp fragment (lanes 1 to 6) from all four species. Forward primer CALF1 and reverse primers Cg-R and Ca-R1 produced amplicons 649 bp (lanes 1 to 4) or 491 bp (lane 5) in length from the *C. gloeosporioides* species complex (*C. siamense* and *C. fructicola*) or *C. acutatum*, respectively. For the second PCR (**B**) primer pair G3F1/C-GAPDH-R produced a 280-bp fragment (lanes 1 to 4) from both *C. siamense* and *C. fructicola*. Primers CL1C and CALR1 produced a 550-bp fragment from *C. fructicola* (lanes 3 and 4) but not from *C. siamense* (lanes 1 and 2).

Acknowledgments

Technical Contribution No. 6350 of the Clemson University Experiment Station. This material is based upon work supported by NIFA/USDA, under project number SC-1700501 and SC Dept. of Agriculture Specialty Crop Block Grant Program.

Literature Cited

- Adaskaveg, J. E., and Hartin, R. J. 1997. Characterization of *Colletotrichum acutatum* isolates causing anthracnose of almond and peach in California. *Phytopathology* 87:979-987.
- Alahakoon, P. W., and Brown, A. E. 1994. Host-range of *Colletotrichum gloeosporioides* on tropical fruit crops in Sri-lanka. *Int. J. Pest Manage.* 40:23-26.
- Bernstein, B., Zehr, E. I., Dean, R. A., and Shabi, E. 1995. Characteristics of *Colletotrichum* from peach, apple, pecan, and other hosts. *Plant Dis.* 79:478-482.
- 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., Buddie, A. G., and Bridge, P. D. 2008. The typification of *Colletotrichum gloeosporioides*. *Mycotaxon* 104:189-204.
- Cannon, P. F., Damm, U., Johnston, P. R., and Weir, B. S. 2012. *Colletotrichum* – current status and future directions. *Stud. Mycol.* 73:181-213.
- 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.
- Denoyes, B., and Baudry, A. 1995. Species identification and pathogenicity study of French *Colletotrichum* strains isolated from strawberry using morphological and cultural characteristics. *Phytopathology* 85:53-57.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783-791.
- Freeman, S., Shalev, Z., and Katan, J. 2002. Survival in soil of *Colletotrichum acutatum* and *C. gloeosporioides* pathogenic on strawberry. *Plant Dis.* 86:965-970.
- Gamagae, S. U., Sivakumar, D., Wijeratnam, R. S. W., and Wijesundera, R. L. C. 2003. Use of sodium bicarbonate and *Candida oleophila* to control anthracnose in papaya during storage. *Crop Prot.* 22:775-779.
- Grabke, A., Williamson, M., Henderson, G. W., and Schnabel, G. 2014. First report of anthracnose on peach fruit caused by *Colletotrichum truncatum* in South Carolina. *Plant Dis.* 98:1154.
- Hartung, J. S., Burton, C. L., and Ramsdell, D. C. 1981. Epidemiological studies of blueberry anthracnose disease caused by *Colletotrichum gloeosporioides*. *Phytopathology* 71:449-453.
- Hu, M. J., Grabke, A., Dowling, M. E., Holstein, H. J., and Schnabel, G. 2015. Resistance in *Colletotrichum siamense* from peach and blueberry to thiophanate-methyl and azoxystrobin. *Plant Dis.* 99:806-814.
- Hyde, K. D., Cai, L., Cannon, P. F., Crouch, J. A., Crous, P. W., Damm, U., Goodwin, P. H., Chen, H., Johnston, P. R., Jones, E. B. G., Liu, Z. Y., McKenzie, E. H. C., Moriwaki, J., Noireung, P., Pennycook, S. R., Pfenning, L. H., Prihastuti, H., Sato, T., Shivas, R. G., Tan, Y. P., Taylor, P. W. J., Weir, B. S., Yang, Y. L., and Zhang, J. Z. 2009a. *Colletotrichum*-names in current use. *Fungal Divers.* 39:147-183.
- Hyde, K. D., Cai, L., McKenzie, E. H. C., Yang, Y. L., Zhang, J. Z., and Prihastuti, H. 2009b. *Colletotrichum*: a catalogue of confusion. *Fungal Divers.* 39:1-17.
- Kim, W. G., and Hong, S. K. 2008. Occurrence of anthracnose on peach tree caused by *Colletotrichum* species. *Plant Pathol. J.* 24:80-83.
- Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. 2007. Clustal W and clustal X version 2.0. *Bioinformatics* 23:2947-2948.
- Liyanage, H. D., McMillan, R. T., and Kistler, H. C. 1992. Two genetically distinct populations of *Colletotrichum gloeosporioides* from citrus. *Phytopathology* 82:1371-1376.
- Moriwaki, J., Sato, T., and Tsukiboshi, T. 2003. Morphological and molecular characterisation of *Colletotrichum boninense* sp. nov. from Japan. *Mycoscience* 44:47-53.
- O'Donnell, K., and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol. Phylogenet. Evol.* 7:103-116.
- Ogawa, J. M., and English, H. 1991. Diseases of Temperate Zone Tree Fruit and Nut Crops. Publ. 3345. University of California, Oakland, CA.
- Ogawa, J. M., Zehr, E. I., Bird, G. W., Ritchie, D. F., Uriu, K., and Uyemoto, J. K., eds. 1995. Compendium of Stone Fruit Diseases. The American Phytopathological Society, St. Paul, MN.
- Penzig, A. 1882. Fungi agrumicoli. Contribuzione allo studio dei funghi parassiti degli agrumi. *Michelia* 2:385-508.
- Phoulivong, S., Cai, L., Chen, H., McKenzie, E. C., Abdelsalam, K., Chuakeatirote, 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. Characterization of *Colletotrichum* species associated with coffee berries in northern Thailand. *Fungal Divers.* 39:89-109.
- Ramsey, G. B., Smith, M. A., and Heiberg, B. C. 1951. Anthracnose of peaches. *Phytopathology* 41:447-455.
- Rojas, E. I., Rehner, S. A., Samuels, G. J., Van Bael, S. A., Herre, E. A., Cannon, P., Chen, R., Pang, J. F., Wang, R. W., Zhang, Y. P., Peng, Y. Q., and Sha, T. 2010. *Colletotrichum gloeosporioides* s.l. associated with *Theobroma cacao* and other plants in Panama: multilocus phylogenies distinguish host-associated pathogens from asymptomatic endophytes. *Mycologia* 102:1318-1338.
- Schnabel, G., Chai, W., and Cox, K. D. 2006. Identifying and characterizing summer diseases on 'Babygold' Peach in South Carolina. *Plant Health Progress*, RS., 10.1094/PHP-2006-0301-01-PubMed
- Shi, Y., Correll, J. C., Guerber, J. C., and Rom, C. R. 1996. Frequency of *Colletotrichum* species causing bitter rot of apple in the southeastern United States. *Plant Dis.* 80:692-696.
- Simmonds, J. H. 1965. A study of the species of *Colletotrichum* causing ripe fruit rot in Queensland. *Queensl. J. Agr. Anim. Sci.* 22:437-459.
- Smith, B. J., and Black, L. L. 1990. Morphological, cultural and pathogenic variation among *Colletotrichum* species isolated from strawberry. *Plant Dis.* 74:69-76.
- Smith, B. J., Magee, J. B., and Gupton, C. L. 1996. Susceptibility of rabbiteye blueberry cultivars to postharvest diseases. *Plant Dis.* 80:215-218.
- Stanley, F., Talma, K., and Ezra, S. 1996. Characterization of *Colletotrichum gloeosporioides* isolates from avocado and almond fruits with molecular and pathogenicity tests. *Appl. Environ. Microbiol.* 62:1014-1020.
- Sutton, B. C. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. Pages 1-26 in: *Colletotrichum Biology, Pathology and Control*. CAB International, Wallingford, UK.
- Tamura, K., Stecher, G., Peterson, D., Filipski, 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. A., 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.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876-4882.
- von Arx, J. A. 1957. Die Arten der Gattung *Colletotrichum* Cda. *Phytopathol. Z.* 29:413-468.
- von Arx, J. A. 1970. A revision of the fungi classified as *Gloeosporium*. *Bibl. Mycol. Res.* 24:1-203.
- Weir, B. S., Johnston, P. R., and Damm, U. 2012. The *Colletotrichum gloeosporioides* species complex. *Stud. Mycol.* 73:115-180.
- Yang, Y. L., Liu, Z. Y., Cai, L., Hyde, K. D., Yu, Z. N., and McKenzie, E. H. C. 2009. *Colletotrichum* anthracnose of *Amaryllidaceae*. *Fungal Divers.* 39:123-149.
- Zehr, E. I., Toler, J. E., and Luszcz, L. A. 1991. Spread and persistence of benomyl-resistant *Monilinia fructicola* in South Carolina peach orchards. *Plant Dis.* 75:590.
- Zhu, F., Bryson, P. K., and Schnabel, G. 2012. Influence of storage approaches on instability of propiconazole resistance in *Monilinia fructicola*. *Pest Manag. Sci.* 68:1003-1009.