Characterization of *Colletotrichum* Species Causing Bitter Rot of Apple in Kentucky Orchards

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Abstract

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Multiple species of *Colletotrichum* can cause bitter rot disease of apple, but the identities and relative representation of the species causing the disease in Kentucky are unknown. In total, 475 *Colletotrichum* isolates were collected from diseased apple fruit in 25 counties and characterized both morphologically and by using various molecular approaches. Multigene sequence analyses revealed that sample isolates belonged to several newly erected species within the *Colletotrichum acutatum* and *C. gloeosporioides* species complexes. The isolates were identified as *C. fiorinae* and *C. nymphaeae*, which reside within the *C. acutatum* species complex, and *C. siamense*, *C. theobromicola*, and *C. fructicola*, which are placed within the *C. gloeosporioides* species complex. *C. fiorinae* was the most common species causing bitter rot in Kentucky, comprising more than 70% of the isolates. Infectivity tests on detached fruit showed that *C. gloeosporioides* species-complex isolates were more aggressive than isolates in the *C. acutatum* species complex. However, isolates within the *C. acutatum*

species complex produced more spores on lesions compared with isolates within the *C. gloeosporioides* species complex. Aggressiveness varied among individual species within a species complex. *C. siamense* was the most aggressive species identified in this study. Within the *C. acutatum* species complex, *C. fiorinae* was more aggressive than *C. nymphaeae*, causing larger, deeper lesions. Apple cultivar did not have a significant effect on lesion development. However, *Colletotrichum* spp. produced more spores on 'Red Stayman Wine-sap' than on 'Golden Delicious'. Fungicide sensitivity tests revealed that the *C. acutatum* species complex was more tolerant to thiophanate-methyl, myclobutanil, trifloxystrobin, and captan compared with the *C. gloeosporioides* species complex. The study also revealed that mycelial growth of *C. siamense* was more sensitive to tested fungicides compared with *C. fructicola* and *C. theobromicola*. These research findings emphasize the importance of accurate identification of *Colletotrichum* spp. within each species complex, because they exhibit differences in pathogenicity and fungicide sensitivity.

Bitter rot is one of the most common and economically important summer fruit rot diseases of apple in Kentucky (Bessin et al. 1994). It can cause significant losses, especially during long periods of warm, wet weather (Shi et al. 1995, 1996; Sutton 1990). Bitter rot of apple can be caused by several different species within the ascomycete fungal genus *Colletotrichum*. The most commonly reported causal species are *Colletotrichum acutatum*, *C. gloeosporioides*, and *Glomerella cingulata* (teleomorph of *C. gloeosporioides*) (González and Sutton 2004; González et al. 2006; Shi et al. 1995, 1996; Sutton 1990). Multiple species are often present within the same orchard or location but prevalence of specific *Colletotrichum* spp. seems to differ from one region to another (González and Sutton 2004; Shi et al. 1995, 1996).

The species causing bitter rot are known to vary in their aggressiveness and in their response to fungicides (Adaskaveg and Hartin 1997; Bernstein et al. 1995; Bragança et al. 2016; Freeman et al. 1998; Peres et al. 2002, 2004; Valero et al. 2010; Velho et al. 2014). Thus, it is important to make an accurate species diagnosis in order to optimize management protocols. However, it can be challenging to identify *Colletotrichum* spp. because they produce similar

symptoms on apple fruit and are often morphologically similar, and the morphology sometimes varies in response to environmental factors in culture (Adaskaveg and Hartin 1997; González et al. 2006; Gunnell and Gubler 1992). Molecular approaches have been widely employed to more effectively define and identify species of *Colletotrichum* (Freeman et al. 1998; Gunnell and Gubler 1992; Smith and Black 1990; Sreenivasaprasad and Talhinhos 2005). Combinations of morphologically based identification and molecular approaches have been demonstrated in some studies to accomplish reliable results for identification of *Colletotrichum* spp. (Brown et al. 1996; Freeman et al. 1998; González and Sutton 2004; Gunnell and Gubler 1992; Jelev et al. 2008; Mills et al. 1992; Sreenivasaprasad and Talhinhos 2005; Sreenivasaprasad et al. 1996).

Multigene sequence-based identification has recently emerged as the preferred method for taxonomy and species identification in the genus *Colletotrichum* (Cai et al. 2009; Cannon et al. 2012; Damm et al. 2012; Du et al. 2005; Sharma et al. 2013; Weir et al. 2012). With the application of multigene-based identification, the number of new *Colletotrichum* spp. has increased dramatically (Cannon et al. 2012; Damm et al. 2012; Weir et al. 2012) as the older species have been resolved into species complexes containing many distinct phylogenetic species. Thus, Weir et al. (2012) identified 22 *Colletotrichum* phylogenetic species within the *C. gloeosporioides* species complex, and Damm et al. (2012) named 31 new species within the *C. acutatum* species complex. Sharma et al. (2013) also proposed that *C. siamense* may be further divided into multiple species. Several of these newly described species have been associated with bitter rot of apple in North and South America and in Europe (Alaniz et al. 2012, 2015; Baroncelli et al. 2014; Ivic et al. 2013; Kou et al. 2014; Velho et al. 2014, 2015). The *C. acutatum* and *C. gloeosporioides* species complexes are known to differ in fungicide sensitivity (Adaskaveg and Hartin 1997; Bernstein et al. 1995; Freeman et al. 1998; Peres et al. 2002, 2004; Valero et al. 2010; Velho et al. 2014, 2015). A recent study by Hu et al. (2015) reported a significant difference in fungicide sensitivity between

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C. siamense and *C. fruticola*, two species within the *C. gloeosporioides* species complex causing anthracnose on peach and blueberry. This suggests that it will be important to identify phylogenetic species within complexes, and to relate these data to pathogenicity and fungicide sensitivity data, in order to optimize management of bitter rot of apple.

The objectives of this study were to identify the *Colletotrichum* spp. associated with bitter rot of apple in Kentucky and to determine which species were prevalent across the state, to determine the phylogenetic relatedness among the species and diversity within the species, and to characterize and compare the colonization potential and fungicide sensitivity of the species.

Materials and Methods

Isolation of *Colletotrichum* spp. from symptomatic apple fruit.

In 2013, 475 *Colletotrichum* isolates were recovered from apple fruit with symptoms of bitter rot collected from commercial orchards or private residences in 25 counties across Kentucky (Table 1). Of approximately 50 commercial orchards across Kentucky, 25 were included in the survey, and diseased fruit from the University of Kentucky Plant Disease Diagnostic Laboratory were collected. Many samples collected from the diagnostic laboratory were submitted by homeowners, and cultivars were mostly unknown. Fruit were stored for up to 7 days at 4°C prior to processing. Fruit were disinfested with 70% ethanol for 30 s and then air dried in a laminar flow hood. Three small pieces of fruit skin and pulp were taken from the outer margin of each lesion and cultured on potato dextrose agar (PDA; Difco) at 25°C with constant light for 7 to 20 days. Monoclonal isolation was performed according to Du et al. (2005), and isolates were stored as conidial suspensions on silica at -80°C (Tuite 1969).

Morphological characterization. Colony color, conidial shape and size, and the ability to produce perithecia in culture were evaluated for each isolate. Colony color was determined after 7 to 15 days on PDA at 23°C in constant light (Gunnell and Gubler 1992). Conidia were harvested from 14-day-old cultures and resuspended in sterile water (Du et al. 2005). Lengths and widths of 50 randomly chosen conidia were measured at ×400 under phase contrast (Du et al. 2005). Data were analyzed with univariate analysis and the Waller-Duncan *K* ratio *t* test available through the SPSS statistical analysis package (IBM, SPSS, Statistic version 21).

Table 1. Sample of *Colletotrichum* isolates from symptomatic fruit in Kentucky orchards

Origin (county)	Year of isolation	Number of isolates ^z				Total isolates
		M-1	M-2	M-3	M-4	
Clay	2013	1	...	...	...	1
Whitley	2013	6	4	...	...	10
Harlan	2013	4	1	1	...	6
Bourbon	2013	150	10	33	18	211
Jessamine	2013	2	...	...	...	2
Martin	2013	19	...	1	...	20
Woodford	2013	27	...	3	...	30
Owen	2013	...	...	...	1	1
Breathitt	2013	14	...	4	...	18
Fayette	2013	34	...	21	...	55
Cumberland	2013	4	...	3	...	7
Madison	2013	12	...	...	...	12
Clinton	2013	...	...	3	...	3
Knott	2013	3	...	...	...	3
Perry	2013	...	...	3	...	3
Nelson	2013	20	...	...	...	20
Bell	2013	11	...	1	...	12
Hancock	2013	4	...	...	...	4
Magoffin	2013	11	...	...	...	11
Lyon	2013	...	...	12	...	12
Marshall	2013	7	...	3	...	10
Montgomery	2013	...	...	17	...	17
Estill	2013	4	...	...	...	4
Allen	2013	2	...	...	...	2
Caldwell	2013	...	...	1	...	1

^z M-1, M-2, M-3, and M-4 indicate morphotypes 1 through 4, respectively.

Isolates were divided into four morphological groups based on colony color and spore shape (Table 1). In total, 26 isolates were selected for further study; these isolates included all four morphotypes and were collected from northern, eastern, central, and western Kentucky (Table 2). One to two randomly selected isolates from each morphotype was selected from each region of the state; in a few cases, some morphotypes were less common or not identified in some regions. Additional isolates of morphotype 1 from commercial orchards were also included because it was most prevalent in commercial orchards where yield losses were high.

Growth rate assay. Growth rate of *Colletotrichum* isolates was measured by using a modified race tube assay (Buiate 2015), originally developed by White and Woodward (1995). Linear mycelial growth was recorded at 3, 5, and 7 days postinoculation (dpi) on PDA in the dark at 18, 21, 23, 25, or 30°C. Three replications of each isolate at each temperature were evaluated. Growth rate was calculated as the 7-day average of mean daily growth rate (millimeters per day) for each culture and temperature. Growth rate data were analyzed with multivariate analysis and the Waller-Duncan *K* ratio *t* test, available through the SPSS statistical analysis package (IBM, SPSS, Statistic version 21).

Isolation of fungal DNA. Conidia from one 2-week-old PDA plate culture were inoculated into 10 ml of potato dextrose broth in 9-by-50-mm petri dishes. Cultures were incubated for 7 to 10 days at 23°C under continuous light. Mycelia were collected and lyophilized, and genomic DNA was extracted from the tissue with the method of Panaccione et al. (1988), as modified by Porebski et al. (1997).

Molecular characterization: Multigene amplification. PCR amplifications were performed for variable regions of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β -tubulin 2 (*TUB2*) genes. These two regions have previously been used to differentiate species within the *C. gloeosporioides* and *C. acutatum* complexes (Damm et al. 2012; Weir et al. 2012). Polymerase chain reaction (PCR) primer pairs were GDF1 (GCCGTCAACGACCCTTCATTGA) and GDR2 (GGGTGGAGTCGTAAGTTCAGCATGT) for *GAPDH* amplification (Templeton et al. 1992) and T1 (AACATGCGTGAGATTGTAAGT) and T2 (TAGTGACCCCTTGCCCAAGTTG) for *TUB2* amplification (O'Donnell and Cigelnik 1997). PCR was performed using the protocols of Weir et al. (2012).

Table 2. Test isolates of *Colletotrichum* spp. isolates from Kentucky orchards

Isolates ^z	Host (cultivar)	Origin (county)	Region	Morphotype
HC 296	Honeycrisp	Bourbon	North	1
KY 228	Unknown	Fayette	North	1
KY 95	Jona Gold	Madison	North	1
KY 123	Jonathan	Bourbon	North	1
HC 557	Honeycrisp	Bourbon	North	1
KY 28	Gibson Golden	Martin	East	1
KY 6	Empire	Harlan	East	1
KY 105A	Unknown	Whitley	East	1
KY 320	Unknown	Allen	Central	1
KY 162	Unknown	Nelson	Central	1
KY 291	Granny Smith	Marshall	West	1
KY 191	Unknown	Hancock	West	1
KY 9	Empire	Harlan	East	2
HC 646	Honeycrisp	Bourbon	North	2
HC 647	Honeycrisp	Bourbon	North	2
KY 263	Pink Lady	Montgomery	North	3
KY 254	Unknown	Lyon	West	3
KY 301	Granny Smith	Marshall	West	3
KY 146	Unknown	Clinton	Central	3
KY 305	Unknown	Cumberland	Central	3
KY 8	Unknown	Harlan	East	3
KY 152	Unknown	Perry	East	3
KY 153	Unknown	Perry	East	3
KY 128	Gold Rush	Bourbon	North	3
HC 540	Honeycrisp	Bourbon	North	4
KY 40	Unknown	Owen	North	4

^z Isolates were selected as representative of each morphotype and region where the isolates were collected.

Sequencing of PCR products. PCR products were purified using Qiagen's gel extraction protocol (Qiagen Inc.). Sequencing reactions were performed using the Sanger dideoxy chain termination method and the BigDye system (PE Applied Biosystems) (Du et al. 2005). Sequencing was performed by the Advanced Genetic Technologies Center, University of Kentucky, on an ABI 310 genetic analyzer (PE Applied Biosystems). Forward and reverse sequences were aligned and edited with Geneious Pro 4.5.7 software (Biomatter Ltd.). Homology searches to confirm species identity for the *GAPDH* and *TUB2* sequences were performed by using megablast against the GenBank nr database. Gene sequences were also submitted to the Q-bank fungal database (www.q-bank.eu) (Damm et al. 2012; Weir et al. 2012).

Phylogenetic analyses. Phylogenetic analyses were performed using MUSCLE for multiple alignment, Gblocks for automatic alignment curation, PhyML for tree building based on approximate likelihood ratio test, and TreeDyn for tree rendering, within the "One Click" mode on the website www.phylogeny.fr (Anisimova and Gascuel 2006; Chevenet et al. 2006; Dereeper et al. 2008, 2010; Edgar 2004; Guindon and Gascuel 2003).

Colonization tests. Colonization tests were performed as an examination of one component of pathogenicity. Experiments were executed in the laboratory with two apple cultivars: 'Golden Delicious', reported to be susceptible to bitter rot (Biggs and Miller 2001), and 'Red Stayman Winesap', reported to be moderately resistant (Roberts and Pierce 1935). Apple fruit used in this study had not been sprayed with fungicides and were obtained from the Horticulture Research Farm, Department of Horticulture, University of Kentucky, located in Lexington. Apple fruit were harvested in August 2014 and stored in the dark at 4°C for up to 2 months before use. Detached fruit were surface disinfested in 10% bleach for 5 min, rinsed in sterile distilled water, then air dried on sterile paper towels at room temperature.

Fruit were wounded with a dissecting needle that had been modified to produce a puncture of 0.5 mm in depth (Adaskaveg and Hartin 1997; Biggs and Miller 2001; Velho et al. 2014, 2015). The wounded fruit were inoculated by placing 6 µl of a conidial suspension (1×10^5 spores harvested from 10-day-old PDA cultures per milliliter of water) on the surface of the wound. Inoculated fruit were incubated in moist chambers for 14 days at room temperature (23 to 24°C in constant light). Lesion diameters were measured daily using a digital caliper. At 14 dpi, fruit were cut through the centers of lesions for measurements of lesion depth. The entire surface area of each lesion was then removed with a scalpel and submerged in 10 ml of sterile water with one drop of Tween 80 (Fisher Scientific) in a 50-ml tube (USA Scientific). Spores were dislodged from the tissue samples by vortexing for 10 s, and then conidia were filtered through a layer of cheesecloth. Spores were counted with a hemocytometer. The experiment was performed twice. The factorial calculation for experiment, replication, cultivar, species complex, species, and isolate was evaluated using an analysis of variance and means were separated using least square means, with Bonferroni correction provided in the statistical algorithms of SAS (version 9.3; SAS Institute).

Fungicide sensitivity assays. *Effect of fungicides on mycelial growth.* Fungicide sensitivity of isolates was tested on PDA amended with fungicides in 25-ml race tubes, as described above. Fourteen isolates were selected from the test isolates above; representative isolates were selected from each documented *Colletotrichum* sp. and from each region of the state. Fungicides included thiophanate-methyl (Topsin M WSB 70 WP; Nippon Soda Company Ltd.; Fungicide Resistance Action Committee [FRAC] 1), myclobutanil (Rally 40 WSP; Dow AgroSciences LLC; FRAC 3), trifloxystrobin (Flint; Bayer CropScience; FRAC 11), and captan (Captan 80 WDG; Arysta LifeScience North America Co.; FRAC M). Concentrations of

Fig. 1. Morphological types found among isolates of *Colletotrichum* from symptomatic fruit collected in several orchards in Kentucky. Characterization was based on colony color, conidial shape, and the ability to produce sexual spores. Colony color of **A** and **E**, morphotype 1; **B** and **F**, morphotype 2; **C** and **G**, morphotype 3; and **D** and **H**, morphotype 4. Colony color was observed from the top (**A**, **B**, **C**, and **D**) and the bottom (**E**, **F**, **G**, and **H**). Conidial shape of **I**, morphotype 1; **J**, morphotype 2; and **K**, morphotype 3; and **L**, asci of morphotype 4. Colonies were 7 days old. Bars = 20 µm.

active ingredients in the amended media were 0.01, 0.1, 1, 10, and 100 µg/ml for Topsin, Rally, and Flint; and active ingredient at 0.1, 1, 10, 100, and 1,000 µg/ml for captan. The linear growth of each test isolate was measured at 10 dpi. Percent inhibition was calculated as [(growth of the control – growth of the experimental)/growth of the control] × 100. Relative growth was calculated as 100 – percent inhibition. Half maximal effective concentration (EC₅₀) was determined based on the relative growth data. Each treatment was replicated three times, and the entire experiment was repeated once. The factorial calculation for experiment, replication, fungicide, species complex, species, and isolate was evaluated using an analysis of variance and means of EC₅₀ were separated using least square means, with the Bonferroni correction provided in the statistical algorithms of SAS (version 9.3; SAS Institute).

Effect of fungicides on conidial germination. Trifloxystrobin (a systemic fungicide) and captan (a protectant fungicide) were tested for their ability to inhibit spore germination. Fungicide solutions and fresh conidial suspensions were mixed with sterile water to a total of 15 ml. The final concentrations of fungicide were 0.001, 0.01, 0.1, 1, and 10 µg/ml, and the final concentration of conidia was 1 × 10⁴ conidia/ml. Three 100-µl droplets of each suspension were placed into an empty 100-mm polystyrene petri dish and the plates were incubated in humidity chambers for 20 to 24 h. Conidia were then observed at ×100 and scored as either nongerminated or germinated according to Chaky et al. (2001) and Vincelli and Dixon (2002). Percent inhibition was calculated as [(germination rate of the control – germination rate of the experimental)/germination rate of the control] × 100. Relative germination rate was calculated as 100 – percent inhibition. EC₅₀ was determined based on the relative germination rate data. Each treatment was replicated three times, and the entire experiment was repeated once.

Results

Morphological characterization. The 475 *Colletotrichum* bitter-rot isolates collected from 25 counties in Kentucky were divided into four morphological types (morphotypes) based on colony color and spore shape. These morphotypes corresponded to the descriptions of the three species most commonly associated with bitter rot of apple: *C. acutatum*, *C. gloeosporioides*, and *G. cingulata* (teleomorph of *C. gloeosporioides*).

Isolates belonging to morphotype 1 were characterized by the production of a pink to red coloration in the media (Fig. 1). Orange conidial masses were submerged in the mycelia. Conidia were predominantly fusiform with pointed ends (Fig. 1). Perithecia were not observed. Based on these morphological characteristics, all of the isolates in morphotype 1 corresponded to *C. acutatum* (González et al. 2006; Shi et al. 1995, 1996).

Morphotype 2 isolates did not produce pigment in the growth medium. Isolates formed white-to-gray mycelia, occasionally with a light yellow tint, and an abundance of orange conidial masses (Fig. 1). Conidia of the isolates in morphotype 2 were mostly fusiform but with ends that were somewhat more rounded than spores of morphotype 1 (Fig. 1). No perithecia were observed in cultures of this morphotype. The conidial shape of the isolates in morphotype 2 corresponded to the description of *C. acutatum* (González et al. 2006; Shi et al. 1995, 1996).

Isolates of morphotype 3 produced gray mycelia, with no coloration in the growth medium (Fig. 1). Some isolates produced abundant aerial mycelia that were white-to-gray. Sectors were observed in the colony mycelia of some isolates of morphotype 3. Conidia of all isolates were cylindrical with rounded ends (Fig. 1). Conidia were produced in small orange masses over the entire colony in most isolates. A few isolates produced larger orange, scattered masses of conidia. No perithecia were observed in culture. The characteristics of the isolates in morphotype 3 were consistent with descriptions of *C. gloeosporioides* (González et al. 2006; Shi et al. 1995, 1996).

The mycelia of the isolates in morphotype 4 were dark gray, with abundant aerial mycelia (Fig. 1). Isolates of morphotype 4 produced no pigmentation in the media. Conidia were cylindrical with rounded ends, similar to those produced by isolates in morphotype 3. However morphotype 4 isolates were distinguished by the production

of perithecia in culture, and asci and ascospores were observed under the microscope (Fig. 1). Based on these morphological characteristics, the isolates in morphotype 4 belonged to *G. cingulata* (teleomorph of *C. gloeosporioides*) (González et al. 2006).

Among the total of 475 isolates, 335 (71%) belonged to morphotype 1, 15 (3%) belonged to morphotype 2, 106 (22%) belonged to morphotype 3, and 19 isolates (4%) belonged to morphotype 4 (Table 1). Twenty-six representative isolates were selected for further study; these isolates included all four morphotypes, and were collected from northern, eastern, central, and western Kentucky (Table 2).

Sizes of conidia produced by each of the 26 selected isolates and two reference isolates were measured and compared (Table 3). Average conidial length and width were significantly different among the four morphotypes ($P < 0.05$). Isolates of morphotype 3 had the longest conidia, while isolates of morphotype 1 had the shortest. Conidia of morphotype 4 were the widest and conidia of morphotype 1 isolates were the narrowest. Conidial length/width ratios of isolates of morphotypes 1 and 4 were significantly smaller than length/width ratios of morphotypes 2 and 3.

Mycelial growth rate. The independent variables morphotype and temperature both had a significant effect on mycelial growth rate ($P < 0.05$). The maximum growth rate occurred at 25°C for all isolates (Table 4). The average growth rate of each morphotype was significantly different from the others ($P < 0.05$). Isolates of morphotype 4 grew the fastest and isolates of morphotype 2 were the slowest (Table 4).

Molecular identification and phylogenetic analysis. *Multigene analyses.* *GAPDH* and *TUB2* DNA fragments were successfully amplified and sequenced from all test isolates (Table 5). The PCR products from the *GAPDH* and *TUB2* amplification were approximately 250 and 700 bp, respectively.

Based on the internal transcribed spacer (ITS) sequence comparison with published sequences in GenBank, all isolates in morphotype 1 and morphotype 2 belonged to the *C. acutatum* species complex.

Table 3. Mean conidial length, width, and length/width ratio among morphotypes^y

Isolate	Morphotype ^z	Length (µm)	Width (µm)	Length/width ratio
HC 296	1 a, a, a	12.40 (0.40)	4.61 (0.30)	2.70 (0.20)
KY 228	1 a, a, a	12.41 (0.46)	4.48 (0.41)	2.79 (0.26)
KY 95	1 a, a, a	12.37 (0.50)	4.78 (0.51)	2.61 (0.27)
KY 123	1 a, a, a	12.26 (0.45)	4.42 (0.71)	2.85 (0.48)
HC 557	1 a, a, a	12.64 (0.52)	4.56 (0.36)	2.79 (0.22)
KY 28	1 a, a, a	12.47 (0.40)	4.61 (0.41)	2.73 (0.31)
KY 6	1 a, a, a	12.41 (0.44)	4.52 (0.35)	2.76 (0.24)
KY 105A	1 a, a, a	12.44 (0.51)	4.74 (0.39)	2.65 (0.26)
KY 320	1 a, a, a	12.51 (0.58)	4.79 (0.64)	2.66 (0.40)
KY 162	1 a, a, a	12.42 (0.65)	4.77 (0.40)	2.63 (0.31)
KY 291	1 a, a, a	12.29 (0.45)	4.47 (0.31)	2.76 (0.21)
KY 191	1 a, a, a	12.59 (0.73)	4.55 (0.51)	2.80 (0.38)
KY 9	2 b, b, b	16.14 (0.62)	4.76 (0.52)	3.43 (0.40)
HC 646	2 b, b, b	15.41 (0.90)	4.94 (0.59)	3.01 (0.42)
HC 647	2 b, b, b	15.57 (0.71)	4.91 (0.37)	3.17 (0.30)
KY 263	3 c, c, b	17.41 (0.84)	6.50 (0.60)	2.70 (0.28)
KY 254	3 c, c, b	17.55 (0.87)	5.60 (0.63)	3.17 (0.38)
KY 301	3 c, c, b	17.86 (0.78)	5.23 (0.54)	3.45 (0.41)
KY 146	4 d	17.66 (0.54)	5.78 (0.50)	3.08 (0.28)
KY 305	4 d	17.78 (0.85)	5.76 (0.60)	3.12 (0.34)
KY 152	4 d	18.21 (0.65)	5.47 (0.48)	3.35 (0.35)
KY 153	4 d	18.90 (1.04)	5.57 (0.43)	3.41 (0.30)
KY 128	4 d	17.87 (0.93)	5.63 (0.53)	3.20 (0.34)
HC 540	4 d	17.10 (0.91)	5.69 (0.76)	3.05 (0.38)
KY 40	4 d	17.41 (0.39)	7.10 (0.55)	2.47 (0.19)
KY 8	4 d	17.81 (0.97)	5.90 (0.45)	3.04 (0.28)

^y Numbers in parentheses represent standard deviation of the mean.

^z Letters represent comparisons for conidial length, conidial width, and length/width ratio, respectively. Letter d refers to asci rather than conidia. Values followed by the same letter were not significantly different based on the Waller-Duncan *K* ratio *t* test ($P < 0.05$).

Results of searches with the *GAPDH* and *TUB2* sequences indicated that all isolates in morphotype 1 (12 isolates) matched *C. fioriniae* and all isolates in morphotype 2 (3 isolates) matched *C. nymphaeae* (Table 4). ITS sequences from morphotype 3 and morphotype 4 placed them in the *C. gloeosporioides* species complex. *GAPDH* and *TUB2* sequences from seven isolates in morphotype 3 matched *C. siamense*, while two isolates matched *C. theobromicola*. All isolates in morphotype 4 corresponded to *C. fructicola*. Species identities returned by querying the Q-bank fungal database (Cannon et al. 2012) with the *GAPDH* and *TUB2* sequences agreed with the results of homology searches against the GenBank nr database.

Molecular phylogenetic analyses based on *GAPDH* and *TUB2* regions in a separate analysis by PhyML grouped the *Colletotrichum* isolates into two major clades, corresponding to the *C. acutatum* species complex and *C. gloeosporioides* species complex (Fig. 2). The *C. acutatum* clade consisted of two separate subclades, which corresponded to *C. fioriniae* and *C. nymphaeae*. The *C. gloeosporioides* clade consisted of three subclades, corresponding to *C. siamense*, *C. theobromicola*, and *C. fructicola* (Fig. 2). Support for all of these clades was greater than 0.7 with both gene sequences. The *GAPDH* sequences revealed additional clades within the *C. siamense* group that also had high levels of statistical support. The *TUB2* sequences did not differentiate these subgroups.

Colonization tests. All 26 isolates of *Colletotrichum* produced typical bitter rot symptoms on Golden Delicious and Red Stayman Winesap fruit. These symptoms included sunken lesions and conical zones of necrotic tissue extending beneath the lesions. Apple cultivar had no significant effect on lesion diameter or on lesion depth ($P > 0.05$; Fig. 3). A significant difference was observed between the two replicate experiments that were performed for each cultivar ($P < 0.05$). The variable of species complex had a significant effect on lesion development ($P < 0.0001$). Isolates belonging to the *C. gloeosporioides* species complex, on average, produced larger and deeper lesions compared with the *C. acutatum* species complex isolates (Fig. 3). The interaction of species complex and apple cultivar had no effect on lesion diameter or on lesion depth ($P > 0.05$; Fig. 3). The variable of fungal species ($P < 0.0001$), as well as the interaction of species and apple cultivar ($P = 0.0135$), had significant effects on lesion development. Isolates of *C. siamense* produced the largest and deepest lesions (Fig. 3). Even though *C. fioriniae* and *C. nymphaeae* belong to the same species complex, *C. fioriniae* caused significantly larger and deeper lesions than *C. nymphaeae* (Fig. 3).

Apple cultivar and species complex both had a significant effect on the sporulation of *Colletotrichum* spp. ($P = 0.0097$ and $P < 0.0001$, respectively). More spores were produced by *Colletotrichum* lesions on Red Stayman Winesap apple, and more spores were produced by members of the *C. acutatum* complex than by isolates belonging to the *C. gloeosporioides* complex (Fig. 4). Lesions caused by *C. fioriniae* produced many more spores than any of the other species. The number of spores produced by *C. nymphaeae*, *C. siamense*, *C. theobromicola*, and *C. fructicola* was not significantly different ($P > 0.05$; Fig. 4).

Sensitivity of *Colletotrichum* isolates to fungicides. The independent variables fungicides (thiophanate-methyl, myclobutanil,

trifloxystrobin, and captan), species complex, species, interaction of fungicide and species complex, and interaction of fungicide and species all had significant effects on the EC_{50} of selected fungicides against mycelial growth of *Colletotrichum* spp. ($P < 0.0001$; Table 6). No differences were observed between the two replicate experiments ($P > 0.05$; Table 6).

The EC_{50} of captan against the mycelial growth of *Colletotrichum* spp. was higher than that of any of the other fungicides in the study (mean $EC_{50} = 299.21$ mg/liter). The EC_{50} of thiophanate-methyl, myclobutanil, and trifloxystrobin were not significantly different from one another ($P > 0.05$; Table 6).

Based on mycelial growth, isolates belonging to the *C. gloeosporioides* complex were more sensitive, on average, to the four fungicides than isolates in the *C. acutatum* species complex. Isolates of *C. fioriniae*, *C. nymphaeae*, and *C. theobromicola* were significantly less sensitive to the four fungicides than isolates of *C. siamense* and *C. fructicola* ($P < 0.05$). The sensitivities of *C. fioriniae*, *C. nymphaeae*, and *C. theobromicola* were not significantly different from one another ($P > 0.05$). Similarly, *C. siamense* and *C. fructicola* did not differ in fungicide sensitivity ($P > 0.05$; Table 6).

The independent variables fungicide (trifloxystrobin and captan), species complex, species, fungicide–species complex interaction, and fungicide–species interaction all had significant effects on the EC_{50} of selected fungicides to the spore germination of each *Colletotrichum* sp. evaluated ($P < 0.05$; Table 7). The EC_{50} of trifloxystrobin was significantly lower than the EC_{50} of captan. The fungicide sensitivity of spores among members of the *C. gloeosporioides* species complex was, on average, significantly higher than the sensitivity of spores of isolates within the *C. acutatum* species complex. The sensitivity of spores of *C. theobromicola* to the two selected fungicides was significantly higher than the sensitivity of spores of the other species ($P < 0.05$; Table 7).

Discussion

Commercial apple growers in Kentucky consider bitter rot to be the most common fruit rot, as well as the most challenging summer disease to control (2015 survey, unpublished). Many orchardists struggle with its management, even when they adhere to recommended

Table 4. Average growth rate of *Colletotrichum* morphotypes at five selected temperatures

Morphotype	Average growth rate (mm/day)					Mean (mm/day) ^z
	18	21	23	25	30	
Morphotype 1	2.59	2.94	4.43	5.69	2.76	3.68 b
Morphotype 2	2.70	3.08	3.32	5.05	3.01	3.43 b
Morphotype 3	2.74	3.83	5.15	6.75	5.30	4.75 a
Morphotype 4	2.79	2.70	6.25	7.42	5.78	4.99 a
Mean (mm/day) ^z	2.67 e	3.25 d	4.69 b	6.12 a	3.90 c	...

^z Values followed by the same letter were not significantly different based on the Waller-Duncan *K* ratio *t* test ($P < 0.05$).

Table 5. Accession numbers for identified *Colletotrichum* isolates using *GAPDH* and *TUB-2* sequences, and Q-Bank online database

Strain	Accession number		Top hit
	<i>GAPDH</i>	<i>TUB2</i>	
HC296	KT777656	KT777681	<i>Colletotrichum fioriniae</i>
HC540	KT777657	KT777682	<i>C. fructicola</i>
HC646	KT777658	KT777683	<i>C. nymphaeae</i>
HC647	KT777659	KT777684	<i>C. nymphaeae</i>
KY105A	KT777660	KT777685	<i>C. fioriniae</i>
KY123	KT777661	KT777686	<i>C. fioriniae</i>
KY128	KT777662	KT777687	<i>C. siamense</i>
KY146	KT777663	KT777688	<i>C. siamense</i>
KY152	KT777664	KT777689	<i>C. theobromicola</i>
KY153	KT777665	KT777690	<i>C. theobromicola</i>
KY162	KT777666	KT777691	<i>C. fioriniae</i>
KY191	KT777667	KT777692	<i>C. fioriniae</i>
KY228	KT777668	KT777693	<i>C. fioriniae</i>
KY254	KT777669	KT777694	<i>C. siamense</i>
KY263	KT777670	KT777695	<i>C. siamense</i>
KY28	KT777671	KT777696	<i>C. fioriniae</i>
KY291	KT777672	KT777697	<i>C. fioriniae</i>
KY301	KT777673	KT777698	<i>C. siamense</i>
KY305	KT777674	KT777699	<i>C. siamense</i>
KY320	KT777675	KT777700	<i>C. fioriniae</i>
KY40	KT777676	KT777701	<i>C. fructicola</i>
KY6	KT777677	KT777702	<i>C. fioriniae</i>
KY8	KT777678	KT777703	<i>C. siamense</i>
KY9	KT777679	KT777704	<i>C. nymphaeae</i>
KY95	KT777680	KT777705	<i>C. fioriniae</i>

Fig. 2. Unrooted **A**, *TUB2*-based and **B**, *GAPDH*-based phylogenetic trees. Trees were produced using PhyML based on approximate likelihood ratio test and TreeDyn for tree rendering. Numbers at each branch indicate percentage of branch support values based on maximum likelihood. Alignment can be found in supplemental materials.

spray schedules. It is widely reported that fungicides are not always reliable. The major findings of this study suggested that variations in the causal pathogen of bitter rot may be one important reason for these disease management challenges.

This study revealed that *Colletotrichum* isolates causing bitter rot of apple in Kentucky orchards belong to five different phylogenetic species: *C. fioriniae*, *C. nymphaeae*, *C. siamense*, *C. theobromicola*, and *C. fructicola*. Furthermore, these species differ in traits relevant to disease epidemiology and management. This study is the first to report pathogenically relevant differences between species within the same species complexes of the bitter rot pathogens.

Among the species identified, only *C. fioriniae* has been reported previously to cause bitter rot of apple in the United States (Kou et al.

2014). However, all five species have been found associated with bitter rot in other countries, including Croatia (Ivic et al. 2013), Brazil, and Uruguay (Velho et al. 2014, 2015). The publications that established and named these five new species included several isolates of *C. fioriniae*, *C. siamense*, and *C. fructicola* recovered from apple in the United States, although it was not reported whether or not these apple fruit had symptoms of bitter rot (Damm et al. 2012; Weir et al. 2012).

The distribution of *Colletotrichum* spp. on apple fruit in Kentucky did not appear to be associated with geography, orchard size, or sub-climate (e.g., elevation or microclimate). Distribution appeared to be essentially random, although a few species (e.g., *C. fructicola*) appeared to be less widely distributed than the others, and *C. fioriniae* was more prevalent across the state, especially in commercial orchards.

Fig. 3. Lesion diameter caused by *Colletotrichum* spp. Species name indicated with CF = *Colletotrichum fioriniae*, CN = *C. nymphaeae*, CS = *C. siamense*, CT = *C. theobromicola*, and CFR = *C. fructicola*. Species name, species complex, and apple cultivar followed by the same superscript letter were not significantly different based on least square means, adjustment for multiple comparison (Bonferroni).

Fig. 4. Spore concentration from the lesion on detached apple infected with *Colletotrichum* spp. Species name indicated with CF = *Colletotrichum fioriniae*, CN = *C. nymphaeae*, CS = *C. siamense*, CT = *C. theobromicola*, and CFR = *C. fructicola*. Species name, species complex, and apple cultivar followed by the same superscript letter were not significantly different based on least square means, adjustment for multiple comparison (Bonferroni).

Multiple species within the same orchard were found in several locations. In the most deeply sampled orchard, multiple species were identified on the same tree, on the same fruit, and even within the same lesion. Thus, individual orchard surveys may be required in order to determine the presence and distribution of *Colletotrichum* spp. in each location.

This study confirmed that classification of species was consistent with known complexes: *C. fiorinae* and *C. nymphaeae* fall within the *C. acutatum* complex while *C. siamense*, *C. theobromicola*, and *C. fructicola* fall within the *C. gloeosporioides* complex. Isolates of *C. fiorinae* consistently produced red pigment on PDA, whereas none of the other species produced the pigment. Thus, this can serve as a reliable diagnostic feature for *C. fiorinae*. Only one species in this study produced perithecia. Previous reports suggested that perithecial and nonperithecial isolates of *C. gloeosporioides* causing bitter rot represented the same strains undergoing different phases of development (González et al. 2006; Shi et al. 1995, 1996; Sutton 1990). However, the present work clearly showed that isolates of *C. gloeosporioides* that formed perithecia belonged to a different species within the *C. gloeosporioides* complex, *C. fructicola*, compared with those that did not form perithecia. Some isolates of *C. fructicola* that had initially formed perithecia lost this ability after subculturing. The loss of fertility in culture is not uncommon in *Colletotrichum* spp. (Vaillancourt et al. 2000). Thus, presence of

perithecia in culture may be a useful trait for diagnosis of *C. fructicola* but their absence is not a reliable indicator for diagnosis of species. None of the other species in this study produced morphological characteristics that were reliable for diagnostics. Individual features such as color of conidia, shape of conidia, and colony characteristics were not consistent measures of species.

Molecular phylogenetic analyses based on *GAPDH* and *TUB2* regions supported the conclusion that five different species (*C. fructicola*, *C. siamense*, *C. theobromicola*, *C. fiorinae*, and *C. nymphaeae*) cause bitter rot symptoms on apple in Kentucky. The molecular analyses were consistent with published work that originally established these five species within the *C. acutatum* and *C. gloeosporioides* complexes (Weir et al. 2012). The *C. acutatum* clade consisted of subclades that corresponded to *C. fiorinae* and *C. nymphaeae*. The *C. gloeosporioides* clade consisted of three subclades corresponding to *C. siamense*, *C. theobromicola*, and *C. fructicola*. Analysis of both sequences, either separately or concatenated, indicated a clear and highly supported differentiation of each species. Although their separation was supported statistically, both sequences suggested a close relatedness of *C. siamense* and *C. fructicola*. Morphological evidence from our study, based on presence or absence of perithecia, supported this separation. Within *C. siamense*, the *GAPDH* sequences suggested additional complexity, with the presence of several highly supported subgroups. This is consistent with the proposal by Sharma

Table 6. Mean half maximal effective concentration (EC₅₀) of mycelial growth of *Colletotrichum* spp. for four select fungicides^z

Isolate	Species complex	Species	EC ₅₀ (mg/liter)			
			Thio a	Mycl a	Trif a	Cap b
HC 296	<i>Colletotrichum acutatum</i> c	<i>C. fiorinae</i> a	6.36 (0.57)	4.24 (0.72)	0.64 (0.14)	526.41 (68.41)
KY 6	<i>C. acutatum</i> c	<i>C. fiorinae</i> a	7.85 (0.35)	3.99 (0.71)	5.88 (2.28)	407.86 (64.79)
KY 320	<i>C. acutatum</i> c	<i>C. fiorinae</i> a	4.34 (1.18)	3.92 (1.09)	0.55 (0.26)	489.42 (32.76)
KY 9	<i>C. acutatum</i> c	<i>C. nymphaeae</i> a	0.85 (0.08)	5.32 (2.01)	0.08 (0.01)	558.33 (229.28)
HC 646	<i>C. acutatum</i> c	<i>C. nymphaeae</i> a	5.75 (2.38)	6.00 (0.50)	7.75 (1.30)	470.28 (25.46)
HC 647	<i>C. acutatum</i> c	<i>C. nymphaeae</i> a	1.75 (0.75)	6.74 (1.25)	0.55 (0.26)	504.55 (113.16)
KY 263	<i>C. gloeosporioides</i> d	<i>C. siamense</i> b	0.01 (0.00)	3.85 (0.84)	57.83 (12.38)	255.00 (77.62)
KY 254	<i>C. gloeosporioides</i> d	<i>C. siamense</i> b	0.01 (0.00)	0.50 (0.26)	52.11 (8.20)	36.88 (2.09)
KY 301	<i>C. gloeosporioides</i> d	<i>C. siamense</i> b	0.01 (0.00)	2.58 (0.60)	80.81 (9.92)	51.83 (11.73)
KY 8	<i>C. gloeosporioides</i> d	<i>C. siamense</i> b	0.01 (0.00)	5.69 (2.48)	0.01 (0.00)	262.50 (21.36)
KY 152	<i>C. gloeosporioides</i> d	<i>C. theobromicola</i> a	0.96 (0.01)	6.95 (0.25)	28.43 (0.43)	457.00 (26.34)
KY 153	<i>C. gloeosporioides</i> d	<i>C. theobromicola</i> a	0.79 (0.01)	6.07 (0.17)	26.07 (3.21)	335.71 (10.71)
HC 540	<i>C. gloeosporioides</i> d	<i>C. fructicola</i> b	0.01 (0.00)	7.30 (1.37)	0.01 (0.00)	89.75 (8.44)
KY 40	<i>C. gloeosporioides</i> d	<i>C. fructicola</i> b	0.13 (0.05)	0.23 (0.13)	0.01 (0.00)	63.50 (15.88)

^z Fungicides thiophanate-methyl (Thio), myclobutanil (Mycl), trifloxystrobin (Trif), and captan (Cap). Numbers in parentheses represent standard error of the mean. Fungicides, species complex, and species name followed by the same letter were not significantly different based on least square means, adjustment for multiple comparison (Bonferroni).

Table 7. Mean half maximal effective concentration (EC₅₀) of spore germination of *Colletotrichum* for two select fungicides^z

Isolate	Species complex	Species	EC ₅₀ (mg/liter)	
			Trifloxystrobin a	Captan b
HC 296	<i>Colletotrichum acutatum</i> c	<i>C. fiorinae</i> a	0.03 (0.00)	0.41 (0.05)
KY 6	<i>C. acutatum</i> c	<i>C. fiorinae</i> a	0.03 (0.00)	0.46 (0.03)
KY 320	<i>C. acutatum</i> c	<i>C. fiorinae</i> a	0.00 (0.00)	0.41 (0.01)
KY 9	<i>C. acutatum</i> c	<i>C. nymphaeae</i> ab	0.01 (0.00)	0.11 (0.03)
HC 646	<i>C. acutatum</i> c	<i>C. nymphaeae</i> ab	0.04 (0.00)	0.51 (0.00)
HC 647	<i>C. acutatum</i> c	<i>C. nymphaeae</i> ab	0.03 (0.01)	0.41 (0.03)
KY 263	<i>C. gloeosporioides</i> d	<i>C. siamense</i> ab	0.01 (0.00)	0.32 (0.06)
KY 254	<i>C. gloeosporioides</i> d	<i>C. siamense</i> ab	0.01 (0.00)	0.24 (0.05)
KY 301	<i>C. gloeosporioides</i> d	<i>C. siamense</i> ab	0.01 (0.00)	0.30 (0.04)
KY 8	<i>C. gloeosporioides</i> d	<i>C. siamense</i> ab	0.01 (0.00)	0.43 (0.04)
KY 152	<i>C. gloeosporioides</i> d	<i>C. theobromicola</i> b	0.01 (0.00)	0.20 (0.03)
KY 153	<i>C. gloeosporioides</i> d	<i>C. theobromicola</i> b	0.01 (0.00)	0.20 (0.05)
HC 540	<i>C. gloeosporioides</i> d	<i>C. fructicola</i> ab	0.00 (0.00)	0.10 (0.02)
KY 40	<i>C. gloeosporioides</i> d	<i>C. fructicola</i> ab	0.05 (0.01)	0.40 (0.02)

^z Numbers in parentheses represent standard error of the mean. Fungicides, species complex, and species name followed by the same letter were not significantly different based on least square means, adjustment for multiple comparison (Bonferroni).

et al. (2013) suggesting that *C. siamense* may consist of a species complex and warrants further investigation.

All tested isolates from all five *Colletotrichum* spp. were pathogenic to wounded apple fruit. On average, isolates within the *C. acutatum* species complex produced smaller lesions than isolates within the *C. gloeosporioides* species complex. However, this study found significant differences in aggressiveness among species within each complex. Thus, *C. siamense* in the *C. gloeosporioides* complex was the most aggressive species overall and was significantly more aggressive than the other two species within that complex. Similarly, within the *C. acutatum* species complex, *C. fioriniae* was more aggressive than *C. nymphaeae*. Isolates of *C. theobromicola* and *C. fructicola* within the *C. gloeosporioides* complex were not significantly different in aggressiveness from the two *C. acutatum* species. Thus, a species-specific diagnosis will be very important for prediction of relative aggressiveness; species complex alone is not a sufficient indicator of pathogenicity or disease risk.

Isolates of *C. fioriniae* produced significantly more spores per unit lesion area than the other four species. It is possible that its relative fecundity is related to its dominance across the state of Kentucky. The dominance of *C. fioriniae* is consistent with previously published research by Shi et al. (1995, 1996) who determined that most of the *Colletotrichum* isolates derived from apple bitter rot in the southeastern United States were chromogenic *C. acutatum*. Although the authors did not perform molecular characterization of their isolates, results of our study suggest that those isolates were probably *C. fioriniae*.

Apple cultivar also had a significant effect on sporulation. On average, significantly more spores were produced on fruit of Red Stayman Winesap than on fruit of Golden Delicious. One possible explanation for this result could be differences in nutrient content (e.g., sugars or amino acids) between the two cultivars. Nour et al. (2010) reported considerable variation in biochemical characteristics and mineral content between various apple cultivars, and nutritional factors are known to influence the sporulation of fungi in vitro (Tandon and Chandra 1962). Evaluation of additional apple cultivars and their unique response to each *Colletotrichum* sp. is necessary for better management of bitter rot within orchards.

Growers in Kentucky typically manage bitter rot and other fungal diseases on apple fruit with timed applications of fungicides that include different modes of action. This study tested the sensitivity of the *Colletotrichum* bitter rot species to some common fungicides used in Kentucky orchards. On average, species within the *C. acutatum* complex were less sensitive to thiophanate-methyl, myclobutanil, trifloxystrobin, and captan than members of the *C. gloeosporioides* species complex. Similar results have been reported previously (Adaskaveg and Hartin 1997; Bernstein et al. 1995; Freeman et al. 1998; Peres et al. 2002, 2004; Valero et al. 2010; Velho et al. 2014, 2015). This may suggest that fungicide use influenced populations within commercial orchards. Specifically, however, fungicide sensitivity varied with species. The *C. acutatum* species *C. fioriniae* and *C. nymphaeae* were both more tolerant of the fungicides used in this study compared with the *C. gloeosporioides* species *C. siamense* and *C. fructicola*. When individual species were considered, only *C. fioriniae* and *C. theobromicola* differed significantly from one another, with *C. fioriniae* being more tolerant. Additionally, *C. theobromicola*, also within the *C. gloeosporioides* complex, did not differ significantly from the two *C. acutatum* isolates in regards to its sensitivity to fungicides; it was significantly more tolerant than the other two *C. gloeosporioides* species. Thus, a diagnosis to species complex is not sufficient to predict fungicide sensitivity among Kentucky bitter rot isolates.

These findings demonstrated the importance of accurate pathogen identification, beyond species complex, for management of bitter rot. Future work will include expansion of isolate collections and analysis of isolates both from Kentucky and beyond its borders. Better understanding of species distribution and individual characteristics of individual species is critical for development of disease management plans for bitter rot in apple. Future work will include orchard surveys and analyses, as well as pathogenicity tests and fungicide efficacy field trials.

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