2020

DIVERSITY AND CROSS-INFECTION POTENTIAL OF COLLETOTRICHUM ON APPLES AND SMALL FRUITS IN KENTUCKY MIXED-FRUIT ORCHARDS

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DIVERSITY AND CROSS-INFECTION POTENTIAL OF *COLLETOTRICHUM* ON APPLES AND SMALL FRUITS IN KENTUCKY MIXED-FRUIT ORCHARDS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

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ABSTRACT OF THESIS

DIVERSITY AND CROSS-INFECTION POTENTIAL OF *COLLETOTRICHUM* ON APPLES AND SMALL FRUITS IN KENTUCKY MIXED-FRUIT ORCHARDS

Fungi in the genus *Colletotrichum* cause apple, blueberry, and strawberry fruit rots, which result in significant losses for Kentucky growers. Most orchards in Kentucky are agritourism-focused and grow multiple fruits in close proximity. These mixed-fruit orchards may facilitate *Colletotrichum* cross-infection, which has serious management implications. Small fruit and apple *Colletotrichum* isolates from Kentucky orchards were characterized by morphotype, phylogenetic species identification, cross-inoculation, genome sequencing, and telomere fingerprinting. The small fruit isolates grouped into seven morphotypes, representing two species complexes: *C. acutatum* and *C. gloeosporioides*. All blueberry isolates belonged to the species *C. fioriniae*, and the majority of strawberry isolates were *C. nymphaeae.* Two other species found less frequently on strawberry were identified as *C. siamense* and *C. fructicola.* The same four species identified on small fruits were also present on apple in Kentucky. Cross-inoculation assays on detached apple, blueberry, and strawberry fruits revealed that all species tested were pathogenic on all three fruits. The genome tree was compared to nine single gene sequence trees, and *CHS* for the *C. acutatum* complex and *ApMat* for *C. gloeosporioides* were identified as superior sequences for species identification. Telomere fingerprinting revealed *C. fioriniae* clonal lineages within three orchards on apple, blueberry, and strawberry, but did not show evidence of cross-infection. Understanding more about *Colletotrichum* in Kentucky orchards will help improve fruit anthracnose management practices.

KEYWORDS: *Colletotrichum*, apple bitter rot, strawberry anthracnose, blueberry ripe rot, cross-infection, genome

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05/08/2020

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ACKNOWLEDGEMENTS

I am sincerely grateful for the direction of my advisors, Dr. Lisa J. Vaillancourt and Dr. Nicole Gauthier. Their knowledge and advice have been invaluable to me, and their patience and encouragement have pushed me beyond what I thought I could achieve. I am also thankful for the constructive input of my third committee member, Dr. Douglas D. Archbold.

I am also thankful for our lab group. Etta Nuckles has been an excellent supervisor in the lab and truly has a servant's heart. My fellow lab mates during my time at the UK Plant Pathology Department, Dr. Franklin Machado, Dr. Aline Vieira de Barros, Renata Belisario, Gabdiel Yulfo Soto, Nathaniel White, and Desiree Szarka, have been a constant source of camaraderie and support. And I would like to thank Dr. Mark Farman, Dr. Mostafa Rahnama, and Rebekah Ellsworth for their unique insight and assistance with my project.

I would like to extend my gratitude to all of the faculty of the UK Plant Pathology Department for their commitment to quality research and training successful students. I also appreciate the dedication of the UK Plant Pathology Department staff, especially Shirley Harris and Cheryl Kaiser, for making students feel welcome and helping them with anything they need.

Lastly, I would like to thank my husband, parents, sister, and friends for their constant love and support, without which none of this would have been possible.

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CHAPTER 1. INTRODUCTION

Fruit production in Kentucky

Fruit production in Kentucky consists of small-scale orchards and farms, many of which focus on local markets. This is largely due to climate and traditional land usage patterns in Kentucky: no area is particularly well-suited for large-scale commercial fruit production. The western part of the state is mainly used for the production of cattle and row crops, including corn and soybean, while the eastern part of the state is too mountainous to allow for significant fruit orchard acreage.

Agritourism is an important and growing market within the state. For example, agritourism contributed $17 million in income for Kentucky farms in 2017 (Census of Agriculture 2019). The Kentucky Department of Agriculture's Kentucky Proud™ campaign promotes the purchase of local produce. Many restaurants try to source Kentucky Proud™ products, and farmers' markets are filled with Kentucky Proud™ merchandise. In order to attract agritourism business, most orchards offer multiple fruit crops to serve as "U-Pick" attractions and provide for local markets. This system serves to extend the season and to capitalize on public marketability by catering to young children with the incorporation of entertainment such as playgrounds and petting zoos. The most common fruit crops in U-Pick orchards are apples, strawberries, and blueberries, which are often planted in close proximity for customer access. These fruit crops work particularly well together because the orchard can remain open from late spring through late autumn with a harvestable U-Pick crop. Strawberries typically ripen in May and June in Kentucky, blueberries in late June and July, and apples can be picked from July through November, depending upon the cultivar.

This arrangement makes good economic sense for an agritourism operation, but it also poses unique management challenges. Farmers must be knowledgeable about multiple fruit crops and must also attend to the business and entertainment sides of the operation. Plant diseases that affect the quality and yield of fruit crops can cause production problems and economic losses that will be especially severe for a small U-

Pick operation. With many fruit crops being grown in close proximity, there is a concern that cross-infection may occur. If the same disease agent is capable of infecting multiple fruits, there is potential for it to move between fruits and increase the disease pressure and risk of yield loss within an orchard. For example, anthracnose fruit rots, which affect multiple fruit species, can become difficult to manage in mixed-fruit orchards. The issue of cross-infection is a phenomenon especially relevant to mixed-fruit orchards and will be discussed in greater depth in later sections.

Apples are the most commonly grown fruit in Kentucky. There are 672 farms that total approximately 1000 apple-producing acres, ranking Kentucky 40th in the U.S. in terms of apple production (Census of Agriculture 2019). The average orchard size is small, ranging from 1 to 40 acres, and the majority of apples are sold within the state to local markets (Gauthier et al. 2017). Apple yields vary year to year, depending upon weather and disease, but average approximately 450,000 to 750,000 bushels per year, with a value of $24.4-40.6 million (Census of Agriculture 2019). Most apple orchards in Kentucky have an agritourism focus and consist of mixed-fruit plantings, containing other fruit crops or specialty produce.

Less marketing data are available for blueberry and strawberry production in Kentucky, but both are popular U-Pick and farmers' market crops. Blueberry acreage for local sale has increased in Kentucky as per capita blueberry consumption has increased, and local blueberry prices have remained stable (Kaiser and Ernst 2018b). Mature blueberry plantings can produce 5 to 11 thousand pounds of fruit per acre in Kentucky, and berries often sell for above the national average price at farmers' markets and U-Pick operations (Strang et al. 2003). The average Kentucky farmers' market price for blueberries from 2014 to 2018 was $4.47 per pint (Wolff and Nang 2019). In the U.S., fresh strawberry consumption has increased by two pounds per capita from 2001 to 2016, and likewise strawberry demand, especially for pre-picked local berries, has increased in Kentucky (Kaiser and Ernst 2018a). Profitable strawberry operations can produce 9,000 to 15,000 quarts per acre, with an optimistic return of over $6,000 per acre (Kaiser and Ernst 2018a; Masabni et al. 2007). The average Kentucky farmers'

market price for strawberries from 2014 to 2018 was $4.74 per quart (Wolff and Nang 2019).

Anthracnose diseases of apple, strawberry, and blueberry

Apple, strawberry, and blueberry are all susceptible to anthracnose diseases caused by fungi within the genus *Colletotrichum*. Apple bitter rot is of principal concern for Kentucky apple production. According to a grower survey, bitter rot caused an average yield loss of approximately 30% during the 2015 to 2016 growing seasons, with some growers reporting losses as high as 90% (Gauthier et al. 2017). It is the second most important apple disease in terms of yield loss and management difficultly in the state, and is only surpassed by fire blight, a bacterial disease caused by *Erwinia amylovora* (Gauthier et al. 2017). Bitter rot is prevalent in regions with a warm, wet climate, which is conducive for disease development and includes Kentucky. Additionally, the top five most common apple cultivars grown in the state range from susceptible to very susceptible to bitter rot, and infection can occur anytime from petal fall through harvest (Gauthier et al. 2017).

The characteristic symptoms of bitter rot are brown, circular, sunken lesions that expand over time. They also form a brown, firm, cone-shaped internal lesion in the fruit pulp that is a diagnostic feature of the disease. Under humid conditions, conidia are produced in pink to orange-colored, sticky masses that arise from acervuli on the exterior lesion surface; they often form diurnal rings. The disease can be well-managed by timely fungicide applications, but cultural practices such as general sanitation, pruning, and clean-picking are critical to effectively manage disease (Gauthier et al. 2017). Munir et al. (2016) found that five species of *Colletotrichum* were responsible for causing bitter rot disease in Kentucky: *C. fioriniae*, *C. nymphaeae*, *C. siamense, C. fructicola,* and *C. theobromicola*. Further information can be found in the section entitled “Previous work on apple in Kentucky” (page 11).

Anthracnose fruit and crown rot of strawberry are also important diseases that cause significant yield loss. Generally, the fruit rot is more common than crown rot in

Kentucky, but the prevalence of the two diseases varies over time and depends upon location (Howard et al. 1992; Jayawardena et al. 2016a; Ward and Hartman 2012). There are no incidence or yield loss data available for the state, but a study from Florida indicated that during the 2012 and 2013 growing seasons, 45% of strawberry growers reported being affected by strawberry anthracnose every year, and that yield loss ranged from 4 to 25% (Borisova et al. 2014). Fruit rot symptoms manifest as circular, firm, sunken lesions that are light tan to black in color and can enlarge to cover entire berries (Howard et al. 1992). As lesions grow, pink-orange conidial masses form and cover the surface (Smith 2008). Crown rot symptoms manifest as wilting, stunting, and eventual death of plants, with red-brown discoloration developing in crown tissues that serves as a diagnostic feature of the disease (Howard et al. 1992). The most effective management strategy for strawberry anthracnose is to begin with clean transplants, but fungicide sprays and proper mulch layers can also help decrease disease pressure (Ward and Hartman 2012). In the United States, *C. nymphaeae* has been reported as the dominant species causing anthracnose fruit rot (Wang et al. 2019).

Ripe rot of blueberry is typically of less concern for Kentucky growers. As the disease name implies, ripe rot does not develop until fruit begin to ripen, usually not becoming evident until after harvest. Because Kentucky blueberries are produced for U-Pick and fresh markets, few blueberries go into long-term storage. Thus, this disease is not a significant management concern for mixed-fruit orchard growers and rarely warrants chemical intervention. However, in the large-scale blueberry industry, ripe rot is a major cause of yield loss. In commercial production, losses of 10 to 20% have been reported in Michigan and 50% in New Jersey. Postharvest losses of up to 100% can occur if storage conditions are poor, including a lack of proper sanitation or sufficiently low temperatures (Milholland et al. 2017). Fruit are infected at bloom, and the fungus remains latent until berry ripening. At that time, soft, sunken spots develop near the calyx, causing shriveling and copious spore production in pustules (Milholland et al. 2017). Effective disease management measures include the use of ripe-rot resistant blueberry cultivars, early and full-bloom fungicide applications, timely harvests, and

immediate postharvest cooling of berries (Milholland et al. 2017; Polashock et al. 2005). The main causal agent of ripe rot has been debated, with earlier reports identifying *C. gloeosporioides* and later reports suggesting *C. acutatum* as the dominant species (Daykin and Milholland 1984; Milholland et al. 2017; Smith et al. 1996).

Colletotrichum taxonomy

The genus *Colletotrichum* is a cosmopolitan group of fungi belonging to the phylum Ascomycota. Almost all plants are susceptible to a disease caused by at least one *Colletotrichum* species (Crouch et al. 2014; da Silva et al. 2020; Dean et al. 2012). *Colletotrichum* is ranked as one of the top ten most important fungal genera in terms of impact both as a plant pathogen and a model research system (Dean et al. 2012). *Colletotrichum* fungi are especially important as fruit and vegetable pathogens, causing both pre- and postharvest economic losses (Børve and Stensvand 2015; Gauthier et al. 2017; Hyde et al. 2009; Kou et al. 2014; Prusky et al. 2013; Sharma and Kulshrestha 2015).

Taxonomy within the genus *Colletotrichum* has been in flux for the past two decades due to advances in molecular sequencing. In 1957, the 100s of existing names for *Colletotrichum* spp., which were established largely based upon host range, were consolidated into 11 different species based solely upon morphological characteristics (von Arx 1957). Molecular approaches have revealed that many of these “species” are actually species complexes containing numerous phylogenetic species (Damm et al. 2012a; Damm et al. 2012b; Damm et al. 2014; Weir et al. 2012). As of 2016, there were 190 currently accepted species divided into 11 species complexes and 23 singleton species (Jayawardena et al. 2016b). The two most important species complexes causing fruit rot diseases are the *C. acutatum* and *C. gloeosporioides* complexes. *Colletotrichum acutatum* was first characterized in 1965 (Simmonds 1965) and was later recognized as a species complex containing 34 individual species based on molecular identification (Damm et al. 2012a; Jayawardena et al. 2016b). The name *C. gloeosporioides* was first used in 1882 (Penzig 1882), but the species was not formalized till 1957 (von Arx 1957);

it was later identified as a species complex containing 38 species (Jayawardena et al. 2016b; Weir et al. 2012).

Accurate species identification within *Colletotrichum* is challenging. Reliance upon morphological features alone has proven to be inadequate for precise identification, as characters like colony color and spore shape can vary depending upon the strain and environment (Adaskaveg 1997; Afanador-Kafuri et al. 2003; Cai et al. 2009; Crouch et al. 2009; Phoulivong et al. 2010). Even some of the early molecular technologies employed are now considered insufficient. For example, sequencing or PCR amplification of the *ITS* region can only differentiate species complexes (Adaskaveg 1997; Freeman 2001; Mills et al. 1992; Sreenivasaprasad et al. 1996) .

The current accepted standard for species identification within the genus *Colletotrichum* is multigene phylogenetic analysis, with an emphasis on a polyphasic approach (Baroncelli et al. 2017; Cai et al. 2009; He et al. 2019; Hyde et al. 2009; Liu et al. 2016; Moreira et al. 2019). For each species complex and for some individual species, the specific set of gene sequences that is the most taxonomically informative has been determined (Damm et al. 2012a; Damm et al. 2012b; Damm et al. 2014; Liu et al. 2015; Weir et al. 2012). The most commonly used genes for the *C. acutatum* and *C. gloeosporioides* species complexes and for species within them are actin (*ACT*), the *Apn2* and MAT1-2-1 intergenic spacer region (*ApMat*), beta-tubulin 2 (*TUB2*), calmodulin (*CAL*), chitin synthase (*CHS*), glutamine synthetase (*GS*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), histone3 (*HIS3*), the internal transcribed spacer region (*ITS*), and manganese-superoxide dismutase (*SOD2*) (Damm et al. 2012a; Jayawardena et al. 2016b; Silva et al. 2012; Weir et al. 2012). A combination of these gene sequences, along with morphological observations, can be used for reliable species identification within these *Colletotrichum* species complexes.

Colletotrichum **disease cycle**

The generalized disease cycle of *Colletotrichum* includes stages of primary infection, spore production and dispersal, secondary infection, and overwintering.

Beginning with primary infection, the lifestyle of *Colletotrichum* can be described as hemibiotrophic, with a short initial biotrophic phase upon infection, followed by destructive necrotrophic growth (Münch et al. 2008; Peres et al. 2005; Wharton and Dieguez-Uribeondo 2004). When a conidium is deposited on a susceptible host, it germinates, forms a germ tube, and then develops a melanized appressorium (Münch et al. 2008). The appressorium utilizes high turgor pressure to force the infection peg through the plant epidermis (Münch et al. 2008). Some *Colletotrichum* spp. also produce cell wall-degrading enzymes to aid in infection, while others have been reported to infect through stomata or wounds without the use of an appressorium (Münch et al. 2008; Wharton and Dieguez-Uribeondo 2004). The fungus then establishes a biotrophic relationship within plant cells by forming infection vesicles and primary hyphae, and eventually, switches to necrotrophy with the formation of secondary hyphae that kill host cells (Münch et al. 2008).

The length of time of the biotrophic phase and the presence or absence of a quiescent infection period differ depending upon the *Colletotrichum* spp. and fruit host. On strawberry, the biotrophic phase is very short or does not happen at all, and there is an immediate switch to necrotrophy (Peres et al. 2005). On apple and blueberry, there is a quiescent infection period that can be shorter or longer, depending upon when initial infection occurs. Disease mainly develops on mature apple and blueberry fruits, so if infection occurs early in the growing season , the quiescent period may be long, and if infection occurs late in the season, the quiescent period is typically short (Peres et al. 2005). *Colletotrichum* also causes postharvest diseases on apple and blueberry, so often infections that occur during the growing season do not manifest till after harvest in storage (Peres et al. 2005; Prusky et al. 2013). When disease develops in the field, conidia produced in acervuli are dispersed by rain splash and wind-blown rain; thus anthracnose diseases are polycyclic (Peres et al. 2005). The production of conidia represents the asexual stage of the fungus. The sexual stage of *C. acutatum* complex species has yet to be observed in nature, but some *C. gloeosporioides* complex species exhibit a sexual stage in the field. This may play some role in the disease cycle, either as

a source of primary inoculum or as overwintering structures (Wharton and Dieguez-Uribeondo 2004), but the importance of ascospore production in the disease cycle is generally unknown (Peres et al. 2005). Once the growing season comes to an end, the fungus becomes dormant and overwinters in the form of appressoria or mycelium on or in plant tissues.

On apple, there are various overwintering sites and sources of primary inoculum. *Colletotrichum acutatum* species complex isolates can be present asymptomatically on apple buds at low levels during dormancy, as well as on leaves during the growing season in Norway (Børve and Stensvand 2007, 2017). This study found that, even though the fungus was only present at low levels, both of these reservoirs could serve as potential inoculum sources. Another study implicated apple buds, bud scales, petals, and fruit as potential sources for inoculum based upon the presence of the fungus (Everett et al. 2010). The most likely source for inoculum also changed with the seasons, with tree canopy sources being important in spring and ground sources, like fallen infected fruitlets, becoming important in summer (Everett et al. 2010). Active *Colletotrichum* has even been recovered in the winter during warm periods from apple tree cankers and buds (Leonberger et al. 2019). Overall, the source for primary inoculum is not obvious on apple because *Colletotrichum* is found to be widespread in orchards, present on both healthy and damaged tissue, and potentially surviving as an epiphyte or endophyte on apple trees or alternative hosts (Peres et al. 2005).

On blueberry, the overwintering site of the fungus has been better elucidated. *Colletotrichum acutatum* was found to survive poorly in mummified berries on the soil surface, but there was a high level of detection in flower buds and twigs (Peres et al. 2005; Verma et al. 2006; Wharton and Dieguez-Uribeondo 2004). For strawberry, overwintering is also more complex because strawberries can be planted as a short-term perennial or as an annual crop. As an annual crop, the most important source for inoculum is the nursery from which the transplants originated. *Colletotrichum* can cause disease on all strawberry plant tissues, and crown, stolon, petiole, and root diseases can occur on transplants (Peres et al. 2005). For both annual and perennial cropping

systems, primary inoculum can also come from the field, as *C. acutatum* has been shown to survive in plant debris or on the soil surface for up to two years (Parikka et al. 2006). During the growing season, *C. acutatum* can survive asymptomatically on strawberry leaves for up to eight weeks and can even cause a leaf spot disease, given suitable temperature and leaf wetness conditions (Leandro et al. 2002).

For all fruit crops, overwintering and survival on alternative hosts is an important potential source of inoculum. Parrika et al. (2006) found that *C. acutatum* was capable of infecting five common weed species found near strawberry fields, and Karimi et al. (2019) recovered *C. nymphaeae* isolates that were capable of causing disease on detached fruit from strawberry field weeds. Other *Colletotrichum* spp. have also been found to survive epiphytically and endophytically on numerous weeds, crops, and forest plants like horseweed, pepper, sugar maple, tomato, vetch, and yellow poplar (Marcelino et al. 2009; Peres et al. 2005).

Colletotrichum cross-infection potential

Many *Colletotrichum* spp. have broad host ranges, and multiple species can cause anthracnose on the same fruit host (Braganca et al. 2016; Grammen et al. 2019; He et al. 2019; Ismail et al. 2015; MacKenzie et al. 2009; Velho et al. 2015). Thus, cross-infection of *Colletotrichum* between fruits has serious management implications. Cross-infection was a common research topic in the 1990s and 2000s when studies were focused on *Colletotrichum* host range, lifestyle, and species characterization on various fruit hosts (Afanador-Kafuri et al. 2003; Alahakoon et al. 1994; Bernstein et al. 1995a; Freeman et al. 2001; Freeman and Shabi 1996; MacKenzie et al. 2009; Sanders and Korsten 2003; Whitelaw-Weckert et al. 2007). However, no definitive conclusions regarding cross-infection were made because some studies reported that fruit-rotting *C. acutatum* and *C. gloeosporioides* species were largely non-host specific (Afanador-Kafuri et al. 2003; Bernstein et al. 1995a; Freeman and Shabi 1996; Lakshmi et al. 2011), while others found evidence of host preference (Alahakoon et al. 1994; Freeman et al. 2001; MacKenzie et al. 2009). For example, Bernstein et al. (1995a) cross-inoculated

peach fruit with apple, peach, and pecan *Colletotrichum* isolates and found that all isolates caused disease on peach and were able to cross-infect in the lab. In contrast, MacKenzie et al. (2009) evaluated the host range of *C. acutatum* from various fruits in Florida and found that isolates were only pathogenic to their original host, except for two isolates that were mildly pathogenic to an alternative host.

One thing all of these early cross-infection studies had in common was a lack of proper *Colletotrichum* species identification, likely because they were published prior to the release of landmark papers describing the *C. acutatum* and *C. gloeosporioides* species complexes (Damm et al. 2012a; Weir et al. 2012). Inadequate species identification makes it difficult to interpret results. Three recent studies neglected to identify isolates to phylogenetic species when investigating cross-infection, making it difficult to draw definitive conclusions about whether or not cross-infection occurs (Harp et al. 2014; Keuete Kamdoum et al. 2018; Lakshmi et al. 2011). Only a few studies on cross-infection have properly identified isolates to phylogenetic species. Phoulivong et al. (2012) identified five *Colletotrichum* spp. causing anthracnose on six different tropical fruits and found that, overall, the species had wide host ranges. Giblin et al. (2010) did not identify species in their initial cross-infection study, but the isolates were identified in a subsequent study, revealing that avocado and mango had only one species in common (Giblin et al. 2018).

The majority of these cross-infection studies were conducted in the laboratory; few tested cross-infection under field conditions. Harp et al. (2014) tested cross-infection of a bell pepper *C. acutatum* s. lat. isolate on bell pepper, strawberry, and tomato in the field and found that the isolate was only pathogenic on pepper, even though *in vitro* it was pathogenic to all hosts. Giblin et al. (2010) tested cross-infectivity of mango and avocado isolates of *C. gloeosporioides* s. lat. on attached avocado fruit in the field and found all isolates were pathogenic with varying levels of aggressiveness. These studies provide conflicting results about cross-infection in the field, emphasizing the complexity of the issue and the need for further research.

Thus far, no study has definitively shown that cross-infection of *Colletotrichum* on fruits occurs in nature. The potential for cross-infection has only been indirectly investigated by examining species host range and characterizing the species present on host fruits. New methods are needed to directly address the issue of cross-infection and to finally answer this 30-year-old question.

Previous work on apple in Kentucky

A large collection of *Colletotrichum* isolates collected from bitter rot lesions in Kentucky has been previously characterized (Munir et al. 2016). The morphology, phylogenetic species identification, pathogenicity, and fungicide sensitivity of the collection were assessed. Isolates were grouped into four morphotypes (M1-M4) based on colony color, spore shape and size, and perithecial production; the four morphotypes aligned with five *Colletotrichum* spp. based on *GAPDH* and *TUB2* phylogenies. Of the 475 isolates collected from across the state, 71% belonged to *C. fioriniae* (M1), 3% to *C. nymphaeae* (M2), 22% to either *C. siamense* or *C. theobromicola* (M3; with *C. theobromicola* being very rare), and 4% to *C. fructicola* (M4) (Munir et al. 2016). *Colletotrichum fioriniae* and *C. nymphaeae* belong to the *C. acutatum* species complex, while *C. siamense, C. theobromicola*, and *C. fructicola* belong to the *C. gloeosporioides* complex. Pathogenicity was tested by using a detached fruit inoculation assay. The *C. gloeosporioides* complex isolates were more aggressive on apple fruit than *C. acutatum* complex isolates; however, *C. acutatum* isolates had a higher reproductive potential and produced more conidia on apple fruit than *C. gloeosporioides* (Munir et al. 2016). There were also differences in pathogenicity and spore production at the species level. Four different fungicides were tested against the five species and, overall, the *C. acutatum* species complex isolates were more tolerant to all four fungicides, and *C. gloeosporioides* isolates were more sensitive (Munir et al. 2016). Within the *C. gloeosporioides* complex, however, *C. theobromicola* was significantly more tolerant to the fungicides than the other two species and was more similar to the *C. acutatum* complex isolates in this trait (Munir et al. 2016). The results of this study indicated the

need for accurate *Colletotrichum* identification to species because individual species within the complexes exhibit important differences in pathogenicity and fungicide sensitivity that impact disease management decisions.

Problems addressed in this study

Kentucky offers a unique opportunity to study anthracnose fruit rot diseases because of the prevalence of mixed-fruit orchards. Bitter rot of apple, anthracnose fruit rot of strawberry, and ripe rot of blueberry are difficult diseases to manage and may cause significant yield losses for growers. Increased understanding of the pathogen and advanced species identification are needed to improve management strategies.

Prior to this study, very little was known about *Colletotrichum* on small fruits in Kentucky. Detailed isolate morphology had not been observed and few species identities had been confirmed. As mentioned above, *Colletotrichum* identification to species is important because species differ in traits like fungicide sensitivity that impact management decisions. Thus, I asked what *Colletotrichum* species were present on small fruits in Kentucky, and what morphological traits were specific to each. Two further questions included how small fruit species compared to the species identified on apple in the state and whether cross-infection of *Colletotrichum* occurred in mixed-fruit orchards. Many studies have previously investigated the occurrence of *Colletotrichum* cross-infection on fruit hosts, but often the results were not clear and studies were contradictory with one another. Given the potential impact that cross-infection may have on disease management, especially in Kentucky where mixed-fruit orchards are common, cross infection was a highly relevant question to explore from multiple angles.

The importance of proper *Colletotrichum* species identification for characterization and analysis also raised questions about best methods for identification. As stated previously, morphology alone cannot be used for reliable taxonomy or species identification, so multigene sequencing followed by phylogenetic analysis is the current standard method for robust identification. However, this method does not translate well to applied plant pathology and diagnostic purposes. There is a

need for a quick, reliable species identification tool for routine *Colletotrichum* diagnosis in order to address management concerns in a specific and timely manner. Therefore, the final question I asked was whether there was a potential for developing an accurate yet diagnostically useful sequence-based species identification tool.

My study had three main objectives. The first objective was to build on the work of Munir et al. (2016) by characterizing the morphology and identifying the phylogenetic species of *Colletotrichum* associated with blueberry and strawberry in Kentucky. The second objective was to determine whether cross-infection played a role in anthracnose disease development in mixed-fruit orchards. The third objective was to investigate the possibility of developing a consolidated sequence-based species identification tool for use in a diagnostic setting. Ultimately, the purpose of this study was to address concerns related to *Colletotrichum* characteristics, identification, and cross-infection because all of these factors are important for anthracnose disease development and management. The information learned about *Colletotrichum* from this study will be valuable for informing management practices and continuing to support fruit growers.

CHAPTER 2. *COLLETOTRICHUM* FRUIT ROTS IN KENTUCKY: INVESTIGATING CROSS-INFECTION POTENTIAL AND DIAGNOSTIC TOOL DEVELOPMENT

Introduction

Colletotrichum is a cosmopolitan plant-associated fungal genus (Crouch et al. 2014; da Silva et al. 2020; Dean et al. 2012). Many species are important fruit pathogens, causing pre- and postharvest yield losses worldwide (Børve and Stensvand 2015; Dean et al. 2012; Gauthier et al. 2017; Hyde et al. 2009; Kou et al. 2014; Prusky et al. 2013; Sharma and Kulshrestha 2015). Taxonomy within the genus has been in flux due to the application of molecular sequence analyses, which has resulted in the naming of multiple individual phylogenetic species from those originally defined by morphology; the former designations are now recognized as species complexes (Damm et al. 2012a; Damm et al. 2012b; Damm et al. 2014; Weir et al. 2012). The two species complexes that are most important as causal agents of anthracnose fruit rot diseases are *C. acutatum* and *C. gloeosporioides* (Damm et al. 2012a; Weir et al. 2012). Many species within these complexes have wide host ranges and, furthermore, multiple species often cause anthracnose on the same fruits (Braganca et al. 2016; Grammen et al. 2019; He et al. 2019; Ismail et al. 2015; MacKenzie et al. 2009; Velho et al. 2015). These factors make anthracnose fruit rot diseases particularly challenging to diagnose and manage.

In the southeastern U.S., *Colletotrichum* species cause three common anthracnose fruit rots: bitter rot of apple (*Malus domestica* Borkh), anthracnose fruit rot of strawberry (*Fragaria x ananassa* Duchesne), and ripe rot of blueberry (*Vaccinium* spp.). Bitter rot is the most important fruit rot disease of apple in the region, causing extensive losses under warm, wet conditions (Gauthier et al. 2017; Rosenberger 2016; Sutton et al. 2014). Anthracnose fruit rot of strawberry is a disease of concern throughout the southeastern U.S., especially in Florida where a major portion of strawberry production is centered (Adhikari et al. 2019; Borisova et al. 2014; Howard et al. 1992; Smith 2008). Ripe rot of blueberry is an economically important disease that causes preharvest and significant postharvest yield losses in all blueberry producing regions (Daykin and Milholland 1984; Milholland et al. 2017; Polashock et al. 2005).

In Kentucky, bitter rot of apple causes an average annual yield loss of 30%, with some reported losses as high as 90% (Gauthier et al. 2017). In a previous study, Munir et al. (2016) identified five *Colletotrichum* species as causal agents of bitter rot in the state: *C. fioriniae* and *C. nymphaeae* in the *C. acutatum* complex; and *C. siamense, C. fructicola,* and *C. theobromicola* in the *C. gloeosporioides* complex. They also reported that the species differed in their aggressiveness on fruit and in their sensitivity to fungicides, demonstrating that identification to species, and not just to complex, is important for disease management (Munir et al. 2016). In contrast with apple, comparatively little is known about *Colletotrichum* spp. that cause anthracnose diseases on strawberry and blueberry in Kentucky. Most of the orchards and farms in the state are small with a focus on the agritourism industry, so multiple fruit crops are often grown in close proximity. This situation raises a concern that cross-infection of *Colletotrichum* strains might occur among different fruit crops within an orchard. Evidence of cross-infection has been reported in previous studies involving other fruit crops, although many of these reports lacked modern species identifications or field confirmation (Afanador-Kafuri et al. 2003; Alahakoon et al. 1994; Bernstein et al. 1995a; Freeman et al. 2001; Freeman and Shabi 1996; Giblin et al. 2010; Harp et al. 2014; Lakshmi et al. 2011; MacKenzie et al. 2009; Phoulivong et al. 2012; Sanders and Korsten 2003). If cross-infection occurs in mixed-fruit orchards in Kentucky, changes in management recommendations will be needed, including modified spray schedules that encompass all of the fruits throughout the season.

Accurate identification of the *Colletotrichum* spp. associated with fruit rots will also improve the application of management strategies, given that different *Colletotrichum* spp. vary in their fungicide sensitivities, levels of aggressiveness, and reproductive potential (Chechi et al. 2019; Chen et al. 2016; He et al. 2019; Hu et al. 2015; Munir et al. 2016; Peres et al. 2004; Yokosawa et al. 2017). Unfortunately, accurate diagnosis of *Colletotrichum* from plant samples is a challenge. The morphological characteristics of species, e.g. spore shape and colony color, can vary depending upon the strain and the environment, and conidial and appressorial size

ranges often overlap. Thus, morphology alone is usually not considered sufficient for accurate identification (Adaskaveg 1997; Afanador-Kafuri et al. 2003; Phoulivong et al. 2010). A diagnostic test based on the *ITS* sequence for members of the *C. acutatum* versus *C. gloeosporioides* species complexes has been widely used (Mills et al. 1992; Sreenivasaprasad et al. 1996), but identification to the species level is necessary for the reasons mentioned above. The current standard for *Colletotrichum* identification is multigene sequencing and phylogenetic analysis including verified voucher sequences (Damm et al. 2012a; Jayawardena et al. 2016b; Weir et al. 2012). However, this standard is not feasible for routine diagnostic purposes. We need a convenient identification tool that allows rapid, accurate diagnosis of *Colletotrichum* fruit rots to the species level in order to adequately address cross-infection and species-specific management concerns. The objective of this study was to work toward this goal by 1) identifying and characterizing the *Colletotrichum* spp. associated with blueberry and strawberry in Kentucky; 2) evaluating the potential for cross-infection by determining whether Kentucky fruit isolates exhibit host specificity or host preference; and 3) investigating the potential for development of an improved sequence-based protocol for diagnosis of *Colletotrichum* species in Kentucky mixed-fruit orchards.

Materials and Methods

Isolate collection

Colletotrichum isolates were collected from blueberry (37) and strawberry (76) with symptoms of anthracnose from mixed-fruit orchards in 16 Kentucky counties between 2013 and 2017 (Table 2.1). A smaller number of isolates from tree fruits other than apple [apricot (1); and peach (3)] were also obtained in these orchards and included in my study. All isolates were single-spored and stored as a permanent collection on silica granules at -80°C (Tuite 1969). Additional apple *Colletotrichum* isolates from Kentucky collected by Munir et al. (2016) were used for morphological

comparison to the small fruit isolates, for the fruit inoculation assays, and genome sequencing.

Morphological observations

Colletotrichum isolates stored as conidial suspensions on silica were applied to 100 x 15 mm Petri plates containing potato dextrose agar (PDA, Difco) and incubated at 23°C under continuous fluorescent light. After 4 d, colonies were subcultured onto PDA and clarified V8 juice agar plates (200 mL clarified V8® juice, 15 g Difco Bacto-agar, 800 mL ultrapure water). After 8 to 10 d, colony color and other notable characteristics on PDA were recorded, and the top and bottom of each PDA culture plate was photographed. Conidia were harvested from 2-wk-old V8 plate cultures by using the method of Du et al. (2005) and photographed with a Zeiss Axioskop and AxioCam HRc with AxioVision 4.8.1 software (Carl Zeiss, Inc. USA). The lengths and widths of at least 50 conidia from each of 30 representative small fruit and apple *Colletotrichum* isolates were measured. The measurement data were grouped by species complex and species and subjected to analysis of variance (ANOVA), and means were compared by Student's t-test ($P < 0.05$). The analysis was performed using R software (version 3.6.1, R Core Team 2019) with the package 'easyanova'.

Preparation of DNA for Polymerase Chain Reaction (PCR)

Colletotrichum isolates were grown for 2 wks on PDA as described above. Fungal mycelium was recovered by scraping the plates gently with a spatula, grinding the mycelium with a pestle in a 1.5 mL Eppendorf tube, and transferring it to a 250 mL flask containing 50 mL of Fries Complete medium (FC; 30 g sucrose, 5 g $C_4H_{12}N_2O_6$, 1 g NH_4NO_3, 1 g KH_2PO_4, 1 g NaCl, 0.48 g $MgSO_4 \bullet 7H_2O$, 0.13 g $CaCl_2 \bullet 2H_2O$, and 1 g Difco yeast extract in 1L ultrapure water). The flask was incubated in a benchtop orbital shaker at 23°C and 175 rpm for 4 d. Fungal mycelium was harvested by vacuum filtration in a Buchner funnel lined with four layers of sterile cheesecloth and washed twice with sterile water. The washed mycelium was blotted dry with sterile paper towels, frozen in liquid nitrogen for at least 10 min, then lyophilized (VirTis SP Scientific) for 48 h.

Freeze-dried tissue was pulverized to a fine powder, and 0.1 g was transferred to a new 15 mL centrifuge tube. DNA was extracted by using a modification of the CTAB extraction method (Thon et al. 2000). The crushed tissue was mixed with 1.4 mL of 65°C CTAB extraction buffer (0.7 M NaCl, 0.1 M Tris, 0.02 M EDTA, 1% CTAB), incubated at 65°C for 30 min, followed by two extractions with phenol:chloroform:isoamyl alcohol (PCI; 25:24:1). The DNA was precipitated by adding 550 µL isopropanol and 90 µL 3 M sodium acetate (pH 5.2) followed by washing with 70% ethanol. DNA was quantified by using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA) and diluted to a final concentration of 10 ng/µL.

Complex-specific PCR

The complex-specific PCR reaction was performed to differentiate members of the *C. acutatum* species complex from members of the *C. gloeosporioides* complex. The complex-specific primers used were CaInt2 (Sreenivasaprasad et al. 1996; GGGGAAGCCTCTCGC), for amplification of members of the *C. acutatum* species complex, and CgInt (Mills et al. 1992; GGCCTCCCGCCTCCGGGCGG), for the *C. gloeosporioides* species complex. The conserved ITS4 primer (TCCTCCGCTTATTGATATGC) was used in conjunction with each complex-specific primer. Each 25 µL reaction contained 10.75 µL of sterile water, 2.5 µL of 10x PCR buffer, 1.5 µL of 50 mM $MgCl_2$, 4 µL of 1.25 mM dNTP mix, 2.5 µL of each 5 µM primer (CaInt2 or CgInt, plus ITS4), 0.25 µL of 5 U/µL Taq polymerase (Invitrogen, Carlsbad, CA), and 1 µL of 10 ng/µL genomic DNA. Samples were processed on a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) under the following conditions: 95°C for 5 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s; followed by 72°C for 60 s. Resulting PCR products, along with a 1 Kb Plus DNA ladder (Invitrogen, Carlsbad, CA), were electrophoresed through a 0.9% agarose gel (Avantor, Radnor, PA) in 1x TAE buffer at 60 V for 2 h. The gel was stained with ethidium bromide for 20 min and imaged using a Gel Doc system (Bio-Rad, Hercules, CA).

Species-specific PCR

To identify individual species within each complex, portions of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and beta-tubulin 2 (*TUB2*) genes were amplified and sequenced. The *GAPDH* sequence was amplified by using the primers GDF1 (GCCGTCAACGACCCCTTCATTGA) and GDR1 (GGGTGGAGTCGTACTTGAGCATGT) (Templeton et al. 1992). Each 30 µL reaction mixture contained 21.9 µL sterile water, 3 µL 10x PCR buffer, 1.2 µL 50 mM $MgCl_2$, 0.6 µL 10 mM dNTP mix, 0.6 µL 10 µM GDF1, 0.6 µL 10 µM GDR1, 0.1 µL 5 U/µL Taq polymerase, and 2 µL 10 ng/µL genomic DNA. Thermocycler parameters were 94°C for 5 min; 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min; followed by 72°C for 7 min. Amplification of *TUB2* was performed using the primers T1 (AACATGCGTGAGATTGTAAGT) and T2 (TAGTGACCCTTGGCCCAGTTG) (O'Donnell and Cigelnik 1997). Each 25 µL reaction mixture contained 12.3 µL sterile water, 2.5 µL 10x PCR buffer, 2.5 µL 50 mM $MgCl_2$, 2.5 µL 12.5 mM dNTP mix, 1 µL 10 µM T1, 1 µL 10 µM T2, 0.2 µL 5U/µL Taq polymerase, and 3 µL 10 ng/µL genomic DNA. The thermocycler parameters were 95°C for 4 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; followed by 72°C for 7 min. The PCR products in both cases were visualized as described above.

PCR products were prepared for sequencing using the DNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA), following the manufacturer's instructions. DNA was quantified with a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA) and sent to Eurofins Genomics (Louisville, KY) for sequencing. *GAPDH* and *TUB2* reads were aligned and manually edited by using Geneious 6.0.6 software (Biomatters, Inc., San Diego, CA). Species identity was determined by using BLASTn sequence similarity searches against the GenBank database (NCBI, Bethesda, MD), and by comparisons with the MycoBank (http://www.mycobank.org/) and Q-bank (https://qbank.eppo.int/fungi/) fungal barcoding databases.

Apple inoculations

Cultivar 'Gala' or 'Fuji' apples that had not been treated with fungicides were harvested from the University of Kentucky Horticulture Research Farm (Lexington, KY). Apples were washed with 10% bleach for 5 min, rinsed twice in sterile deionized water for 5 min, and air-dried. Apples were arranged in a randomized complete block design in humidity boxes on wire mesh with damp germination paper beneath. Experiments included either 21 (trial 1) or 18 (trial 2) representative *Colletotrichum* isolates, comprising all six species collected from Kentucky orchards (Table 2.2), including two species found on apple but not on small fruits: *C. theobromicola* (Munir et al. 2016) and an unidentified member of the *C. kahawae* clade (McCulloch et al. 2020), as well as one water control and one negative control (*C. graminicola*, a maize pathogen). Each trial was repeated twice, with five technical replicates per treatment. Inoculum was prepared by harvesting conidia from 2- to 3-wk-old PDA plates as described above and diluting to a concentration of 1×10^5 conidia/mL (Munir et al. 2016). Apples were punctured to a depth of 2 mm with a sterilized dissecting probe, and 10 µL of each treatment solution was applied to the surface of each wound. To confirm spore viability, 100 µL of each spore suspension were plated onto PDA, and fungal growth was confirmed after 4 d. Outer lesion diameter measurements were taken every 2 d, beginning at 4 dpi. At 14 dpi, final outer lesion diameter measurements were documented, and then apples were sliced in half through the middle of the wound and the inner lesion depth was measured.

Strawberry inoculations

Strawberries were purchased from the local supermarket during harvest season and used the same day of purchase. Berries were washed gently with 10% bleach, rinsed twice with sterile deionized water for 5 min, and thoroughly air-dried. Berries were arranged in a randomized complete block design in humidity boxes on wire mesh with damp germination paper beneath. The treatments were the same as above. Trial 1 was repeated twice, with nine technical replicates per treatment. Trial 2 was repeated once,

with ten technical replicates per treatment. Conidia were harvested from 3 wk PDA plates as described above and diluted to a concentration of 5 x 10^6 conidia/mL (Freeman et al. 2001). Berries were inoculated by producing a puncture wound 2 mm deep with a sterilized dissecting probe and pipetting 5 µL of each treatment solution onto each wound. Spore suspensions were also applied to PDA plates to confirm viability. Berries were observed daily, and any exhibiting signs of contamination (e.g. *Botrytis* or *Rhizopus*) were discarded immediately. Lesion diameters were measured on the remaining berries at 7 dpi.

Blueberry inoculations

Ripe 'Blue crop' cultivar blueberries were harvested from a local orchard in season. Blueberries were stored at 4°C before use and were inoculated either 4 d or 3 wks after picking. Berries were washed with 10% bleach, rinsed twice with sterile deionized water, and air-dried. Berries were arranged in a randomized complete block design in humidity boxes with PCR multiplates and damp germination paper. The blueberry inoculations were only performed with the trial 1 *Colletotrichum* strains (Table 2.2) plus controls, and the experiment was repeated once. Each experiment included three berries per treatment per technical replicate and nine technical replicates per experiment. Conidia were harvested from 3 wk PDA plates as described above and diluted to a concentration of 5 x 10^5 conidia/mL [adapted from Miles et al. (2012)]. Berries were inoculated by making a 2 mm-deep puncture wound in the calyx with a sterilized dissecting probe and pipetting 10 µL of each treatment solution onto the wound. Spore suspensions were also plated onto PDA plates to confirm viability. The number of berries showing signs (disease incidence) was recorded daily, and after 10 d, the berries were rated for disease severity on a scale of 0-4 based on fungal sporulation level: zero = no sporulation; 1 = 1-10% spore coverage; 2 = 11-49% spore coverage; 3 = 50-99% spore coverage; and 4 = 100% spore coverage (Figure 2.3 G-K).

Fruit inoculation statistics

Fruit inoculation data for the apple, strawberry, and blueberry detached fruit assays were analyzed by one-way analysis of variance (ANOVA), grouping individual treatment data points by species complex, species, and original fruit host. Post-hoc comparisons were made by using the Tukey-Kramer Test. All analyses were performed in SAS 9.4 Proc Glimmix (SAS Institute, Cary, NC).

Maxi-prep of high quality DNA for whole genome sequencing

Conidia were harvested from 3 wk-old PDA plates as described above, and the concentration was adjusted to 1 x 10^6 conidia/mL. A 1 liter flask containing 500 mL of Fries Complete medium was inoculated with 1 mL of the 1 x 10^6 conidia/mL suspension and incubated in a benchtop orbital shaker at 200 rpm and 30°C for 2 to 6 d. Mycelium was harvested after 2 to 4 d, as soon as microcolonies became visible, by vacuum filtration through a Buchner funnel lined with 4 layers of sterile cheesecloth. The mycelium was rinsed three times with sterile water, blotted on sterile paper towels, and homogenized in a Waring blender cup in 30 mL of Fries Complete medium. Five milliliters of the slurry were used to inoculate 250 mL of fresh Fries Complete medium in a 1-liter flask that was incubated at 175 rpm and 25°C for 2-3 d. The culture was harvested by vacuum filtration as described above and the mycelium was weighed, flash-frozen in liquid nitrogen, and stored at -20°C until processing.

Two grams of frozen tissue were used for DNA extraction. The tissue was ground to a fine powder in a mortar and pestle under liquid nitrogen, transferred to a 50 mL tube with 4 mL of 65°C 1x CTAB, and incubated at 65°C for 1 h. After adding 4 mL of PCI, the tube was rolled on an Orbitron Rotator I (Boekel Scientific, Feasterville, PA) for 5 min, and centrifuged at 3000 rpm for 30 min. The aqueous phase was transferred to a new tube and re-extracted with 4 mL of PCI as described above. The aqueous phase was transferred to a new tube and re-extracted with 4 mL of chloroform as described above. The aqueous phase from the chloroform extraction was layered onto 4 mL of isopropanol in a fresh tube, and centrifuged at 13,000 rpm and 4°C for 30 min. The DNA

pellet was washed twice with 5 mL 95% ethanol, centrifuging at 13,000 rpm and 4°C for 10 min. The pellet was air-dried for 10 min, then resuspended in 1 mL 1x TE buffer and 5 µL RNase A overnight at 4°C. The resuspended pellet was rolled on the Orbitron for 30 min, then aliquoted into 1.5 mL Eppendorf tubes, leaving enough room to add half the volume of 7.5 M ammonium acetate. The tubes were rolled on the Orbitron for 30 min, then centrifuged at 13,000 rpm for 25 min. The supernatant was transferred to a new Eppendorf tube and an equal volume of chloroform was added. The tubes were rolled on the Orbitron for 5 min, then centrifuged at 6000 rpm for 15 min. The aqueous phase was transferred to a new Eppendorf tube, two volumes of cold 95% ethanol were added, and the tubes were centrifuged at 13,000 rpm for 30 min. The pellet was washed twice with 1 mL of 70% ethanol, centrifuging at 13,000 rpm for 3 min, then air-dried for 20 min and re-suspended in 100 µL sterile DNA-grade water at 4°C overnight.

The DNA was quantified by using a Qubit fluorometer and submitted to the Duke Center for Genomic and Computational Biology (Durham, NC) for sequencing. The Illumina NextSeq 500 platform was used to sequence 300 bp DNA insert libraries, generating 150 bp paired-end reads at 50x coverage.

Whole genome assembly

Whole genomes of 28 representative *Colletotrichum* strains were assembled by using a custom command line-based bioinformatics pipeline, "BioPipe". Paired Illumina read input files were merged into a single file and trimmed with Trimmomatic V0.32 in paired-end mode, specifying a minimum length of 20. Nextera adaptor sequences were removed with ILLUMINACLIP. Forward and reverse paired output files were interleaved using the interleave.py script (https://github.com/jorvis/biocode/blob/master/fasta/interleave_fasta.py) and assembled by using Velvet (Zerbino and Birney 2008) via the VelvetOptimiser wrapper (https://github.com/tseemann/VelvetOptimiser). Parameters for VelvetOptimiser were as follows: start kmer value:83, end kmer value:115, step size:2, and with the -shortPaired setting. Contigs generated by VelvetOptimiser were screened for bacterial

contamination by applying Kraken (Wood and Salzberg 2014), using default settings and a custom reference database consisting of the *Colletotrichum* reference genome set from GenBank (Table 2.3). Contaminant contigs were removed from the assemblies by a custom script. Cleaned assemblies were input to another custom pipeline, "iSNPcaller" (https://github.com/drdna/iSNPcaller) that called Single Nucleotide Polymorphisms from BLAST alignments of repeat-masked sequences. The pairwise distance matrix generated by iSNPcaller was input into MEGAX to generate a similarity cladogram using the Neighbor-Joining program.

Phylogenetic analysis

Phylogenetic analysis of *ACT, ApMat, CAL, CHS, GAPDH, GS, ITS, SOD2*, and *TUB2* gene sequences was conducted according to the methods of Damm et al. (2012a) and Weir et al. (2012). *Colletotrichum gloeosporioides* and *C. acutatum* sequences from GenBank (NCBI, Bethesda, MD) (Table 2.4) were used to identify homologs via BLASTn from whole genome assemblies. BLAST output sequences were imported into MEGA7 (Version 7.0.26, https://www.megasoftware.net/) and aligned with MUSCLE (default parameters). Alignments were manually edited and trimmed. Phylograms were constructed using the PhyML Approximate Likelihood-Ratio Test and TreeDyn for rendering in Phylogeny.fr (Dereeper et al. 2010; Dereeper et al. 2008).

Results

Morphology and Species Identification

The *Colletotrichum* isolates from small fruits were classified into seven groups based on colony morphology, aka morphotype (Table 2.1, Figure 2.1). The first four of these groups corresponded with morphotypes 1 to 4 (M1 to M4) originally described in Munir et al. (2016) for isolates of *Colletotrichum* causing apple bitter rot in Kentucky. As previously defined, M1 was characterized by a smooth gray-green upper surface and production of a distinctive red pigment on the reverse. M2 exhibited a dark gray to

white upper surface with a white border and was orange on the reverse. M3 had a fluffy, white to gray-green upper and a mottled tan or brown reverse. The upper and lower surfaces of M4 were both dark gray with white borders. Three additional morphotypes were recognized among the small fruit isolates examined in this study. M5 was similar to M1 on the upper surface, but the reverse lacked the red pigment and was instead orange-brown. M6 was a brighter pink on the reverse than M1, and the upper surface was also pink, sometimes exhibiting a central orange zone and a distinctive speckled appearance. M7 was similar to M2 on the reverse, but the top was darker with a thin white border (Figure 2.1).

Application of the complex-specific sequence analysis of 54 representative small fruit isolates demonstrated that M1, M2, M5, M6, and M7 all belonged to the *C. acutatum* species complex, while M3 and M4 belonged to *C. gloeosporioides* (Suppl. Table 2.1).

Amplification of *GAPDH* and/or *TUB2* sequences from representative strains for each morphotype, and comparison of the sequences with NCBI, Q-bank, and Mycobank DNA databases, indicated that M1, M5, and M6 all belonged to the species *C. fioriniae* (Table 2.5). All members of M2 and M7 were identified as *C. nymphaeae* (Table 2.5). The three representatives of M3 were *C. siamense*, while the *GAPDH* sequence of the single M4 isolate matched *C. fructicola* (Table 2.5). The *C. gloeosporioides* isolates and some *C. nymphaeae* isolates produced double bands for the *TUB2* product, thus the *TUB2* sequence of these isolates was not included in our analysis. For *C. gloeosporioides*, *the* off-target band may have been the *TUB1* gene, which has a similar sequence (Buhr and Dickman 1993; Maymon et al. 2006), but it was unclear why some of the *C. nymphaeae* isolates also consistently produced double bands. Nonetheless, even when only one gene was available, comparison with the databases produced a single, unambiguous match in each case.

For each fruit host, the isolates grouped by morphotype could then be assigned to species. All of the blueberry isolates in this study were identified as *C. fioriniae* (Figure 2.2). Most (74%) belonged to M1, 10% were M5, and 16% belonged to M6. The single

isolate from apricot and two of the three isolates from peach were also identified as *C. fioriniae*, all M1. Most (91%) of the strawberry isolates belonged to *C. nymphaeae* (M2 and M7). The M2 morphotype was more common, comprising 88.5% of the collection. The remainder of the isolates from strawberry were identified as *C. fioriniae* (M1, 4%), *C. siamense* (M3, 4%), and *C. fructicola* (M4, 1%) (Figure 2.2). Twelve of the 76 strawberry isolates in the collection originated from diseased crowns: nine of these isolates (75%) were *C. nymphaeae*, while the remainder were *C. siamense*. One of the three isolates from peach also belonged to *C. siamense*.

Conidia representing all four *Colletotrichum* spp. recovered from strawberry and blueberry, and one isolate of *C. theobromicola* [aka. *C. fragariae*, originally collected from strawberry in Mississippi (Du et al. 2005; Smith and Black 1990)], were compared with conidia recovered from isolates of the same species from apple (Table 2.6). *Colletotrichum fioriniae* conidia were fusiform in shape. *Colletotrichum nymphaeae* conidia were also fusiform, but with slightly less pronounced points on the ends than *C. fioriniae*. Spores of *C. siamense* and *C. fructicola* were cylindrical with rounded ends. *Colletotrichum theobromicola* conidia varied from ovoid to cylindrical with one tapered end. Conidia of strains within each species were similar in shape and size, regardless of their original fruit host. There were some significant differences in average size between fungal species in different species complexes, but the ranges within species were broad and overlapped between species (Table 2.6).

Fruit inoculation assays

Results of multiple inoculation studies with representative isolates of all six *Colletotrichum* species demonstrated that they were all capable of causing typical anthracnose symptoms on apple, blueberry, and strawberry fruits, regardless of their original fruit host (Figure 2.3). The *C. graminicola* negative control did not cause disease symptoms or sporulate on any of the fruits (not shown). Mock-inoculated (water control) apple and strawberry fruits also resulted in no symptoms (not shown). Some of

the control blueberry fruits did develop ripe rot during the incubation: disease incidence and severity were evaluated in comparison with this baseline of natural infection.

As previously reported by Munir et al. (2016), *C. gloeosporioides* was more aggressive than *C. acutatum* on apples when isolates were grouped by species complex (Figure 2.4A, Suppl. Table 2.2). This was not the case for small fruits, however. Depending upon the trial, *C. acutatum* was more aggressive or similarly aggressive when compared with *C. gloeosporioides* on strawberries, and *C. acutatum* was more aggressive on blueberries (Figure 2.4A, Suppl. Table 2.2).

There was a broad range of aggressiveness on all three fruits among isolates within the same species (Figure 2.4B). On apple, *C. siamense*, *C. fructicola*, and *C. kahawae* clade were the most aggressive, while *C. theobromicola* and the two *C. acutatum* complex species were the least aggressive (Figure 2.4B, Suppl. Tables 2.3 and 2.5). The results were different for strawberries, in which *C. fioriniae* and *C. nymphaeae* were similar to *C. siamense* and *C. fructicola*, while *C. kahawae* clade and *C. theobromicola* were much less aggressive. For the blueberry inoculations, we collected both disease incidence and severity data, and the two measures were correlated (Suppl. Figure 2.1), so only incidence data is reported in Figure 2.4. The two *C. acutatum* species were the only ones that differed statistically from the controls on blueberry. Overall, the results indicate that the *C. acutatum* species *C. fioriniae* and *C. nymphaeae* were more aggressive on small fruits than on apples, while the *C. kahawae* clade isolates were more aggressive on apples than on small fruits. *Colletotrichum siamense* and *C. fructicola* were aggressive both on apples and strawberries, and *C. theobromicola* was less aggressive than the *C. acutatum* species complex and other members of the *C. gloeosporioides* complex on both apples and strawberries. When isolates were grouped by host origin (Figure 2.4C), there was no evidence for host preference. Isolates of the same species from different hosts generally performed similarly when inoculated on the same fruit.

Genome sequencing and gene trees

Genomes of 28 representative *Colletotrichum* strains covering all six species from apple, strawberry, and blueberry were sequenced and assembled (Table 2.7). Assemblies were aligned and numbers of SNPs were compared in order to produce a whole genome tree (Suppl Table 2.7, Figure 2.5). The genome tree resolved all described species, as well as the novel morphotype subgroups within the species *C. fioriniae* (M6) and *C. nymphaeae* (M7). In most cases, the genome and other single gene species identifications agreed with the preliminary *GAPDH* and/or *TUB2* identifications, but for three isolates in the *C. gloeosporioides* complex the nine single genes matched (> 98% similarity) more than one species. Two of these isolates were identified as unknown members of the *C. kahawae* clade; the *GAPDH* sequence alone had identified them as *C. camelliae* (McCulloch et al. 2020). The third isolate was the single M4 strawberry isolate that matched *C. fructicola* based on *GAPDH* alone, but the other single gene sequences matched multiple other species, and the genome separated it from *C. fructicola* and grouped it more closely with an endophyte of an unknown species within the *C. gloeosporioides* complex (Rytas Vilgalys, personal communication). The reference *C. siamense* genome was separated from the Kentucky isolates. No single gene tree was capable of capturing the full resolution of the genome tree for both species complexes (Suppl. Figures 2.2-2.8). However, specific genes were able to differentiate species and subgroups within each species complex. For the *C. acutatum* complex, the *CHS* sequence efficiently delineated the *C. fioriniae* and *C. nymphaeae* subgroups present in the genome tree (Figure 2.6). For the *C. gloeosporioides* complex, *ACT, ApMat, CAL, GAPDH, GS*, and *SOD2* were all able to delineate species within the complex and separate *C. fructicola* from the unknown species present in the genome tree (Figure 2.7, Suppl. Figures 2.2, 2.4, 2.5, 2.6, and 2.8). *ApMat* provided the clearest distinction between species (Figure 2.7).

Discussion

In this study, the four species of *Colletotrichum* associated with strawberry anthracnose and blueberry ripe rot in Kentucky (*C. fioriniae, C. nymphaeae, C. siamense*, and *C. fructicola*) were confirmed to be the same species causing apple bitter rot in the same orchards studied by Munir et al. (2016). *Colletotrichum fioriniae* was the dominant species on both apple (70%) and blueberry (100%). In contrast, the most abundant species on strawberry was *C. nymphaeae* (95%), which is the most commonly reported causal agent of strawberry anthracnose in the U.S. and worldwide (Baroncelli et al. 2015; Damm et al. 2012a; Jayawardena et al. 2016a; Karimi et al. 2016; Wang et al. 2019).

Differences in relative abundance suggest that cross-infection may occur less frequently between strawberries and the other fruits. The timing of fruit development may help explain this, as strawberries ripen in May and June, blueberries from June to July, and apples from July through November. Furthermore, apples and blueberries are both long-term perennial crops that remain in the orchards for long periods, whereas strawberries are mostly grown as either annual (plasticulture) or short-term perennial (matted row) crops. Species common on strawberry may reflect populations that may be introduced on transplants.

Most reports suggest that fruit-rotting *C. acutatum* and *C. gloeosporioides* species are largely non-host specific (Afanador-Kafuri et al. 2003; Bernstein et al. 1995a; Freeman and Shabi 1996; Lakshmi et al. 2011), although a few have found evidence of host preference (Alahakoon et al. 1994; Freeman et al. 2001; MacKenzie et al. 2009). In my study, all fruit isolates, regardless of original host or species, were pathogenic on all three fruits tested. Furthermore, there was no significant difference in aggressiveness among *Colletotrichum* strains of the same species that originated from different hosts, suggesting a lack of host adaptation of the individual strains within a species. However, there were significant differences in aggressiveness on different fruits between the two species complexes, as well as among some species within complexes, highlighting the importance of proper species identification.

Traditionally, phenotypic characters such as culture color, conidium shape and size, presence of setae or perithecia, and growth rate have been used to identify *Colletotrichum* species (Sutton 1992; von Arx 1957). Even though *Colletotrichum* morphology is generally an unreliable tool for identification, Munir et al. (2016) found that when isolates were categorized by 'morphotype' based on specific colony and conidial characteristics under defined cultural and environmental conditions, the morphological groups were closely correlated with molecular species identifications. Likewise, under consistent, controlled environmental conditions, the majority of the small fruit isolates could be grouped into morphotypes originally described by Munir et al. (2016), and morphotype predicted species identity with a high degree of accuracy. The new variant morphotypes M6 and M7 could be recognized as distinct branches near the other members of the same species in the genome tree, confirming consistent genetic distinctions among these morphotypes. This reliable connection between morphotype under controlled conditions and phylogenetic relatedness may be useful for making preliminary *Colletotrichum* spp. identifications for quick diagnosis.

The currently accepted standard for *Colletotrichum* species identification is multigene sequencing with an emphasis on a polyphasic approach (Baroncelli et al. 2015; Braganca et al. 2016; Cai et al. 2009; Chechi et al. 2019; Chen et al. 2016; Damm et al. 2012a; Grammen et al. 2019; He et al. 2019; Hyde et al. 2009; Jayawardena et al. 2016b; Liu et al. 2015; Moreira et al. 2019; Park et al. 2018; Wang et al. 2016; Weir et al. 2012). Previous studies have also determined that some genes are more informative than others for the identification of species within the *C. acutatum* and *C. gloeosporioides* complexes (Damm et al. 2012a; Liu et al. 2015; Weir et al. 2012). Damm et al. (2012a) found that *GAPDH* and *TUB2* were the most informative genes for the *C. acutatum* complex, and, in our study, these worked well for species prediction within that complex, but they did not capture the variant M6 or M7 morphotypes, and the *C. gloeosporioides* resolution was poor. In comparing our whole genome tree to single gene trees, our goal was to identify the most informative gene(s) for our isolate collection that also captured morphotypic variation. For the *C. acutatum*

complex, *CHS* was the only gene that was able to recapitulate the genome tree in its entirety, including the novel morphotypes. For the *C. gloeosporioides* complex, *ApMat* provided the clearest resolution, including resolution of the novel endophytic species from *C. fructicola* and *C. siamense*. This finding is in agreement with several studies that have focused on *C. gloeosporioides* and also recommended the use of *ApMat* for identification (Liu et al. 2015; Sharma et al. 2013; Silva et al. 2012; Wang et al. 2016; Yokosawa et al. 2017). These gene sequences may be used to further develop diagnostic PCR primers to species level, similar to those used for *C. acutatum* and *C. gloeosporioides* complex identification (Mills et al. 1992; Sreenivasaprasad et al. 1996).

While *CHS* and *ApMat* may work well for our Kentucky isolate collection, single gene and multi-gene sequence analysis should still be used with caution. Multi-locus sequencing and Genealogical Concordance Phylogenetic Species Recognition (GCPSR) have improved *Colletotrichum* taxonomy, but it is critical to still incorporate other information and to use a polyphasic approach to delineate species (Cai et al. 2009; Liu et al. 2016). An example of this point is the confusion about the species *C. siamense*. Using multi-locus sequencing and largely relying on *ApMat* sequence data alone, it was determined that *C. siamense* in the *C. gloeosporioides* complex was itself a species complex (Prihastuti et al. 2009; Sharma et al. 2013; Sharma et al. 2014). However, Liu et al. (2016) tested the *C. siamense* species boundary using GCPSR with six or five genes, as well as three coalescent molecular analysis methods, and found that the existence of a *C. siamense* species complex was not supported. Furthermore, mating tests and morphological analysis did not support the existence of a species complex (Liu et al. 2016). Even though the reference *C. siamense* strain (ICMP18578) seemed to be distant from our Kentucky *C. siamense* strains in the genome tree, they may still belong to the same phylogenetic species given that *C. siamense* is a very diverse group. Additional analyses with more *C. siamense* genomes from a wide range of hosts and locations are necessary to determine the taxonomic status of *C. siamense* at a genome level.

Ultimately, stable *Colletotrichum* taxonomy and reliable species identification are crucial for the implementation of effective disease management strategies. Species

differ in their fungicide sensitivity, virulence, and host range, all of which impact chemical control, horticultural practices, resistance breeding, and biosecurity. Many studies have shown that *Colletotrichum* isolates differ in their fungicide sensitivities at a species level (Chechi et al. 2019; Chen et al. 2016; Munir et al. 2016), and in some cases, certain species have developed fungicide resistance (Hu et al. 2015; Yokosawa et al. 2017). A few publications have mentioned the potential management impact of *Colletotrichum* cross-infection on a cropping system (Freeman et al. 1998; Harp et al. 2014; Lakshmi et al. 2011; Phoulivong et al. 2012), although none have outlined possible mitigation strategies. Knowing which species are present within a production system, their sensitivities, and the likelihood for cross-infection, can help improve fungicide and general management recommendations. Furthermore, *Colletotrichum* species identification can reveal previously unsuspected yet important differences in behavior and pathogenicity of species present within an orchard. The foundation for sequence-based diagnostic identification developed in this study provides a starting point for determining whether variability in disease development or response to fungicides in the field is associated with species identity.

Table 2.1 *Colletotrichum* isolates collected from blueberry and strawberry fruits, and from apricot and peach, in Kentucky by county and morphotype.

Host	County	Morphotype							Total
		M1	M2	M3	M4	M5	M6	M7	
Apricot	Christian	1	-	-	-	-	-	-	1
Blueberry	Bourbon	15	-	-	-	4	-	-	19
Blueberry	Scott	9	-	-	-	-	5	-	14
Blueberry	Simpson	3	-	-	-	-	-	-	3
Blueberry	Webster	1	-	-	-	-	-	-	1
Peach	Daviess	1	-	-	-	-	-	-	1
Peach	Henderson	1	-	-	-	-	-	-	1
Peach	Woodford	-	-	1	-	-	-	-	1
Strawberry	Adair	-	4	-	-	-	-	-	4
Strawberry	Christian	-	2	-	1	-	-	-	3
Strawberry	Daviess	-	20	-	-	-	-	-	20
Strawberry	Fayette	-	4	-	-	-	-	-	4
Strawberry	Graves	-	-	1	-	-	-	-	1
Strawberry	Grayson	-	2	-	-	-	-	-	2
Strawberry	Marshall	-	4	-	-	-	-	-	4
Strawberry	Muhlenberg	-	1	-	-	-	-	-	1
Strawberry	Pulaski	-	2	-	-	-	-	-	2
Strawberry	Scott	1	21	-	-	-	-	-	22
Strawberry	Todd	-	1	-	-	-	-	-	1
Strawberry	Woodford	2	4	2	-	-	-	3	11
Strawberry	Unknown	-	1	-	-	-	-	-	1
Total		34	66	4	1	4	5	3	117

Table 2.2 Kentucky *Colletotrichum* isolates used for apple, blueberry, and strawberry fruit inoculation assays.

Isolate	Host	Origin (County)	Species	Trial[z]
HC 25	Apple	Bourbon	*C. fioriniae*	2
HC 278	Apple	Bourbon	*C. kahawae* clade	1
HC 282	Apple	Bourbon	*C. kahawae* clade	2
HC 291	Apple	Bourbon	*C. kahawae* clade	2
HC 292	Apple	Bourbon	*C. kahawae* clade	1
HC 296	Apple	Bourbon	*C. fioriniae*	2
HC 533	Apple	Bourbon	*C. fructicola*	2
HC 540	Apple	Bourbon	*C. fructicola*	1
HC 646	Apple	Bourbon	*C. nymphaeae*	2
KY 146	Apple	Clinton	*C. siamense*	2
KY 152	Apple	Perry	*C. theobromicola*	2
KY 254	Apple	Lyon	*C. siamense*	2
KY 540	Apple	Fayette	*C. siamense*	2
KY 6	Apple	Harlan	*C. fioriniae*	1, 2
KY 639	Apple	Harlan	*C. fioriniae*	2
KY 8	Apple	Harlan	*C. siamense*	2
KY 650	Apricot	Christian	*C. fioriniae*	1
KY 116	Blueberry	Bourbon	*C. fioriniae*	1
KY 118	Blueberry	Bourbon	*C. fioriniae*	1
KY 119	Blueberry	Bourbon	*C. fioriniae*	2
KY 640	Blueberry	Webster	*C. fioriniae*	1
KY 646	Blueberry	Scott	*C. fioriniae*	2
KY 648	Blueberry	Scott	*C. fioriniae*	1
KY 655	Blueberry	Bourbon	*C. fioriniae*	1
KY 657	Blueberry	Bourbon	*C. fioriniae*	1
KY 777	Peach	Woodford	*C. siamense*	1
KY 332	Strawberry	Christian	*C. gloeosporioides* s.l.	1
KY 509	Strawberry	Woodford	*C. nymphaeae*	2
KY 521	Strawberry	Marshall	*C. nymphaeae*	2
KY 522	Strawberry	Marshall	*C. nymphaeae*	1
KY 563	Strawberry	Fayette	*C. nymphaeae*	1
KY 567	Strawberry	Christian	*C. nymphaeae*	1
KY 613	Strawberry	Woodford	*C. nymphaeae*	1
KY 615	Strawberry	Woodford	*C. fioriniae*	1, 2
KY 687	Strawberry	Graves	*C. siamense*	1
KY 745	Strawberry	Woodford	*C. nymphaeae*	1
KY 748	Strawberry	Woodford	*C. siamense*	1

[z] Only trial 1 isolates were used for the blueberry fruit inoculation assay.

Table 2.3 Reference *Colletotrichum* genomes used for the whole genome and single gene trees.

Isolate	Species	Host	Source	GenBank accession number
1104-7	*C. fructicola*	*Malus domestica*	Liang et al. 2018	MVNS00000000.1
CBS 122122	*C. simmondsii*	*Carica papaya*	Baroncelli et al. 2016	JFBX00000000.1
CBS 607.94	*C. salicis*	*Salix* sp.	Baroncelli et al. 2016	JFFI00000000.1
MH 18	*C. fioriniae*	*Populus* sp.	JGI, used with permission	
GC 23	*C. gloeosporioides* s.l.	*Populus* sp.	JGI, used with permission	
ICMP18578	*C. siamense*	*Coffea arabica*	Meng et al. 2019	RJJI00000000.1
IMI 309357	*C. orchidophilum*	*Phalaenopsis* sp.	Baroncelli et al. 2018	MJBS00000000.1
KC05	*C. scovillei*	*Capsicum* sp.	Han et al. 2016	LUXP00000000.1
Nara gc5	*C. fructicola*	*Fragaria* x *ananassa*	Gan et al. 2013	ANPB00000000.1
SA-01	*C. nymphaeae*	*Fragaria* x *ananassa*	Baroncelli et al. 2016	JEMN00000000.1
SMCG1#C	*C. gloeosporioides* s.s.	*Cunninghamia lanceolata*	Huang et al. 2019	QFRH00000000.1

Table 2.4 Reference genes used for BLASTn against *Colletotrichum* whole genome assemblies.

Gene	*C. gloeosporioides* complex	*C. acutatum* complex
ACT	JX009491	JQ949620
ApMat	MN915014	-
CAL	JX009675	KJ954727
CHS	JX009772	JQ948960
GAPDH	JX009998	JQ948629
GS	JX010077	KJ955025
ITS	JX010167	JQ948299
SOD2	JX010310	-
TUB2	JX010388	JQ949950

Table 2.5 Species identifications of representative small fruit *Colletotrichum* isolates from Kentucky based on *GAPDH* and *TUB2* gene sequences with accession numbers.

Isolate	Host	County	Morphotype	Species	*GAPDH*	*TUB2*
KY 650	Apricot	Christian	1	*C. fioriniae*	MN412421	MN412455
KY 116	Blueberry	Bourbon	5	*C. fioriniae*	MN412412	MN412453
KY 118	Blueberry	Bourbon	1	*C. fioriniae*	MN412413	MN412445
KY 640	Blueberry	Webster	6	*C. fioriniae*	MN412443	MN412446
KY 644	Blueberry	Scott	1	*C. fioriniae*	MN412414	MN412451
KY 647	Blueberry	Scott	6	*C. fioriniae*	MN412416	MN412454
KY 648	Blueberry	Scott	6	*C. fioriniae*	MN412444	MN412449
KY 655	Blueberry	Bourbon	1	*C. fioriniae*	MN412415	MN412450
KY 657	Blueberry	Bourbon	5	*C. fioriniae*	MN412418	MN412448
KY 673	Blueberry	Bourbon	1	*C. fioriniae*	MN412419	MN412452
KY 761	Blueberry	Bourbon	5	*C. fioriniae*	MN412422	MN412456
KY 765	Blueberry	Scott	6	*C. fioriniae*	MN412420	MN412457
KY 771	Blueberry	Simpson	1	*C. fioriniae*	MN412417	MN412458
KY 777	Peach	Woodford	3	*C. siamense*	MN412437	-
KY 332	Strawberry	Christian	4	*C. fructicola*[z]	MN412435	-
KY 516	Strawberry	Scott	2	*C. nymphaeae*	MN412424	MN412459
KY 522	Strawberry	Marshall	2	*C. nymphaeae*	MN412434	MN412460
KY 563	Strawberry	Fayette	2	*C. nymphaeae*	MN412436	MN412461
KY 567	Strawberry	Christian	2	*C. nymphaeae*	MN412438	MN412467
KY 569	Strawberry	Daviess	2	*C. nymphaeae*	MN412429	MN412462
KY 576	Strawberry	Adair	2	*C. nymphaeae*	MN412430	MN412463
KY 585	Strawberry	Scott	1	*C. fioriniae*	MN412423	MN412447
KY 613	Strawberry	Woodford	7	*C. nymphaeae*	MN412439	MN412464
KY 628	Strawberry	Scott	2	*C. nymphaeae*	MN412431	MN412465
KY 631	Strawberry	Scott	2	*C. nymphaeae*	MN412432	MN412468
KY 635	Strawberry	Fayette	2	*C. nymphaeae*	MN412425	MN412466
KY 687	Strawberry	Graves	3	*C. siamense*	MN412440	-
KY 740	Strawberry	Daviess	2	*C. nymphaeae*	MN412426	MN412469
KY 745	Strawberry	Woodford	7	*C. nymphaeae*	MN412441	-
KY 746	Strawberry	Daviess	2	*C. nymphaeae*	MN412427	-
KY 748	Strawberry	Woodford	3	*C. siamense*	MN412442	-
KY 753	Strawberry	Scott	2	*C. nymphaeae*	MN412428	-
KY 756	Strawberry	Pulaski	2	*C. nymphaeae*	MN412433	-

[z] Based on *GAPDH* sequence alone; identified as *C. gloeosporioides* s.l. (unknown species)

Table 2.6 Mean conidial measurements of representative individual *Colletotrichum* isolates from apple, blueberry, and strawberry, and mean measurements of isolates combined by species (in gray).

Isolate	Species	Host	Length (µm)[y]	Width (µm)[y]	L/W ratio
HC 25	*C. fioriniae*	Apple	15.04 (1.54)	5.24 (0.42)	2.87
KY 6	*C. fioriniae*	Apple	14.36 (1.56)	4.99 (0.48)	2.87
KY 323	*C. fioriniae*	Apple	14.83 (1.03)	5.22 (0.57)	2.84
KY 650	*C. fioriniae*	Apricot	14.38 (1.66)	5.25 (0.58)	2.74
KY 116	*C. fioriniae*	Blueberry	14. 12 (2.15)	5.23 (0.56)	2.70
KY 118	*C. fioriniae*	Blueberry	14.33 (1.64)	4.98 (0.40)	2.88
KY 640	*C. fioriniae*	Blueberry	14.31 (1.73)	5.30 (0.52)	2.70
KY 648	*C. fioriniae*	Blueberry	14.07 (1.31)	5.12 (0.51)	2.75
KY 655	*C. fioriniae*	Blueberry	14.09 (1.21)	5.17 (0.51)	2.73
KY 657	*C. fioriniae*	Blueberry	14.12 (1.53)	5.27 (0.51)	2.68
KY 615	*C. fioriniae*	Strawberry	13.71 (1.56)	5.03 (0.56)	2.73
	C. fioriniae		14.37 (1.55) b, c[z]	5.18 (0.52) b, c	2.78
HC 646	*C. nymphaeae*	Apple	14.04 (1.07)	4.79 (0.42)	2.93
HC 647	*C. nymphaeae*	Apple	14.72 (1.50)	4.93 (0.39)	2.99
KY 9	*C. nymphaeae*	Apple	15.37 (2.12)	4.94 (0.46)	3.11
KY 522	*C. nymphaeae*	Strawberry	16.00 (2.29)	4.98 (0.54)	3.21
KY 563	*C. nymphaeae*	Strawberry	15.73 (2.17)	5.05 (0.55)	3.11
KY 567	*C. nymphaeae*	Strawberry	15.28 (1.98)	4.74 (0.58)	3.22
KY 613	*C. nymphaeae*	Strawberry	13.91 (1.84)	4.84 (0.44)	2.87
KY 745	*C. nymphaeae*	Strawberry	14.28 (1.26)	5.04 (0.58)	2.83
	C. nymphaeae		14.81 (1.97) b, b	4.91 (0.50) b, d	3.02
KY 8	*C. siamense*	Apple	16.03 (2.58)	4.89 (0.47)	3.28
KY 146	*C. siamense*	Apple	16.30 (2.68)	5.22 (0.60)	3.12
KY 254	*C. siamense*	Apple	16.64 (1.85)	5.47 (0.34)	3.04
KY 777	*C. siamense*	Peach	15.97 (1.17)	5.84 (0.64)	2.73
KY 687	*C. siamense*	Strawberry	16.54 (1.40)	6.08 (0.40)	2.72
KY 748	*C. siamense*	Strawberry	15.30 (1.25)	5.29 (0.56)	2.89
	C. siamense		16.13 (1.96) a, a	5.46 (0.64) a, b	2.95
HC 540	*C. fructicola*	Apple	11.18 (2.31)	4.58 (0.66)	2.44
KY 332	*C. fructicola*[x]	Strawberry	17.89 (1.51)	5.89 (0.83)	3.04
	C. fructicola		14.18 (3.91) a, c	5.17 (0.98) a, c	2.74

Table 2.6 (continued).

Isolate	Species	Host	Length (µm)	Width (µm)	L/W ratio
KY 152	*C. theobromicola*	Apple	15.45 (1.93)	5.13 (0.50)	3.01
KY 153	*C. theobromicola*	Apple	17.44 (1.88)	5.58 (0.49)	3.13
CF 75	*C. theobromicola*	Strawberry	13.13 (3.91)	6.57 (0.74)	2.00
	C. theobromicola		15.81 (2.95) a, a	5.63 (0.76) a, a	2.81

[x] Based on *GAPDH* sequencing alone; identified as *C. gloeosporioides* s.l. (unknown species)

[y] Mean length and width with standard deviation in parentheses

[z] Values with the same letter are not statistically different ($P \leq 0.05$) according to Student's t-test analysis; the first letter is for species complex comparison; the second letter is for species comparison.

Table 2.7 Genome assembly statistics and accession numbers for Kentucky apple, blueberry, and strawberry *Colletotrichum* isolates.

Isolate	Species	Host	Assembly Size	Contigs	N50	Accession Number
HC 25	*Colletotrichum fioriniae*	Apple	49.9 Mb	1801	64086	JABGMZ000000000
HC 296	*C. fioriniae*	Apple	49.8 Mb	2378	50913	JABGMX000000000
KY 116	*C. fioriniae*	Blueberry	50.3 Mb	2044	53604	JABGMN000000000
KY 119	*C. fioriniae*	Blueberry	49.9 Mb	2288	49414	JABGMM000000000
KY 323	*C. fioriniae*	Apple	49.7 Mb	1294	84077	JABGMO000000000
KY 6	*C. fioriniae*	Apple	49.8 Mb	2266	47212	JABGMQ000000000
KY 615	*C. fioriniae*	Strawberry	50 Mb	3500	48042	JABGMD000000000
KY 640	*C. fioriniae*	Blueberry	49.7 Mb	2735	48577	JABGML000000000
KY 646	*C. fioriniae*	Blueberry	50.1 Mb	2198	53012	JABGMK000000000
KY 648	*C. fioriniae*	Blueberry	50.5 Mb	3134	84455	JABGMJ000000000
HC 540	*C. fructicola*	Apple	57.1 Mb	2411	107635	JABGMW000000000
KY 332	*C. gloeosporioides* s.l.	Strawberry	58 Mb	2140	77145	JABGMH000000000
HC 278	*C. kahawae* clade	Apple	53.2 Mb	11879	8471	JABSTW000000000
HC 292	*C. kahawae* clade	Apple	54.6 Mb	5057	29983	JABGMY000000000
HC 646	*C. nymphaeae*	Apple	51.3 Mb	3384	67103	JABGMV000000000
KY 563	*C. nymphaeae*	Strawberry	50.4 Mb	1727	131269	JABGMG000000000
KY 567	*C. nymphaeae*	Strawberry	51.6 Mb	3076	127617	JABGMF000000000
KY 613	*C. nymphaeae*	Strawberry	50.6 Mb	873	138789	JABGME000000000
KY 745	*C. nymphaeae*	Strawberry	50.5 Mb	1431	84829	JABGMB000000000
KY 146	*C. siamense*	Apple	58.1 Mb	2037	59576	JABGMU000000000
KY 254	*C. siamense*	Apple	58.5 Mb	1907	67367	JABGMS000000000
KY 540	*C. siamense*	Apple	58.4 Mb	2319	59953	JABGMR000000000
KY 687	*C. siamense*	Strawberry	57.4 Mb	697	207360	JABGMC000000000
KY 748	*C. siamense*	Strawberry	57.1 Mb	1276	114013	JABGMA000000000
KY 777	*C. siamense*	Peach	57.7 Mb	975	179466	JABGMI000000000
KY 8	*C. siamense*	Apple	57.6 Mb	1594	77820	JABGMP000000000
CF75	*C. theobromicola*	Strawberry	55.9 Mb	1275	123512	JABGLZ000000000
KY 152	*C. theobromicola*	Apple	56.5 Mb	5122	23897	JABGMT000000000

Supplementary Table 2.1 Kentucky small fruit *Colletotrichum* isolates used for complex-specific PCR analysis.

Isolate	Host	Morphotype	Species Complex
KY 650	Apricot	1	*C. acutatum*
KY 118	Blueberry	1	*C. acutatum*
KY 406	Blueberry	1	*C. acutatum*
KY 644	Blueberry	1	*C. acutatum*
KY 655	Blueberry	1	*C. acutatum*
KY 672	Blueberry	1	*C. acutatum*
KY 673	Blueberry	1	*C. acutatum*
KY 771	Blueberry	1	*C. acutatum*
KY 782	Blueberry	1	*C. acutatum*
KY 116	Blueberry	5	*C. acutatum*
KY 657	Blueberry	5	*C. acutatum*
KY 761	Blueberry	5	*C. acutatum*
KY 640	Blueberry	6	*C. acutatum*
KY 647	Blueberry	6	*C. acutatum*
KY 648	Blueberry	6	*C. acutatum*
KY 649	Blueberry	6	*C. acutatum*
KY 765	Blueberry	6	*C. acutatum*
KY 329	Peach	1	*C. acutatum*
KY 662	Peach	1	*C. acutatum*
KY 777	Peach	3	*C. gloeosporioides*
KY 585	Strawberry	1	*C. acutatum*
KY 619	Strawberry	1	*C. acutatum*
KY 409	Strawberry	2	*C. acutatum*
KY 508	Strawberry	2	*C. acutatum*
KY 509	Strawberry	2	*C. acutatum*
KY 516	Strawberry	2	*C. acutatum*
KY 522	Strawberry	2	*C. acutatum*
KY 563	Strawberry	2	*C. acutatum*
KY 565	Strawberry	2	*C. acutatum*
KY 567	Strawberry	2	*C. acutatum*
KY 569	Strawberry	2	*C. acutatum*
KY 572	Strawberry	2	*C. acutatum*
KY 573	Strawberry	2	*C. acutatum*
KY 574	Strawberry	2	*C. acutatum*

Supplementary Table 2.1 (continued).

Isolate	Host	Morphotype	Species Complex
KY 576	Strawberry	2	*C. acutatum*
KY 621	Strawberry	2	*C. acutatum*
KY 624	Strawberry	2	*C. acutatum*
KY 628	Strawberry	2	*C. acutatum*
KY 631	Strawberry	2	*C. acutatum*
KY 635	Strawberry	2	*C. acutatum*
KY 735	Strawberry	2	*C. acutatum*
KY 738	Strawberry	2	*C. acutatum*
KY 740	Strawberry	2	*C. acutatum*
KY 746	Strawberry	2	*C. acutatum*
KY 753	Strawberry	2	*C. acutatum*
KY 756	Strawberry	2	*C. acutatum*
KY 775	Strawberry	2	*C. acutatum*
KY 687	Strawberry	3	*C. gloeosporioides*
KY 747	Strawberry	3	*C. gloeosporioides*
KY 748	Strawberry	3	*C. gloeosporioides*
KY 332	Strawberry	4	*C. gloeosporioides*
KY 613	Strawberry	7	*C. acutatum*
KY 618	Strawberry	7	*C. acutatum*
KY 745	Strawberry	7	*C. acutatum*

Supplementary Table 2.2 Most aggressive *Colletotrichum* species complex on apple, strawberry, and blueberry for inoculation trials 1 and 2.

Trial	Fruit	Species complex	*P*-value
1	Apple	*C. gloeosporioides*	< 0.0001*
	Strawberry	N/A	0.6355
	Blueberry	*C. acutatum*	0.0036*
2	Apple	*C. gloeosporioides*	< 0.0001*
	Strawberry	*C. acutatum*	< 0.0001*

*indicates significant difference ($P < 0.05$).

Supplementary Table 2.3 Comparison of *Colletotrichum* isolate aggressiveness as grouped by species for Trial 1 apple, strawberry, and blueberry fruit inoculations.

Host		*C. fioriniae*	*C. nymphaeae*	*C. siamense*	*C. fructicola*	*C. kahawae* clade	Control
Apple	***C. fioriniae***	-	0.8631	< 0.0001*	< 0.0001*	< 0.0001*	-
	C. nymphaeae	0.8631	-	< 0.0001*	< 0.0001*	< 0.0001*	-
	C. siamense	< 0.0001*	< 0.0001*	-	0.0353*	0.0955	-
	C. fructicola	< 0.0001*	< 0.0001*	0.0353*	-	0.9931	-
	***C. kahawae* clade**	< 0.0001*	< 0.0001*	0.0955	0.9931	-	-
Strawberry	***C. fioriniae***	-	0.1559	0.004*	0.8721	0.0002*	-
	C. nymphaeae	0.1559	-	0.6992	0.0875	< 0.0001*	-
	C. siamense	0.004*	0.6992	-	0.0053*	< 0.0001*	-
	C. fructicola	0.8721	0.0875	0.0053*	-	0.0154*	-
	***C. kahawae* clade**	0.0002*	< 0.0001*	< 0.0001*	0.0154*	-	-
Blueberry	***C. fioriniae***	-	0.4826	0.0369*	0.3147	0.06	0.0005*
	C. nymphaeae	0.4826	-	0.5641	0.9659	0.5554	0.0131*
	C. siamense	0.0369*	0.5641	-	0.9881	0.9999	0.2846
	C. fructicola	0.3147	0.9659	0.9881	-	0.9715	0.1563
	***C. kahawae* clade**	0.06	0.5554	0.9999	0.9715	-	0.4774
	Control	0.0005*	0.0131*	0.2846	0.1563	0.4774	-

*indicates significant difference ($P < 0.05$).

Supplementary Table 2.4 Comparison of *Colletotrichum* isolate aggressiveness as grouped by original host for Trial 1 inoculation assays.

Host		Apple	Strawberry	Blueberry	Peach	Apricot
Apple	Apple	-	0.9846	< 0.0001*	0.013*	0.0445*
	Strawberry	0.9846	-	< 0.0001*	0.0019*	0.0612
	Blueberry	< 0.0001*	< 0.0001*	-	< 0.0001*	0.9993
	Peach	0.013*	0.0019*	< 0.0001*	-	< 0.0001*
	Apricot	0.0445*	0.0612	0.9993	< 0.001*	-
Strawberry	Apple	-	0.0002*	0.0357*	< 0.0001*	1
	Strawberry	0.0002*	-	0.4896	0.181	0.4618
	Blueberry	0.0357*	0.4896	-	0.0236*	0.7828
	Peach	< 0.0001*	0.181	0.0236*	-	0.0808
	Apricot	1	0.4618	0.7828	0.0808	-
Blueberry	Apple	-	0.9911	0.1416	1	0.6256
	Strawberry	0.9911	-	0.1594	0.9999	0.7611
	Blueberry	0.1416	0.1594	-	0.6833	1
	Peach	1	0.9999	0.6833	-	0.8460
	Apricot	0.6256	0.7611	1	0.8460	-

*indicates significant difference ($P < 0.05$).

Supplementary Table 2.5 Comparison of *Colletotrichum* isolate aggressiveness as grouped by species for Trial 2 inoculation assays.

Host		*C. fioriniae*	*C. nymphaeae*	*C. siamense*	*C. fructicola*	*C. theobromicola*	*C. kahawae* clade
Apple	C. fioriniae	-	0.4879	< 0.0001*	0.0092*	< 0.0001*	0.7213
	C. nymphaeae	0.4879	-	< 0.0001*	0.0005*	0.0008*	0.1042
	C. siamense	< 0.0001*	< 0.0001*	-	0.1531	< 0.0001*	< 0.0001*
	C. fructicola	0.0092*	0.0005*	0.1531	-	< 0.0001*	0.2829
	C. theobromicola	< 0.0001*	0.0008*	< 0.0001*	< 0.0001*	-	< 0.0001*
	C. kahawae *clade*	0.7213	0.1042	< 0.0001*	0.2829	< 0.0001*	-
Strawberry	C. fioriniae	-	0.9771	0.9998	0.5829	< 0.0001*	< 0.0001*
	C. nymphaeae	0.9771	-	0.952	0.3848	< 0.0001*	< 0.0001*
	C. siamense	0.9998	0.952	-	0.7199	< 0.0001*	< 0.0001*
	C. fructicola	0.5829	0.3848	0.7199	-	< 0.0001*	< 0.0001*
	C. theobromicola	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	-	0.9907
	C. kahawae *clade*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.9907	-

*indicates significant difference ($P < 0.05$).

Supplementary Table 2.6 Comparison of *Colletotrichum* isolate aggressiveness as grouped by original host for Trial 2 inoculation assays.

Host		Apple	Strawberry	Blueberry
Apple	**Apple**	-	0.0043*	0.0085*
	Strawberry	0.0043*	-	0.9570
	Blueberry	0.0085*	0.9570	-
Strawberry	**Apple**	-	0.0251*	0.3505
	Strawberry	0.0251*	-	0.7123
	Blueberry	0.3505	0.7123	-

*indicates significant difference ($P < 0.05$).

Supplementary Table 2.7 Pairwise comparisons of single nucleotide polymorphisms (SNPs) for Kentucky and reference *Colletotrichum* genome assemblies.

	KY640	MH18	HC292	KY119	KC05	GC23	KY615	KY540	KY563	KY152	KY745	HC296	KY646	KY6	SA-01	SMCG1#C	KY648	HC25	KY8	KY748
KY640		1290	161000	2843	87721	163250	1503	157193	87073	157612	87082	2716	8087	2643	87150	162829	8087	2831	156597	158764
MH18			162963	2726	88096	162965	2132	162633	87049	162975	87344	2712	8095	2489	87144	163608	8073	2696	163076	162976
HC292				163072	162802	80976	160393	80776	162621	97318	162685	161558	160860	162859	162763	81525	162534	162954	80708	80611
KY119					87656	163355	2197	162321	87050	162122	86960	1761	6999	1995	87129	163109	6984	2049	162883	162672
KC05						162236	87681	161725	31589	161953	31112	87664	87608	87753	31616	162021	87577	87702	161483	161836
GC23							162771	42254	162058	98820	162286	163350	163207	163452	162126	61671	163076	163287	42290	42352
KY615								155170	86991	156166	87016	2641	7482	2702	87070	163257	7491	2836	153195	155963
KY540									161648	98053	161146	159789	159110	162464	161735	62415	162295	162186	14576	15109
KY563										162160	21176	86961	87000	86991	18	162453	86974	86974	161664	161591
KY152											161510	159955	158498	162555	162262	99241	161844	162102	97952	97839
KY745												86923	86953	86943	21193	161779	86939	86961	161117	161354
HC296													7774	2029	87048	162950	7782	1489	160191	161634
KY646														7850	87064	162795	10	8013	159422	160660
KY6															87068	163696	7839	2157	162967	162960
SA-01																162441	87046	87067	161776	161669
SMCG1#C																	162726	162917	62432	61830
KY648																		8021	162725	162497
HC25																			162732	162470
KY8																				14817
KY748																				

Supplementary Table 2.7 (continued).

	KY116	KY323	KY567	1104-7	Naragc5	KY146	KY332	KY687	KY777	CBS122122	ICMP18578	CF75	HC278	IMI309357	HC540	KY613	KY254	HC646	CBS607.94
KY640	2823	2478	87101	161041	158762	156391	161635	157078	159538	87537	160654	161792	159623	126542	159486	87110	156735	87026	104957
MH18	2590	2555	87079	162913	163031	162724	163316	162831	163080	87851	162850	163636	161854	126658	162972	87359	162855	87257	105122
HC292	163136	162818	162659	80831	81198	80623	80985	80663	80663	163030	80772	97929	595	164158	80810	162784	80734	161361	162945
KY119	2354	2901	87074	162667	162252	162428	163421	162497	162871	87507	163086	162843	161980	126607	162508	86996	162623	87090	105032
KC05	87598	87745	31610	161768	162097	161448	162326	161680	161900	49423	162319	162452	161870	126952	161855	31131	161603	31524	106556
GC23	163328	163277	162077	32823	33065	42482	1095	42295	42172	162631	42718	99022	79184	164090	32837	162191	42191	161965	163235
KY615	2389	2894	87004	161232	157940	153025	160861	154676	157131	87444	159914	161756	158976	126445	159427	87039	153292	86936	104960
KY540	162118	162157	161647	39016	39232	14540	42225	14831	11742	161610	31008	98408	78903	162702	38811	161074	14984	159343	163099
KY563	86971	87080	5	162101	162104	161520	162147	161451	161907	48926	162463	162893	161752	126948	162290	21208	161916	4669	106283
KY152	162129	161968	162166	98475	98770	97793	98670	97726	98038	161668	98402	14499	95601	163661	98330	161509	98293	160500	161663
KY745	86911	87026	21263	161575	161395	161259	162382	161243	161711	48879	162442	162200	161928	126819	161418	9	161561	21080	106460
HC296	2492	2566	86996	161491	160292	159794	162165	160121	161949	87499	161081	162506	160411	126556	160264	86931	159873	86954	105051
KY646	7598	8226	87026	161341	159879	158696	161730	159277	161351	87424	160903	162217	159479	126439	159920	86976	158610	86971	105010
KY6	2575	1988	87021	162987	162641	162612	163238	162579	163174	87523	162977	163467	161794	126568	162732	86979	162637	87052	104986
SA-01	87033	87155	16	162127	162074	161584	162290	161640	161987	48966	162496	162923	161859	127066	162370	21227	162014	4684	106286
SMCG1#C	162885	163058	162420	61820	62059	62495	61638	61943	62269	161014	62297	99292	79676	164417	61680	161754	62483	162389	163667
KY648	7593	8237	86999	162345	162079	162438	163203	162543	162637	87358	162883	162588	161306	126480	162306	86952	162296	87060	105003
HC25	2801	2240	87015	162365	162017	162429	163427	162339	162588	87463	162940	162821	161823	126611	162385	86984	162411	87064	105018
KY8	162786	162660	161686	38974	39143	12004	42324	14062	14234	161503	30754	98400	78832	163006	38965	161110	12249	159217	163500
KY748	162613	162602	161577	38864	39131	15343	42288	4771	7360	161591	30404	98282	78792	163296	38817	161369	14644	161081	163170
KY116		2842	86994	162467	162167	162248	163349	162465	162705	87397	162917	162978	162038	126544	162404	86905	162482	87044	105000
KY323			87117	162434	162016	162295	163250	162302	162792	87482	163025	162979	161740	126508	162149	87061	162330	87160	105024
KY567				162103	162076	161566	162182	161511	161914	48980	162493	162853	161789	127145	162276	21283	161947	4692	106300
1104-7					1960	39216	32731	38862	38902	161686	38996	98901	79094	163780	1946	161505	38900	161242	163522
Naragc5						39338	33012	39129	39155	162022	39059	99220	79395	164269	1713	161355	39166	160826	162806
KY146							42497	13873	15332	161166	30938	98390	78719	162614	39001	161184	13366	159119	163200
KY332								42172	42114	162882	42694	98789	79243	164003	32711	162258	42266	160963	163258
KY687									9129	161379	30428	98276	78776	162918	38833	161173	14198	159448	163372
KY777										161871	30654	98374	78836	163257	38742	161611	13670	161445	163622
CBS122122											162183	162053	161997	127091	161830	48895	161889	49037	106530
ICMP18578												98564	78979	163875	38725	162474	30942	160771	163801
CF75													95898	164639	98672	162161	98551	162540	161491
HC278														163122	79013	161998	78869	160505	162088
IMI309357															163677	126839	162709	126943	123684
HC540																161424	38780	160091	163301
KY613																	161559	21105	106479
KY254																		159571	163313
HC646																			106322
CBS607.94																			

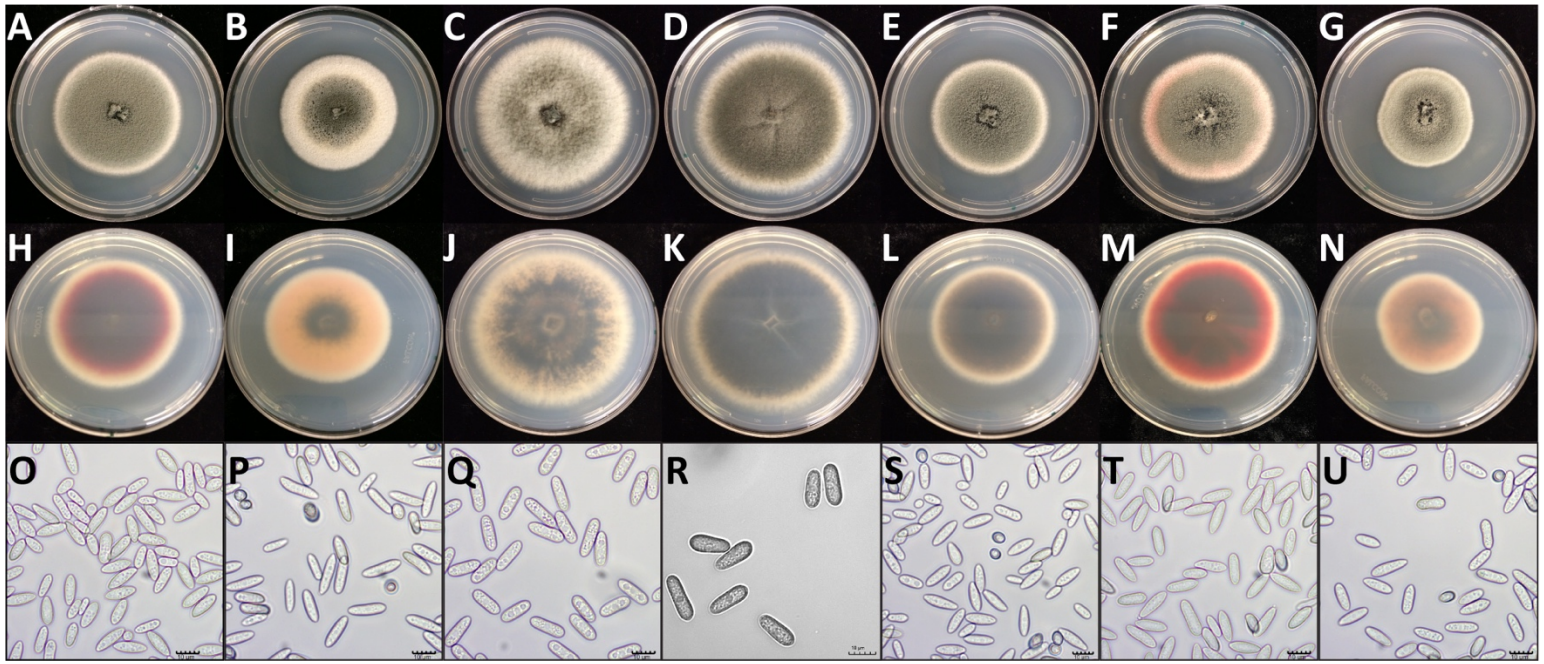


Figure 2.1 Colony and conidium morphology of Kentucky small fruit *Colletotrichum* isolates. Isolates were grown on potato dextrose agar for colony morphology and V8 juice agar for conidium morphology. (A-G) Top of plates; (H-N) underside of plates; (O-U) conidia. (A, H, O) Represent morphotype 1 (M1); species *C. fioriniae*. (B, I, P) Represent M2; species *C. nymphaeae*. (C, J, Q) Represent M3; species *C. siamense*. (D, K, R) Represent M4; species *C. fructicola.* (E, L, S) Represent M5; species *C. fioriniae*. (F, M, T) Represent M6; species *C. fioriniae*. (G, N, U) Represent M7; species *C. nymphaeae.* Scale bar for conidia is 10 µm.

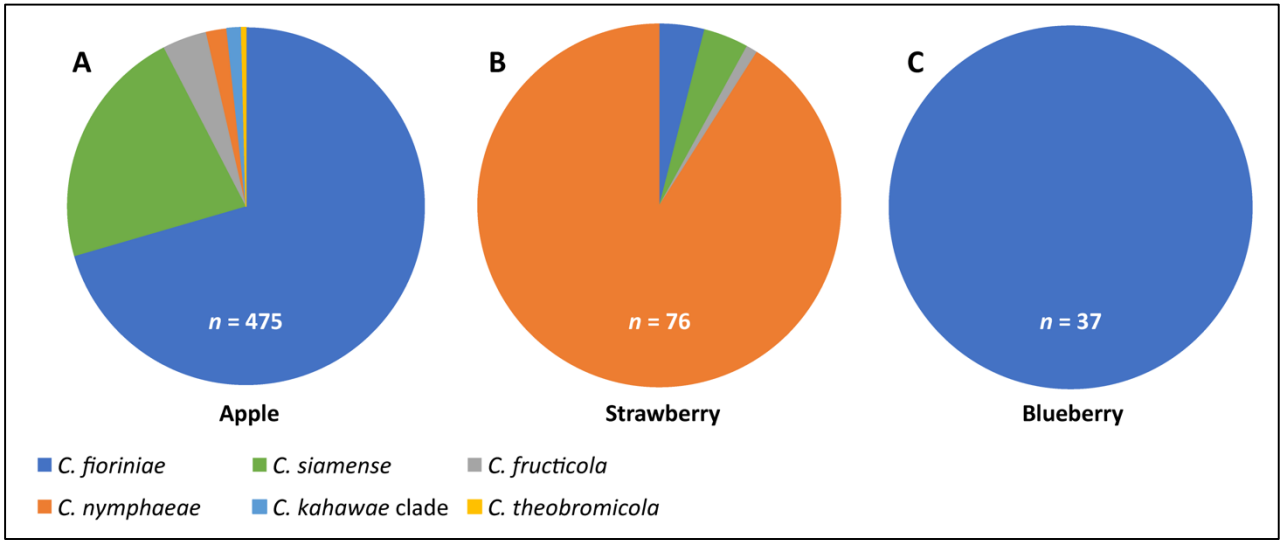


Figure 2.2 Comparison of (A) apple, (B) strawberry, and (C) blueberry field distributions of *Colletotrichum* species in Kentucky orchards. The apple data (A) were adapted from Munir et al. (2016).

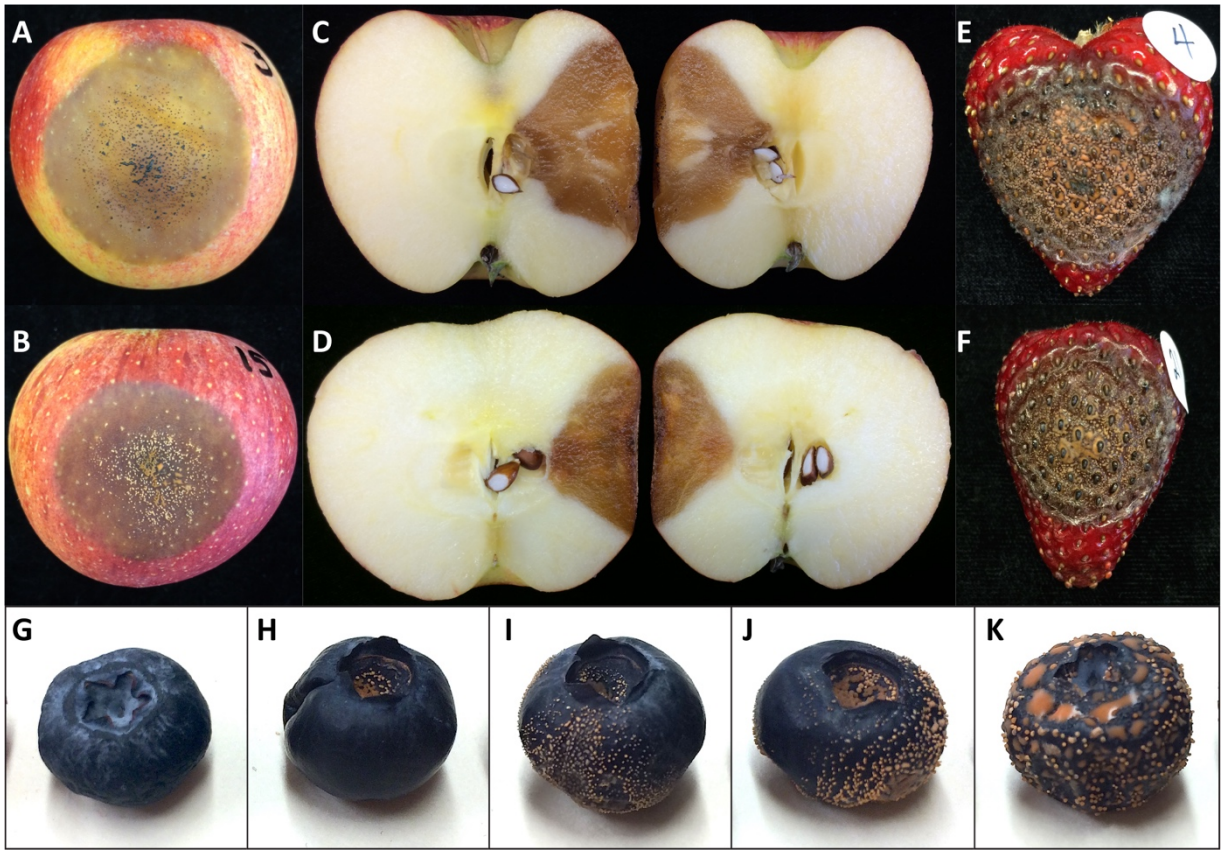


Figure 2.3 *Colletotrichum* fruit inoculation symptoms on apple (A-D), strawberry (E and F), and blueberry (G-K). Fruits were inoculated with *C. siamense* (A, C, E; *C. gloeosporioides* complex); *C. fioriniae* (B and D; *C. acutatum* complex); and *C. nymphaeae* (F; *C. acutatum*). (G-K) Blueberry symptoms severity scale: G = 0%, H = 1-10%, I = 11-49%, J = 50-99%, K = 100% fungal spore coverage.

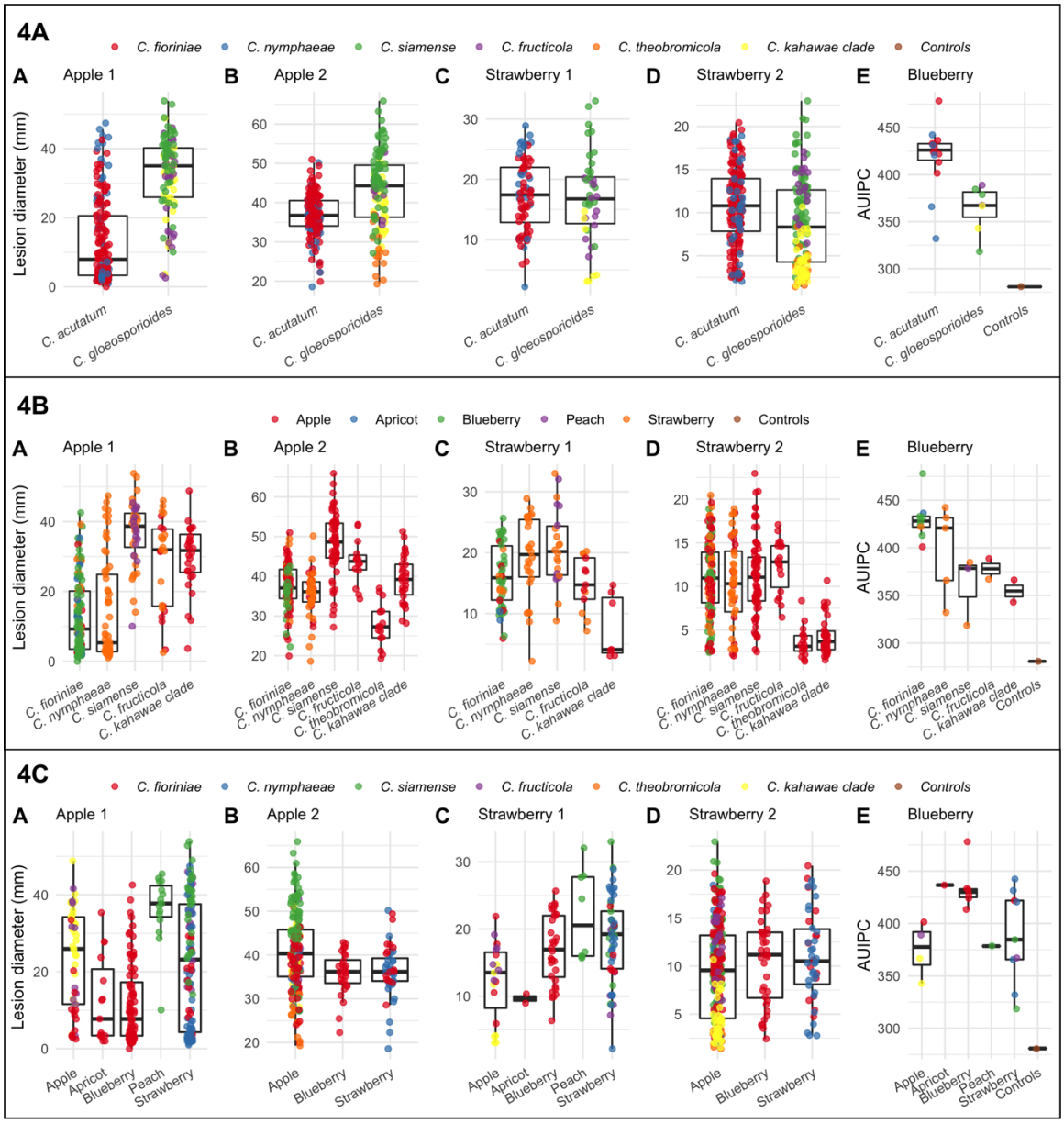


Figure 2.4 Anthracnose lesion diameter on apple (A and B) and strawberry (C and D), and AUDPC (disease incidence) on blueberry (E) for *Colletotrichum* isolates as grouped by species complex (4A), species (4B), and original fruit host (4C). Apple 1, strawberry 1, and blueberry trials were conducted with the same set of *Colletotrichum* isolates, and apple 2 and strawberry 2 were conducted with the same set of isolates (Table 2.2).

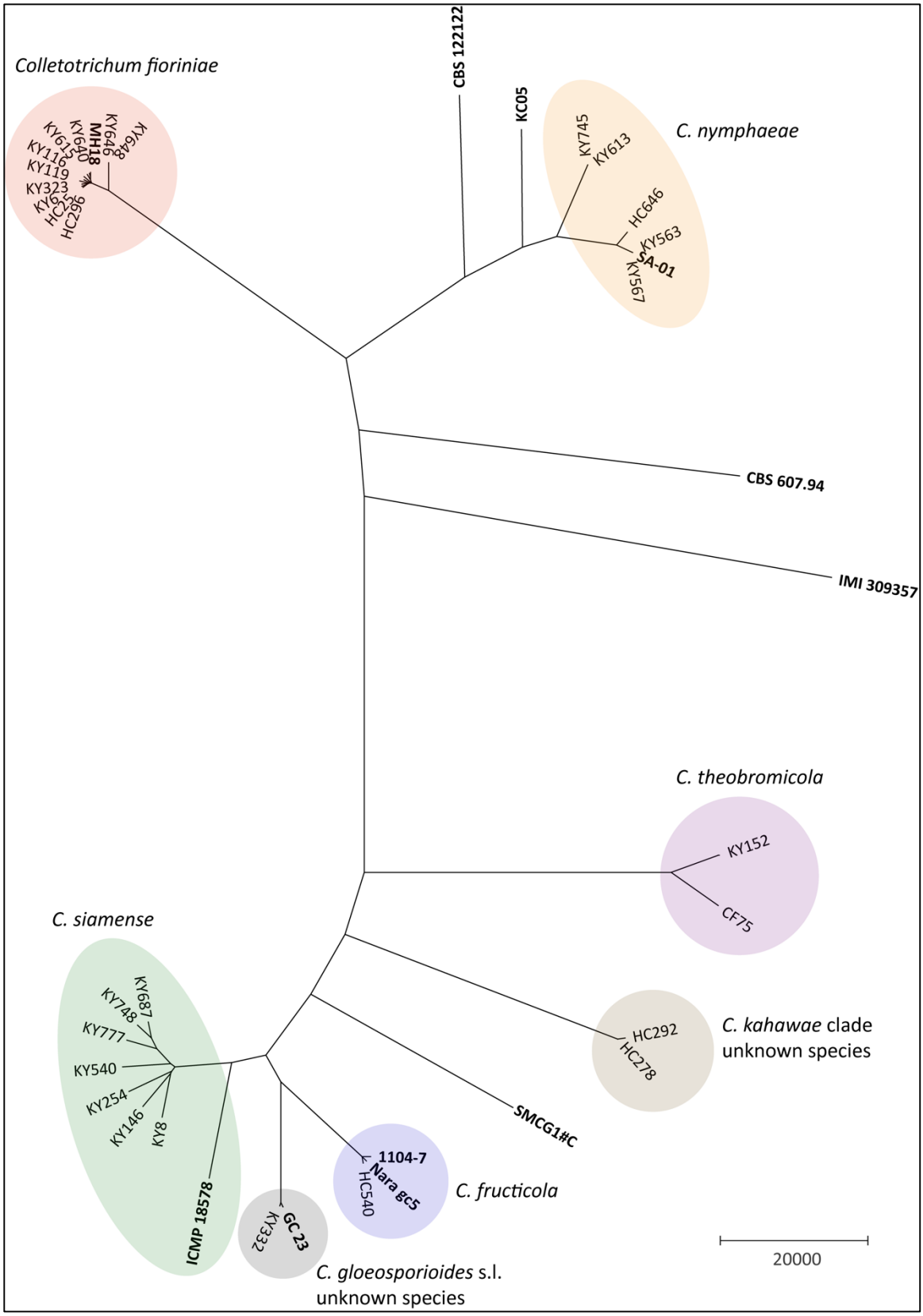

Colletotrichum fioriniae
CBS 122122
KC05
C. nymphaeae
KY745
KY613
HC646
KY563
SA-01
KY567
KY649
KY648
MH18
KY640
KY615
KY116
KY119
KY323
KY621
HC296
CBS 607.94
IMI 309357
C. theobromicola
KY152
CF75
C. siamense
KY687
KY748
KY777
KY540
KY254
KY146
KY8
ICMP 18578
HC292
HC278
C. kahawae clade
unknown species
SMCG1#C
1104-7
Nara gc5
HC540
C. fructicola
GC 23
KY232
C. gloeosporioides s.l.
unknown species
20000

Figure 2.5 Whole genome phylogenetic tree of Kentucky fruit and reference (in bold, Table 2.3) *Colletotrichum* isolates based on SNP comparisons (Suppl. Table 2.7). Calculation of bootstrap values is not possible because the tree is based on pairwise distance data.

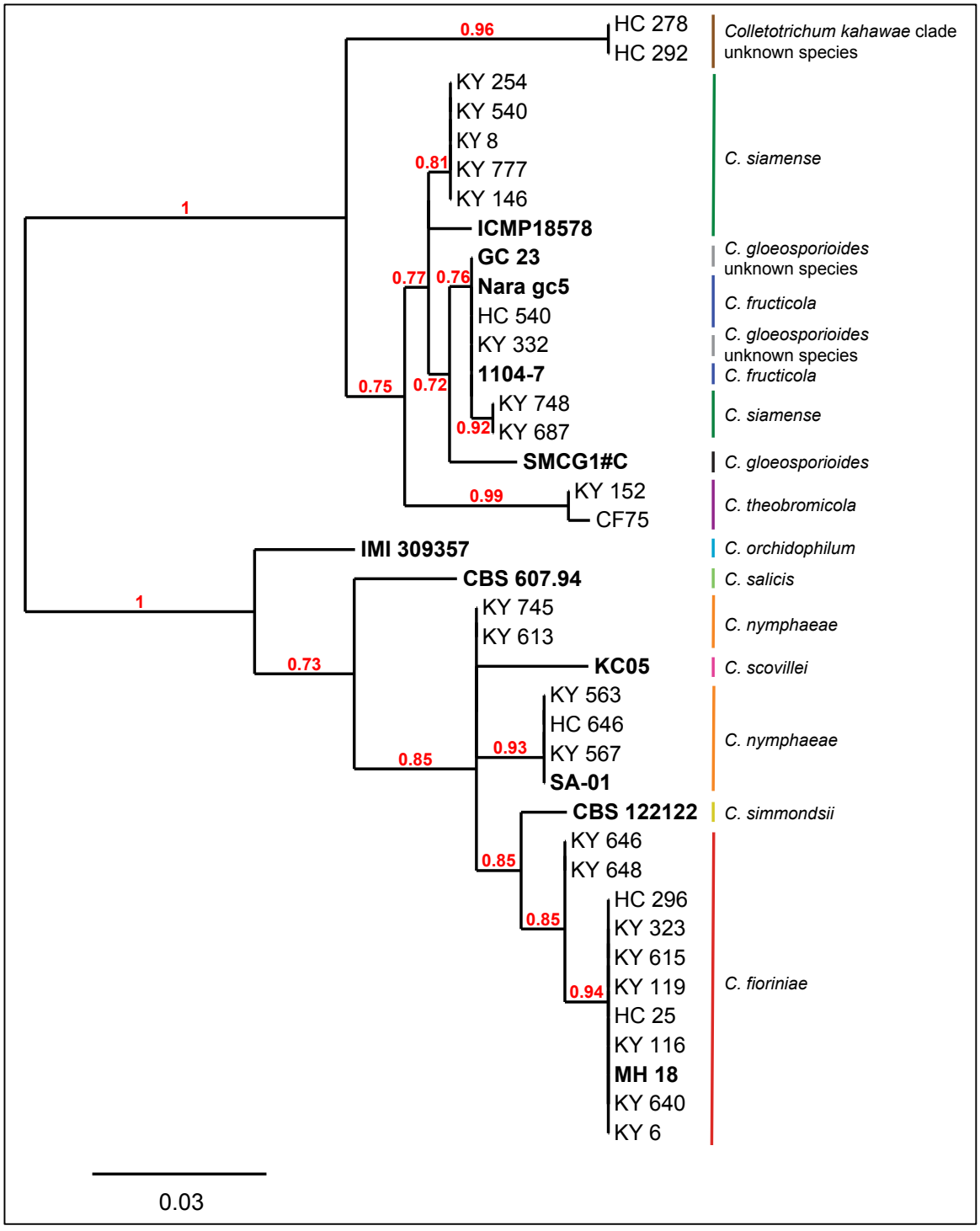


Figure 2.6 PhyML Approximate Likelihood-Ratio Test tree for the chitin synthase (*CHS*) gene alignment indicating phylogenetic affinities for Kentucky fruit and reference *Colletotrichum* isolates. The tree is midpoint rooted, and bootstrap values are shown in red. Reference isolates are shown in bold.

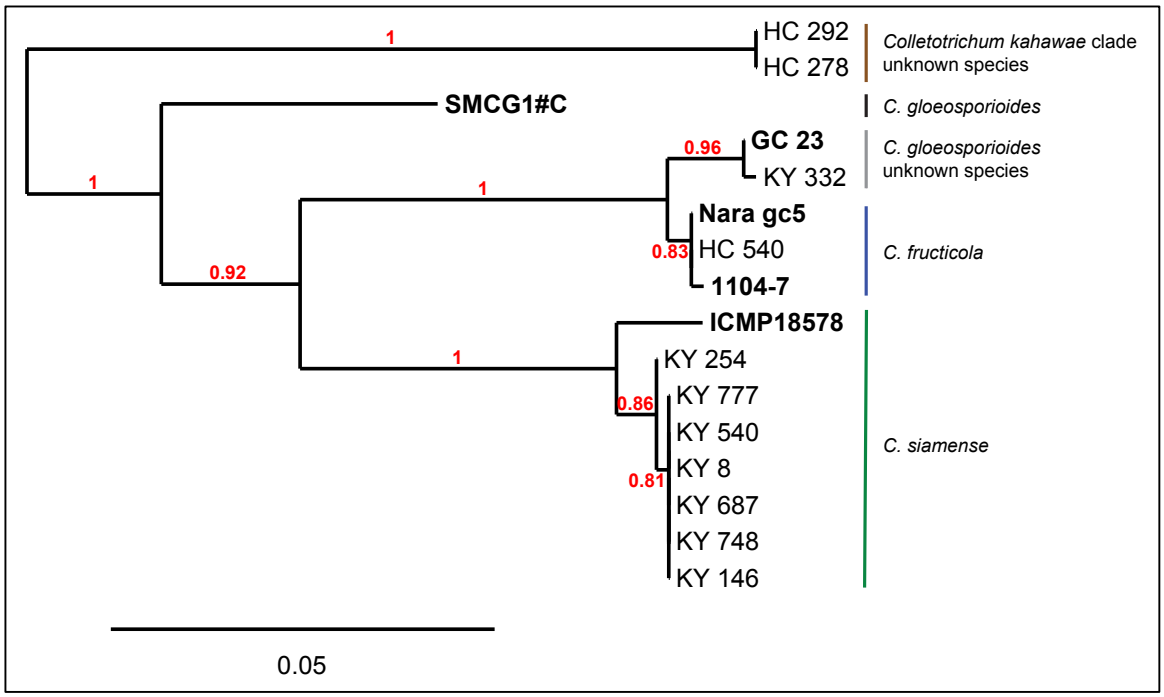
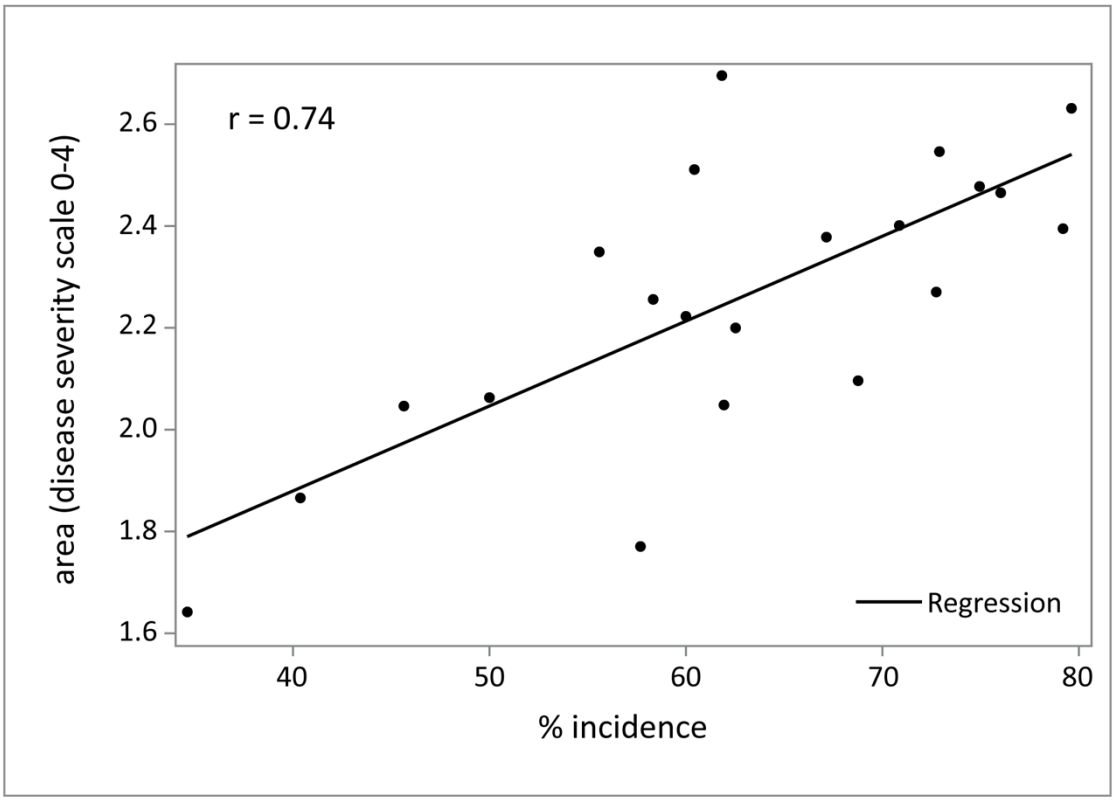
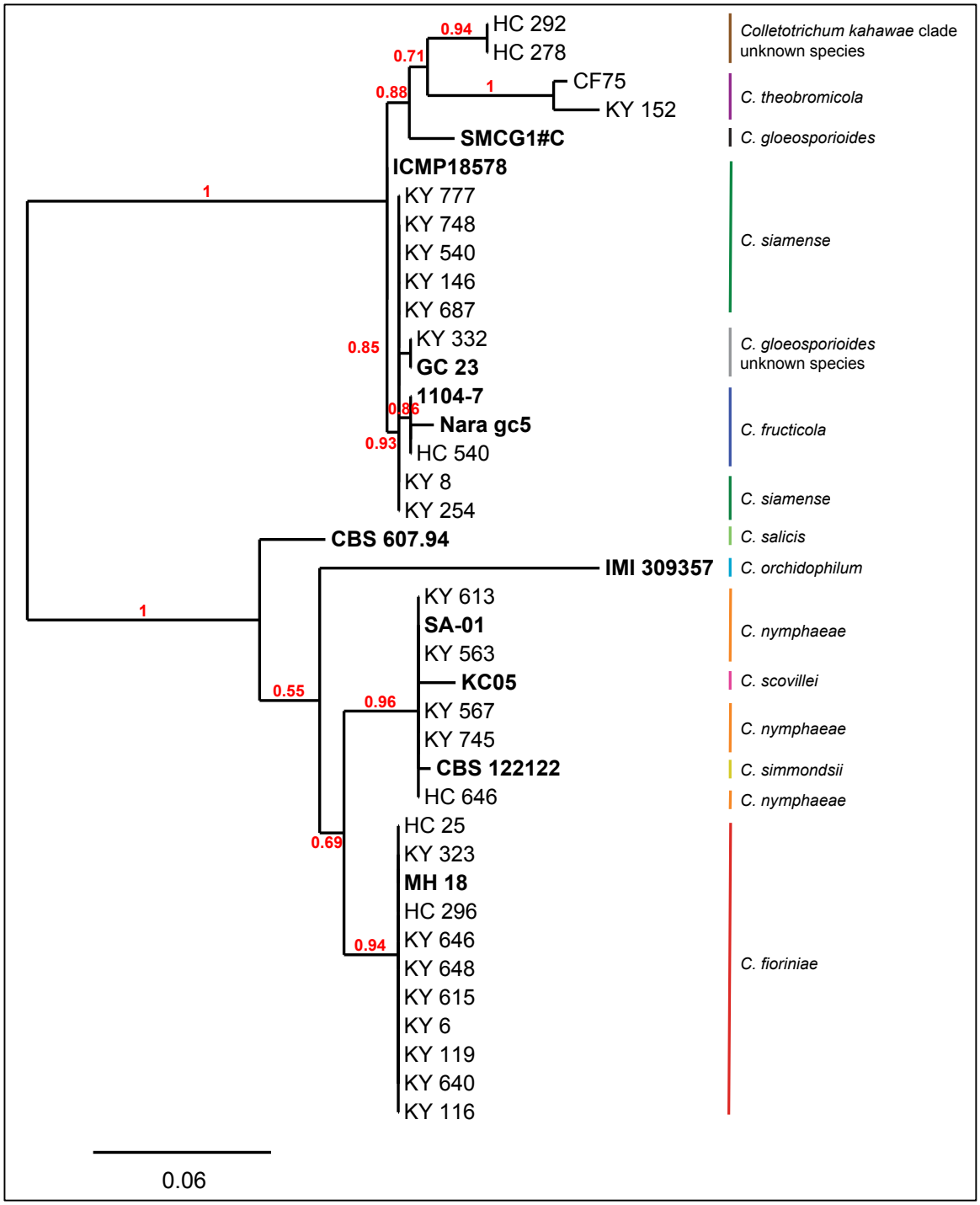


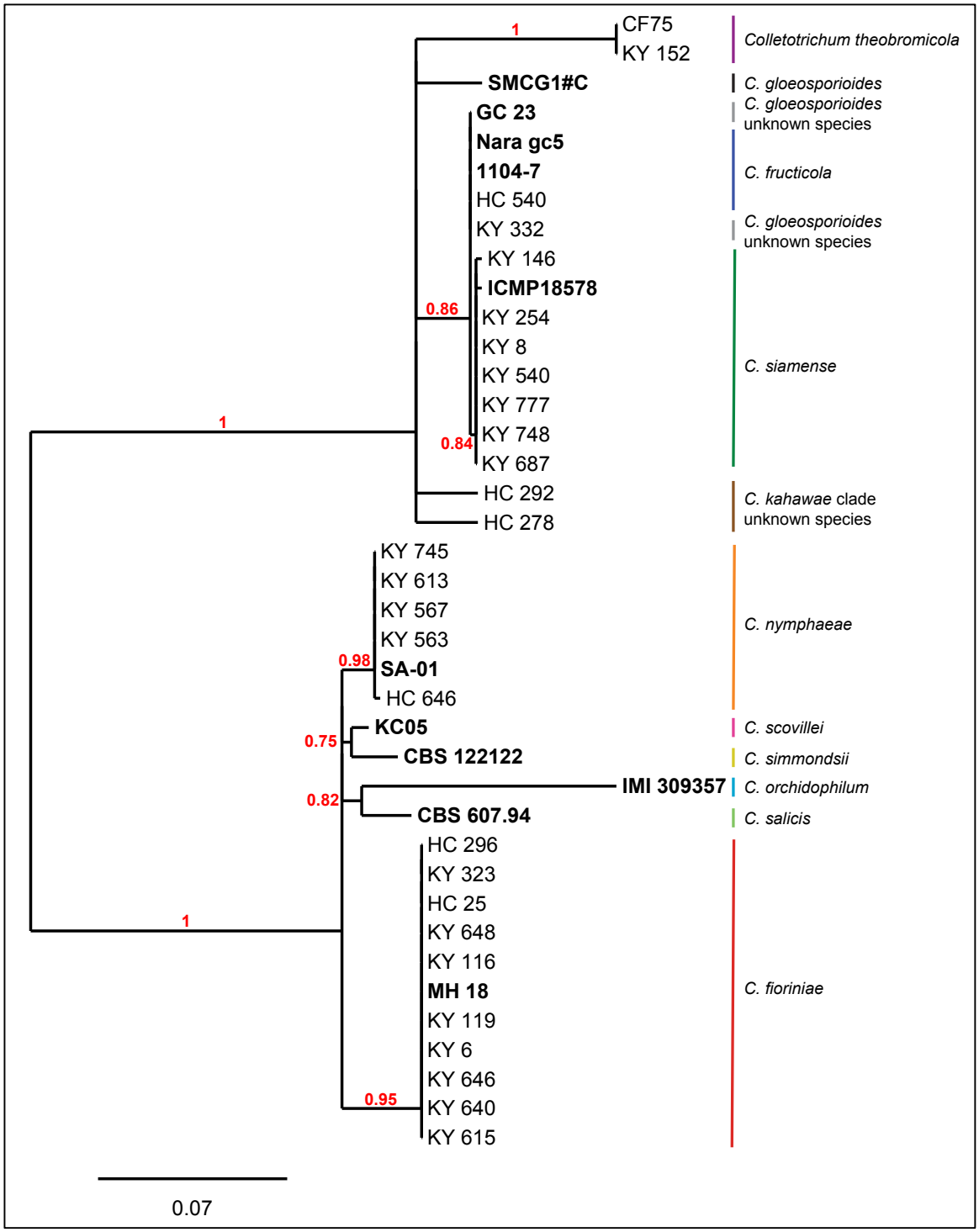
Figure 2.7 PhyML Approximate Likelihood-Ratio Test tree for the *Apn2* and MAT1-2-1 intergenic spacer region (*ApMat*) alignment indicating phylogenetic affinities for Kentucky fruit and reference *Colletotrichum gloeosporioides* complex isolates. The tree is midpoint rooted, and bootstrap values are shown in red. Reference isolates are shown in bold.



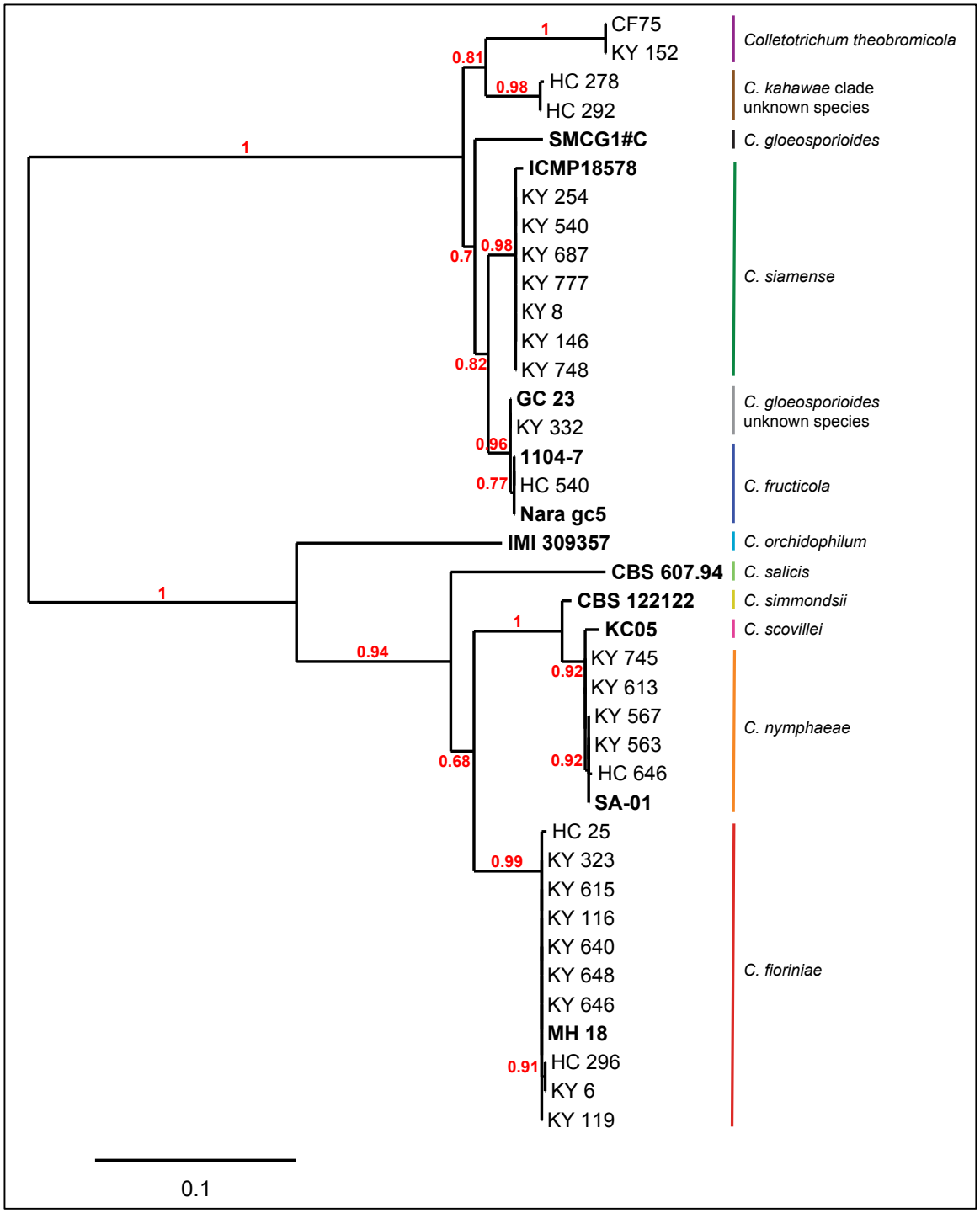
Supplementary Figure 2.1 Linear regression of average percent disease incidence (% incidence) versus average disease severity (area) for *Colletotrichum* blueberry fruit inoculation experiment 2. Pearson correlation coefficient r = 0.74.



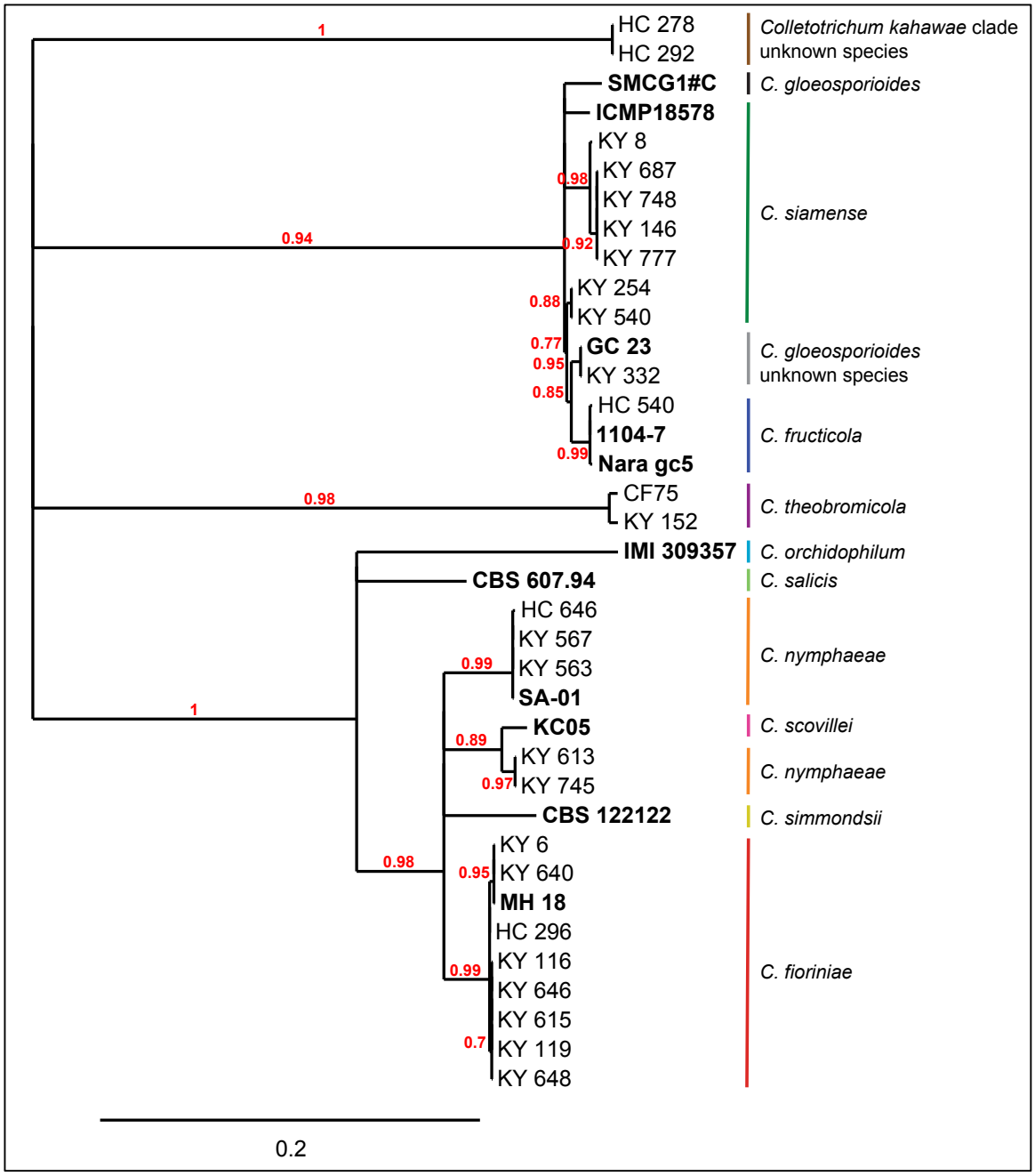
Supplementary Figure 2.2 PhyML Approximate Likelihood-Ratio Test tree for the actin (*ACT*) gene alignment indicating phylogenetic affinities for Kentucky fruit and reference *Colletotrichum* isolates. The tree is midpoint rooted, and bootstrap values are shown in red. Reference isolates are shown in bold.



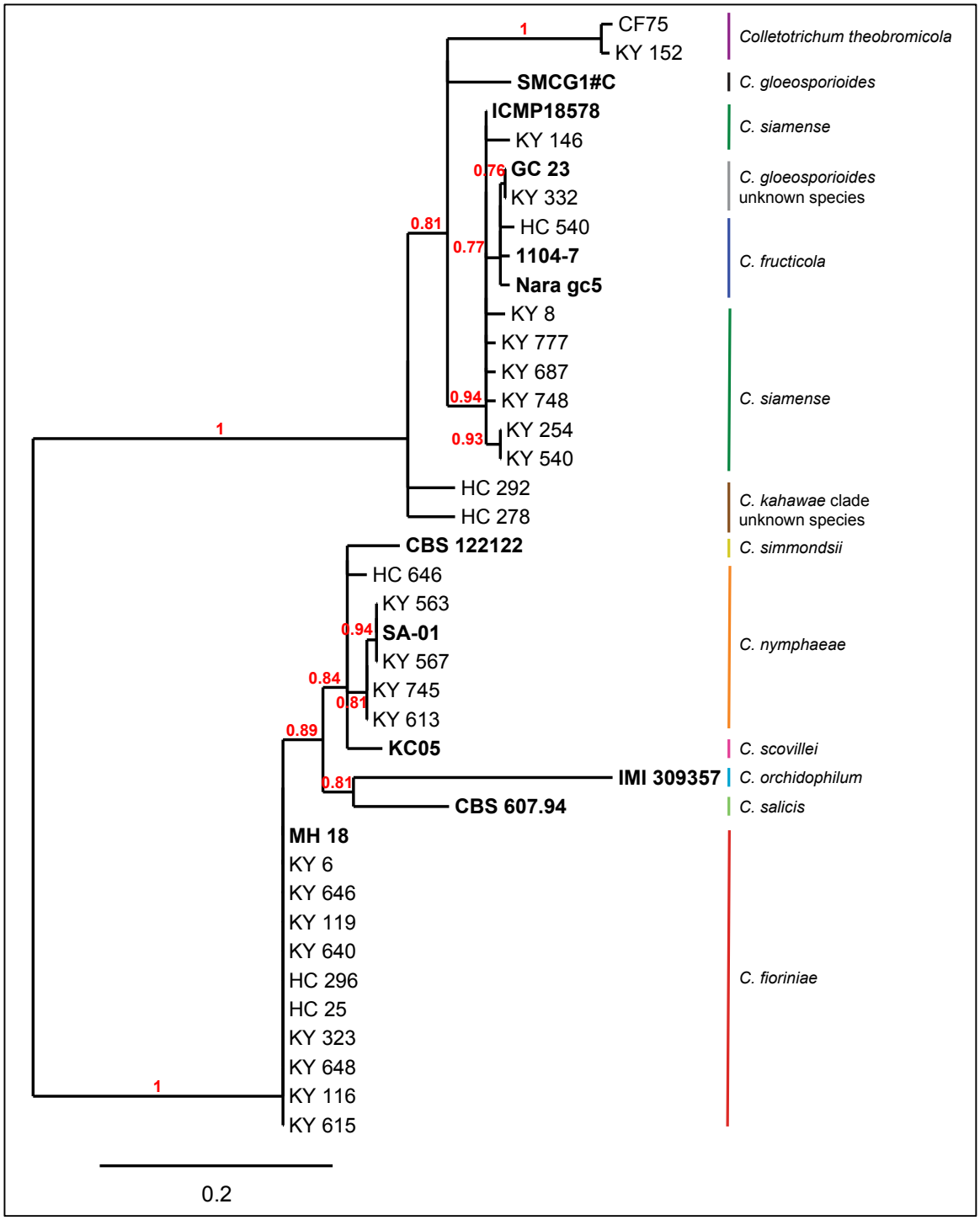
Supplementary Figure 2.3 PhyML Approximate Likelihood-Ratio Test tree for the beta-tubulin (*TUB2*) gene alignment indicating phylogenetic affinities for Kentucky fruit and reference *Colletotrichum* isolates. The tree is midpoint rooted, and bootstrap values are shown in red. Reference isolates are shown in bold.



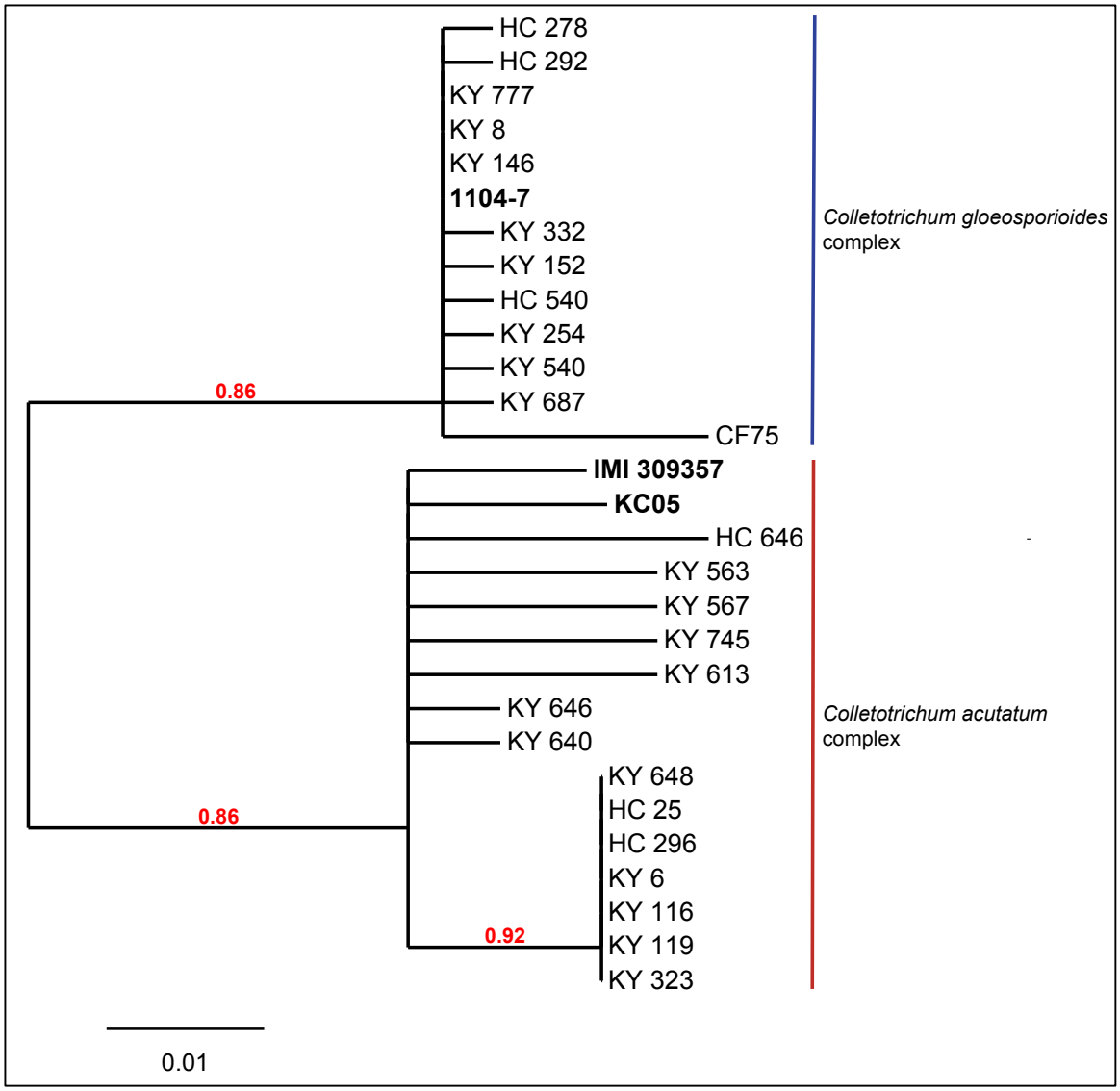
Supplementary Figure 2.4 PhyML Approximate Likelihood-Ratio Test tree for the calmodulin (*CAL*) gene alignment indicating phylogenetic affinities for Kentucky fruit and reference *Colletotrichum* isolates. The tree is midpoint rooted, and bootstrap values are shown in red. Reference isolates are shown in bold.



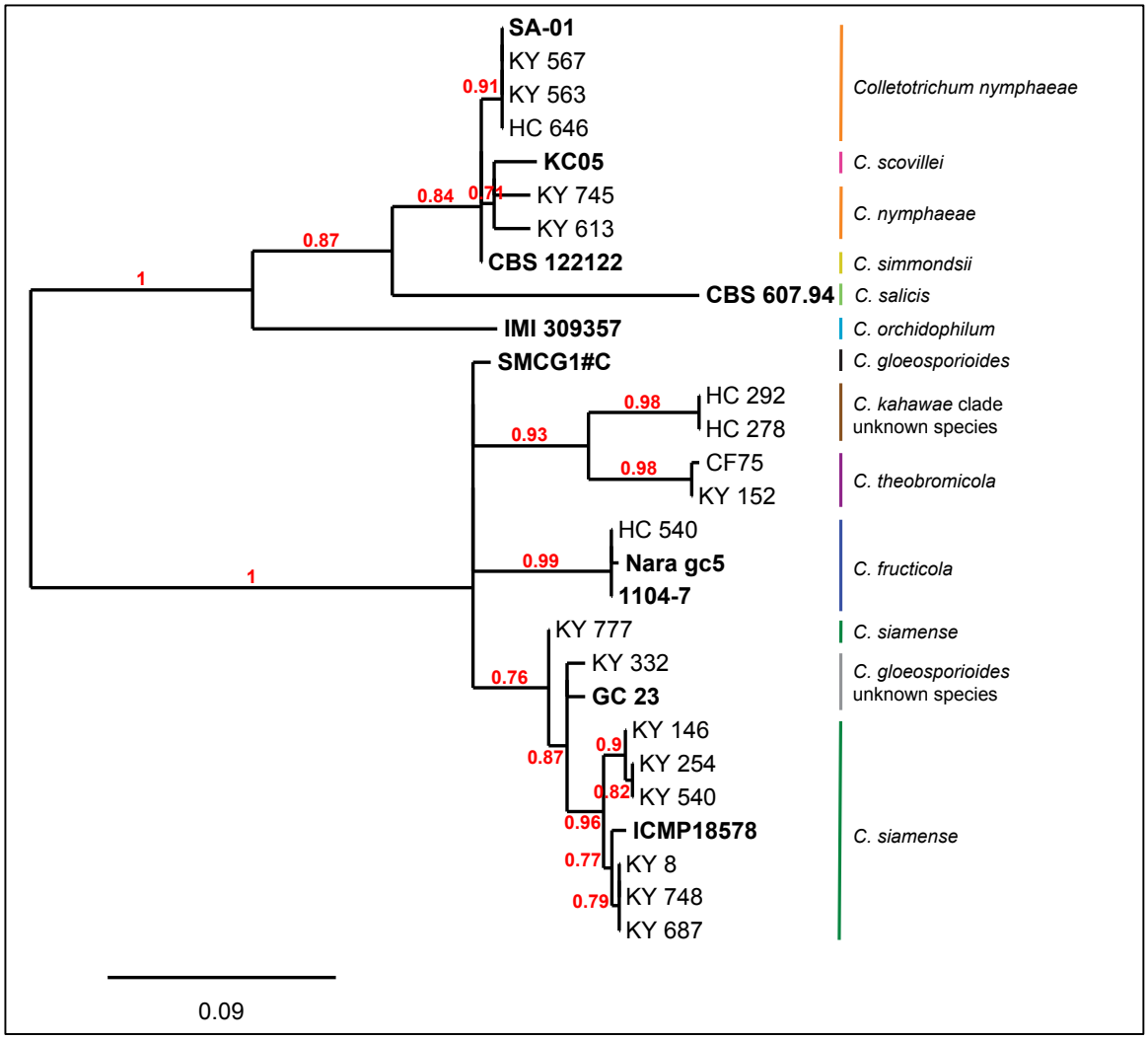
Supplementary Figure 2.5 PhyML Approximate Likelihood-Ratio Test tree for the glutamine synthetase (*GS*) gene alignment indicating phylogenetic affinities for Kentucky fruit and reference *Colletotrichum* isolates. The tree is midpoint rooted, and bootstrap values are shown in red. Reference isolates are shown in bold.



Supplementary Figure 2.6 PhyML Approximate Likelihood-Ratio Test tree for the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene alignment indicating phylogenetic affinities for Kentucky fruit and reference *Colletotrichum* isolates. The tree is midpoint rooted, and bootstrap values are shown in red. Reference isolates are shown in bold.



Supplementary Figure 2.7 PhyML Approximate Likelihood-Ratio Test tree for the internal transcribed spacer region (*ITS*) alignment indicating phylogenetic affinities for Kentucky fruit and reference *Colletotrichum* isolates. The tree is midpoint rooted, and bootstrap values are shown in red. Reference isolates are shown in bold. Not all isolates are represented because the BLASTn search of genome assemblies with a reference *ITS* sequence failed to return a viable sequence in some cases.



Supplementary Figure 2.8 PhyML Approximate Likelihood-Ratio Test tree for the manganese-superoxide dismutase (*SOD2*) gene alignment indicating phylogenetic affinities for Kentucky fruit and reference *Colletotrichum* isolates. The tree is midpoint rooted, and bootstrap values are shown in red. Reference isolates are shown in bold. No *C. fioriniae* isolates are included because the BLASTn search failed to return a homologous sequence.

CHAPTER 3. *COLLETOTRICHUM FIORINIAE* CLONE IDENTIFICATION IN MIXED-FRUIT ORCHARDS USING TELOMERE FINGERPRINTING

Introduction

Colletotrichum fungi are cosmopolitan plant pathogens that are well known for causing anthracnose fruit rot diseases, which result in significant yield losses worldwide (Børve and Stensvand 2015; da Silva et al. 2020; Dean et al. 2012; Gauthier et al. 2017; Hyde et al. 2009; Prusky et al. 2013). Three anthracnose fruit rots of particular concern in the southeastern U.S. are bitter rot of apple (*Malus domestica* Borkh), anthracnose fruit rot of strawberry (*Fragaria x ananassa* Duchesne), and ripe rot of blueberry (*Vaccinium* spp.). Bitter rot is the most important fruit rot disease of apple in the southeastern region and can cause average annual yield losses of 30% (Gauthier et al. 2017; Rosenberger 2016; Sutton et al. 2014). Anthracnose fruit rot of strawberry is also a disease of concern across the region, especially in Florida where a major portion of strawberry production is centered (Borisova et al. 2014; Howard et al. 1992; Smith 2008). Ripe rot of blueberry is an economically important disease that can cause devastating yield losses as a postharvest pathogen (Daykin and Milholland 1984; Milholland et al. 2017; Polashock et al. 2005).

In Kentucky, it is common for apple, blueberry, and strawberry to be grown within the same orchard and in close proximity, as most of the orchards in the state are small and cater to the agritourism industry. This situation raises the concern that cross-infection of *Colletotrichum* spp. may occur among different fruit crops within a mixed-fruit orchard. Evidence of cross-infection has been reported in many studies where *Colletotrichum* spp. were found to have broad host ranges and were pathogenic to many hosts, with variable levels of aggressiveness (Afanador-Kafuri et al. 2003; Bernstein et al. 1995a; Freeman and Shabi 1996; Giblin et al. 2010; Lakshmi et al. 2011; Phoulivong et al. 2012; Whitelaw-Weckert et al. 2007). In contrast, other studies have shown evidence of host preference of some isolates on some fruit crops and low risk for cross-infection (Alahakoon et al. 1994; Freeman et al. 2001; Harp et al. 2014; MacKenzie et al. 2009). Few of these cross-infection studies have adequately identified

the test isolates to phylogenetic species, and the majority only assessed *in vitro* cross-inoculation potential. Therefore, whether or not cross-infection occurs in the field at an impactful level is still largely unknown. From a management perspective, awareness of cross-infection incidence may greatly influence management decisions pertaining to spray schedules, orchard layout, and crop rotation.

Results reported in Chapter 2 suggest a potential for cross-infection in mixed-fruit orchards. *In vitro* cross-inoculations indicated that all *Colletotrichum* isolates, regardless of species or original host, were pathogenic on apple, blueberry, and strawberry fruits. A survey of the field distribution of *Colletotrichum* spp. also suggested a high level of species overlap between the apple, blueberry, and strawberry strains from mixed-fruit orchards in Kentucky. The most commonly identified species was *C. fioriniae*, which accounted for 70% of the isolates from apple (Munir et al. 2016), 100% from blueberry, and 4% from strawberry, making *C. fioriniae* the only species shared across all three fruits. This makes *C. fioriniae* a promising candidate species for more directly evaluating cross-infection in Kentucky. If strains of the same clonal lineage of *C. fioriniae* can be identified from two different fruits within a single orchard, then it can be inferred that cross-infection has occurred.

Putative clones can be identified by various fingerprinting methods including microsatellite analyses, amplified fragment length polymorphisms (AFLP), or restriction fragment length polymorphisms (RFLP). A previous study on *Colletotrichum* from apple in Kentucky investigated strain diversity with random amplified polymorphic DNA (RAPD) fingerprinting, but the population of *C. fioriniae* was rather homogenous with this technique (Munir 2015). Telomere RFLP fingerprinting was chosen for the present study because telomeres are known to be hypervariable regions of the genome that often reveal intraspecies differentiation in fungi (Farman and Kim 2005; Starnes et al. 2012; Xavier 2016).

The main objective of this study was to further investigate the occurrence of cross-infection in mixed-fruit orchards in Kentucky by focusing on clonal lineage identification of *C. fioriniae* strains. Strain collections from three deeply sampled mixed-

fruit orchards that contained at least two fruit crops (apple, blueberry, or strawberry) were utilized for Southern blot telomere fingerprint analysis. The goals of this research were to validate telomere fingerprinting as a viable method for *C. fioriniae* clonal lineage identification, to evaluate the fingerprints for each orchard strain collection for diversity and presence of clonal lineages, and to infer the potential risk for cross-infection by *Colletotrichum* in Kentucky mixed-fruit orchards.

Materials and Methods

Colletotrichum fioriniae *strain collections*

Colletotrichum fioriniae strains comprising a control group were collected between 1995 and 2016. They were chosen to be geographically diverse, and included strains from diseased apple (5), blueberry (2), and strawberry (2) fruits, representing two countries and five different U.S. states (Tables 3.1 and 3.2). The purpose of the control group was to confirm polymorphism of telomere fingerprints within the species. The experimental *C. fioriniae* strain groups were isolated from symptomatic apple, blueberry, and strawberry fruits from three different mixed-fruit orchards in Kentucky. The strains were collected between 2013 and 2018 (Tables 3.1 and 3.2). All strains were single spored and stored as a permanent collection on silica granules at -80°C (Tuite 1969). Strains were previously identified as *C. fioriniae* based on morphology (morphotypes M1, M5 and M6; Table 3.1; Suppl. Figures 3.1-3.3) and/or multigene phylogenetic analysis (see Chapter 2). The Orchard 1 experimental group included apple (7), blueberry (5), and strawberry (1) strains; the Orchard 2 experimental group included apple (13) and strawberry (2) strains; and the Orchard 3 experimental group included apple (8) and blueberry (13) strains. The strains used for analysis were specifically selected from different fruits growing in close proximity in efforts to maximize the chance of identifying clonal lineages.

Preparation of genomic DNA

Colletotrichum strains stored as conidial suspensions on silica were applied to Petri plates containing potato dextrose agar (PDA, Difco) and ampicillin (50 mg/mL), and were incubated at 23°C under continuous fluorescent light. After 4 d, colonies were subcultured onto small clarified V8 juice agar plates (200 mL clarified V8® juice, 15 g Difco Bacto-agar, 800 mL ultrapure water). Conidia were harvested from 2-wk-old V8 plate cultures by using the method of Du et al. (2005). Conidia were quantified using a hemocytometer, and the volume of conidial suspension necessary for 1 x 10^5 spores/mL in 50 mL of media was calculated. A 250 mL flask containing 50 mL of Fries Complete medium (30 g sucrose, 5 g $C_4H_{12}N_2O_6$, 1 g NH_4NO_3, 1 g KH_2PO_4, 1 g NaCl, 0.48 g $MgSO_4 \bullet 7H_2O$, 0.13 g $CaCl_2 \bullet 2H_2O$, and 1 g Difco yeast extract in 1L ultrapure water) was then inoculated with the conidial suspension. There were two flasks per *C. fioriniae* strain, and all flasks were incubated in a benchtop orbital shaker at 23°C and 175 rpm for 4 d. Fungal mycelium was harvested by vacuum filtration in a Buchner funnel lined with four layers of sterile cheesecloth, and the mycelium was washed thrice with sterile water. It was then blotted dry with sterile paper towels, frozen in liquid nitrogen for 10 min, and lyophilized (VirTis SP Scientific) for 48 h.

Freeze-dried tissue was pulverized to a fine powder, and 0.1 g was transferred to a new 15 mL centrifuge tube. DNA was extracted using a modified CTAB extraction protocol (Thon et al. 2000). The crushed tissue was mixed with 1.2 mL of room temperature CTAB extraction buffer (0.7 M NaCl, 0.1 M Tris, 0.02 M EDTA, 1% CTAB) and incubated at room temperature for 15 min. Two extractions with an equal volume of phenol:chloroform:isoamyl alcohol (PCI; 25:24:1) were performed, with the second utilizing a 5PRIME Phase Lock Gel Heavy tube (Quantabio, Beverly, MA). A third extraction with an equal volume of chloroform was then performed. The DNA was precipitated with 500 µL isopropanol and 90 µL 3 M sodium acetate (pH 5.2). DNA was quantified by using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA).

Southern blot analysis

The genomic DNA concentration was adjusted to 100 ng/µL, and 1 µg of genomic DNA was digested with *Pst*I (New England Biolabs, Ipswich, MA) according to the protocol of Farman (2011). Twenty microliters of digested DNA and 5 µL of Gel Loading Dye, Purple (6X) (New England Biolabs, Ipswich, MA) were loaded into a 0.7% agarose gel (20 cm long) with 0.5x TBE buffer and run at 20 V for 38 to 42 h. The gel was stained in an ethidium bromide and 0.5x TBE solution for 30 min, de-stained in 0.5x TBE for 30 min, and then imaged using a Gel Doc system (Bio-Rad, Hercules, CA). The DNA was then transferred to a positively charged nylon membrane (Amersham Hybond™-N^+, GE Healthcare, Piscataway, NJ) and cross-linked following the electroblotting protocol of Starnes et al. (2012).

The telomere probe was prepared using a template-less PCR reaction with complementary primers Mg Tel F (5'-TTAGGGTTAGGGTTAGGG-3') and Mg Tel R (5'-CCCTAACCCTAACCCTAA-3'). Each PCR reaction contained 20 pmol of each primer, 5 µL 10x PCR buffer, 2 µL 50 mM $MgCl_2$, 2 µL 10 mM dNTP mix, 0.2 µL 5 U/µL Taq polymerase (Invitrogen, Carlsbad, CA), and DNA-grade water to a total volume of 50 µL. The PCR parameters were: 94°C for 1 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min; followed by 72°C for 5 min. The reaction products were separated by gel electrophoresis on a 0.7% agarose gel at 30 V for 6 h. Fragments of 1.6 to 2 kb were excised and purified using a QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). DNA-grade water was added to 25 ng of probe DNA to a total volume of 17.5 µL, the probe was boiled for 2 min to denature, and then it was removed to ice. The probe was radioactively labeled by adding 5 µL labeling buffer (2.5X Random Primers Solution plus 10X dNTP Mixture, Invitrogen, Carlsbad, CA), 1 µL BSA, 1 µL α^{32}P dCTP, and 0.5 µL Klenow fragment (Invitrogen, Carlsbad, CA) to the probe DNA, and the reaction was incubated for 1 h at 37°C. The labeling reaction was stopped by adding 50 µL of Dye Stop solution (10 mM Tris-HCl, pH 8; 10 mM EDTA, pH 8; 0.8% dextran blue; 0.04% orange G), and the probe was cleaned via an illustra MicroSpin G-50 column with

Sephadex (GE Healthcare, Piscataway, NJ). Then, the probe was denatured with 12 µL 2N NaOH for 8 min and then neutralized with 12 µL 1M Tris-HCl (pH 7.4).

The membrane was pre-hybridized for 30 min at 65°C with 5 mL hybridization solution (0.125 M Na_2HPO_4, pH 7.2; 7% SDS; 1 mM EDTA). The pre-hybridization solution was discarded, and 10 mL fresh solution were added to the membrane with 50 µL of radioactively labeled probe to hybridize overnight at 65°C. The membrane was washed twice with 60 mL 2x SSC and once with 60 mL 0.1% SSC/0.1% SDS for 20 min each at 65°C. Then, the membrane was blotted dry, wrapped in plastic wrap, and exposed to a Phosphor Screen for 3 d. The Southern blot was imaged using a Typhoon FLA 9500 biomolecular imager (GE Healthcare, Piscataway, NJ).

Quantification of fingerprint variability

The formula of Nei and Li (1979) as used by Farman and Kim (2005) was used to calculate the similarities of all pairwise strain comparisons within each experimental orchard group. Telomere fingerprint similarity was calculated as Similarity (S_{xy}) = $2N_{xy}$/ ($N_x + N_y$), where N_x and N_y are the number of fragments per strain *x* and strain *y*, and N_{xy} is the number of shared fragments. The pairwise distance was calculated as $D_{xy} = 1 - S_{xy}$ (Suppl. Tables 3.1-3.3). The similarity matrices were then used to build strain similarity phylograms for each experimental orchard group in MEGA7 (Version 7.0.26) using the Neighbor-Joining tree function.

Results

Colletotrichum fioriniae *control group*

Nine *C. fioriniae* strains representing a diverse geographic, temporal, and pathogenicity range were selected for telomere fingerprinting to test whether or not the method revealed intraspecies differentiation (Tables 3.1 and 3.2). Visual comparison of the telomere fingerprints indicated that there was a high level of band pattern diversity, and none of the fragments appeared to be shared by all nine strains (Figure

3.1). This indicated that telomere fingerprinting would potentially be useful for clonal lineage identification.

Orchard 1

Telomere fingerprints of 13 *C. fioriniae* strains from Orchard 1 collected over a 4-year period from apple, blueberry, and strawberry were analyzed. Overall, the apple strains were more diverse than the blueberry strains, even though all apple strains were collected the same year, and blueberry strains were collected across three years. Within the apple strains, KY 549 and KY 522 had nearly identical fingerprints. This is particularly interesting because they were isolated from two different apple cultivars (Figure 3.2, Table 3.2). One apple strain, KY 550, grouped more closely with the blueberry strains, but was not a clone of them (Figure 3.3). The five blueberry strains formed two clonal lineage clades. KY 646 and KY 648, collected the same year and only differing by one band, made up one clonal lineage. Both of these strains belonged to the M6 morphotype (Table 3.1). KY 763, KY 774, and KY 832, all belonging to the M1 morphotype, comprised a second lineage (Table 3.1, Figures 3.2 and 3.3). These three strains were collected across two years, indicating persistence of clones on blueberry within Orchard 1 (Table 3.2). KY 763 and KY 774 were more closely related to each other than to KY 832, collected the following year, suggesting that telomere fingerprints can experience minor band shifts over time within a clonal lineage (Figure 3.3). The single strawberry strain was more similar to the apple strains than to the blueberry strains, but was not clonal (Figure 3.3, Table 3.2).

Orchard 2

Telomere fingerprints of 15 *C. fioriniae* strains from Orchard 2 collected over a 4-year period from apple and strawberry were analyzed. Overall, the strains from Orchard 2 were more homogenous than the group from Orchard 1, as the apple strains formed four separate clonal lineages (Figures 3.4 and 3.5). Each lineage included two or three strains isolated from the same lesion (Table 3.2, Figures 3.4 and 3.5). The two

strawberry strains that were collected the same year from different plants were clonal, and grouped closely with apple strains KY 41 and KY 42, which may indicate a common ancestry (Table 3.2, Figures 3.4 and 3.5). Interestingly, although the apple strains KY 41 and KY 42 belonged to a rare morphotype (M5), the strawberry strains, like all of the other apple strains, belonged to the common morphotype M1 (Table 3.1, Figure 3.5).

Orchard 3

Telomere fingerprints of 21 *C. fioriniae* strains from Orchard 3, collected over a 6-year period from apple and blueberry, were analyzed. Overall, the apple strains were more diverse than the blueberry strains, but strains from the two fruits were more related to one another compared to the groups of isolates from Orchard 1 and Orchard 2 (Figure 3.7). One blueberry strain (KY 668) was included in a clade that contained most of the apple strains. One apple strain (KY 7007) was a sister to the clade containing most of the blueberry strains. Apple strains KY 18 and KY 846, collected five years apart from the same apple cultivar, formed the only apple clonal lineage (Table 3.2, Figure 3.7). The blueberry strains formed three clonal lineages, all of them spanning multiple collection years. KY 114 and KY 119 had identical fingerprints and formed a lineage with KY 122 and KY 655 (Figures 3.6 and 3.7). All were collected the same year, except KY 655 which was collected three years later (Table 3.2). KY 760 and KY 821 were collected one year apart, and differed by only one band (Table 3.2, Figure 3.6). KY 116, KY 120, and KY 761 formed another blueberry clonal lineage, and together with KY 657, they formed a group of morphotype M5 strains that was separate from the M1 strains (Table 3.1, Figure 3.7). The single M5 apple strain, KY 17, grouped closely with the M5 blueberry strains, indicating they may share a common ancestor (Table 3.1, Figure 3.7).

Discussion

Colletotrichum fioriniae strains from apple, blueberry, and strawberry fruits from three Kentucky mixed-fruit orchards were analyzed by Southern blot telomere fingerprinting for clone identification and assessment of cross-infection incidence. Clonal lineages were identified from each orchard on the same fruit, but there was no evidence for cross-infection among different fruits. This does not confirm an absence of cross-infection, even though *C. fioriniae* strains were chosen from each orchard in order to optimize the likelihood of clone detection. Cross-infection could have been present, but at a level that was too low to detect in this study. If cross-infection was not a factor, or if incidence was low, the resulting risk of *Colletotrichum* strains moving among fruit crops would have been reduced. From a disease management perspective, this would be a positive outcome because no drastic changes to management practices would be necessary. While this observation may apply to Kentucky orchards, definitive conclusions about cross-infection incidence cannot be drawn for other locations, fruit crops, or *Colletotrichum* spp.

This study revealed other interesting information about *C. fioriniae* population dynamics. Across the three orchard collections, the blueberry *C. fioriniae* strains included more clonal lineages than the apple strains. One potential explanation for this observation is that the two anthracnose diseases were managed quite differently within the orchards. Bitter rot is a serious disease on apple in Kentucky, and most growers proactively manage the disease by applying fungicides and other cultural control practices (Gauthier et al. 2017). In contrast, ripe rot is not a disease of concern in Kentucky because blueberry storage is uncommon, so growers often do not manage this disease. These differing approaches may affect the *C. fioriniae* population structure on the two fruits. On blueberry, clones of a few strains may be widespread due to the limited selection pressure, whereas on apple, a more heterogeneous population may be present because of the control pressure applied. If growers were to actively manage ripe rot, it may be possible to track how the *C. fioriniae* population changes over time under new conditions.

Few *C. fioriniae* strains were recovered from diseased strawberry fruits, indicating that this species does not play a major role in the incidence and spread of anthracnose fruit rot on strawberry in Kentucky. This was also the case across the United States and in other strawberry producing countries, where *C. nymphaeae* (also in the *C. acutatum* complex) has been identified as a dominant species (Jayawardena et al. 2016a; Karimi et al. 2016; Wang et al. 2019). The *C. fioriniae* strains recovered in Kentucky were more closely related to the apple strains than to the blueberry strains, but they were not clones. The strains may have been introduced into the orchard on the strawberry transplants, or they could have come from the apple trees, but additional research is necessary to answer this question.

One significant conclusion that can be drawn from this study is that *C. fioriniae* strains persist within an orchard over multiple years, implying that the fungus is able to overwinter within the orchard and then contribute to disease development from year to year. In the group of isolates from Orchard 3, the single apple clonal lineage consisted of strains isolated five years apart, and one of the blueberry clonal lineages included strains isolated four years apart. *Colletotrichum fioriniae* strains may still be moving into orchards from alternative hosts or from greater distances, but my data show that strains can survive within orchards for long periods of time. This finding lends emphasis to the importance of orchard sanitation and cultural practices for reduction of primary inoculum within the orchard. Furthermore, in the Orchard 1 group, strains of a clonal lineage were identified on apples from different cultivars. This suggests a lack of strain specialization to particular apple cultivars. *In vitro* cross-inoculations using a variety of apple cultivars support this finding (Munir et al. 2016; Chapter 2).

Another interesting observation from this study was that culture morphology and morphotype of *C. fioriniae* strains largely matched the telomere fingerprint groupings. A majority of the strains belonging to the two variant *C. fioriniae* morphotypes M5 and M6 had distinct telomere fingerprints and formed clonal lineages separate from the remaining strains.

Even though Southern blot telomere fingerprinting revealed important information about *C. fioriniae* strain diversity and movement within an orchard, there were limitations to this approach that precluded concrete evidence of cross-infection. First, the number of strains selected for this study was limited, so it is possible that *C. fioriniae* clones were present on multiple fruit crops within an orchard, but they escaped detection because of inadequate sample depth. Additionally, most strains lacked detailed collection data, like specific crop row, plant, fruit, isolation lesion, or geographic coordinate data. This information would have given further insight into how strains move within an orchard and how position determines relatedness.

My goal in this study was to validate the Southern blot telomere fingerprinting method for clone detection. Questions included 1) does this method identify clones, and 2) how similar do fingerprints need to be. My conclusion for the first question is that the method does identify clones. Two strains from the Orchard 1 group (KY 646 and KY 648) and two from Orchard 3 (KY 116 and KY 119) were used for whole genome sequencing in Chapter 2. The whole genome sequencing and SNP comparisons indicated that KY646 and KY648 were clones of one another, while KY116 and KY119 were not. The telomere fingerprints were consistent with these results. The second question is more complex. In order to know how similar telomere fingerprints must be for strains to form a clonal lineage, prior knowledge of chromosome and telomere structure is useful. This information is lacking for *C. fioriniae* specifically, but many studies have explored this topic with other *Colletotrichum* spp. and the related Sordariomycete *Magnaporthe oryzae*. One study on *C. acutatum* s. lat. genome organization, and another study on the cytological chromosome structure of three *Colletotrichum* species revealed that chromosome number can range from 6 to 13, due to the presence of expendable B-chromosomes, aka mini-chromosomes (Garrido et al. 2009; Taga et al. 2015). The number of *C. fioriniae* telomere bands in my study fits within this chromosomal range.

Telomeres are highly variable genomic regions because they often undergo rearrangements, insertions, deletions, and mutations caused by telomere instability, repeat-induced point mutation (RIP), or transposon movement (Baird 2018; Farman

2007; Wu et al. 2009). These phenomena lead to changes in telomere and sub-telomeric region size and sequence, which cause band shifts and affect the telomere fingerprint. However, telomere instability can vary between species and even within species, depending on telomere structure. For example, in the fungus *M. oryzae*, it has been shown that strains can have very stable or unstable telomere fingerprints over multiple generations depending upon the telomere structure (Farman 2007; Farman and Kim 2005; Starnes et al. 2012). Starnes et al. (2012) found that stable strains lacked certain transposons called MoTeRs in or near telomeres, whereas MoTeRs were abundant in the telomeres of unstable strains, indicating that transposon action contributes to telomere instability. In this situation, strains that are technically clonal can have telomere fingerprints that differ by multiple bands. The exact structure of *Colletotrichum* telomeres has not been explored in depth, but one detailed study on the genome of *C. higginsianum* mapped transposons to 23 of the 24 telomeres on 10 core chromosomes and two mini-chromosomes (Dallery et al. 2017). Therefore, *Colletotrichum* spp. may potentially have an increased level of telomere instability because transposons are present in telomeres, and telomere fingerprint variability between clones of a strain can be expected. More research is required to confirm the structure of *C. fioriniae* telomeres, but for the purpose of this study, I am confident in considering strains that differ by three or fewer bands as belonging to a clonal lineage.

For further confirmation of clone identification, additional methods should be utilized or developed. For select strains, whole genome sequencing may be employed, but a threshold number of SNPs should also be determined for clone consideration. A PCR-based gene sequencing clone identification tool can also be developed from genome sequence data. The seven genes commonly used for *Colletotrichum* multigene sequencing cannot be used for clone differentiation (Chapter 2), but genome data including clonal and non-clonal *C. fioriniae* strains can be mined to identify loci that are polymorphic at an intraspecies level and correspond to clonal lineages that have already been established with telomere fingerprinting. Moving forward, a unique clone identifiable via the PCR-based tool can be released within an orchard, and its movement

tracked throughout the orchard to observe cross-infection in real time. Even though no definitive conclusion could be made concerning *Colletotrichum* cross-infection in Kentucky mixed-fruit orchards in the present study, our knowledge of how *C. fioriniae* strains move within an orchard has increased and has brought us closer to answering this important question.

Table 3.1 Morphotype and origin of *Colletotrichum fioriniae* strains used for Southern blot telomere fingerprint analysis.

Strain	Group	Host	Morphotype[y]	Origin[z]
HC 296	Control	Apple	1	Bourbon Co.
HSP 11	Control	Apple	1	Tennessee
KY 670	Control	Apple	1	New York
PA HC89	Control	Apple	1	Pennsylvania
RI 1-4-57	Control	Apple	1	Rhode Island
ERL 1379	Control	Blueberry	1	New Jersey
KY 657	Control	Blueberry	5	Bourbon Co.
2.7.15	Control	Strawberry	1	New Zealand
KY 615	Control	Strawberry	1	Woodford Co.
KY 547	Orchard 1	Apple	1	Scott Co.
KY 548	Orchard 1	Apple	1	Scott Co.
KY 549	Orchard 1	Apple	1	Scott Co.
KY 550	Orchard 1	Apple	1	Scott Co.
KY 551	Orchard 1	Apple	1	Scott Co.
KY 552	Orchard 1	Apple	1	Scott Co.
KY 561	Orchard 1	Apple	1	Scott Co.
KY 646	Orchard 1	Blueberry	6	Scott Co.
KY 648	Orchard 1	Blueberry	6	Scott Co.
KY 763	Orchard 1	Blueberry	1	Scott Co.
KY 774	Orchard 1	Blueberry	1	Scott Co.
KY 832	Orchard 1	Blueberry	1	Scott Co.
KY 585	Orchard 1	Strawberry	1	Scott Co.
KY 41	Orchard 2	Apple	5	Woodford Co.
KY 42	Orchard 2	Apple	5	Woodford Co.
KY 44	Orchard 2	Apple	1	Woodford Co.
KY 45	Orchard 2	Apple	1	Woodford Co.
KY 46	Orchard 2	Apple	1	Woodford Co.
KY 51	Orchard 2	Apple	1	Woodford Co.
KY 52	Orchard 2	Apple	1	Woodford Co.
KY 53	Orchard 2	Apple	1	Woodford Co.
KY 54	Orchard 2	Apple	1	Woodford Co.
KY 55	Orchard 2	Apple	1	Woodford Co.
KY 558	Orchard 2	Apple	1	Woodford Co.
KY 559	Orchard 2	Apple	1	Woodford Co.
KY 560	Orchard 2	Apple	1	Woodford Co.
KY 615	Orchard 2	Strawberry	1	Woodford Co.
KY 619	Orchard 2	Strawberry	1	Woodford Co.

Table 3.1 (continued).

Strain	Group	Host	Morphotype[y]	Origin[z]
KY 17	Orchard 3	Apple	5	Bourbon Co.
KY 18	Orchard 3	Apple	1	Bourbon Co.
KY 124	Orchard 3	Apple	1	Bourbon Co.
KY 135	Orchard 3	Apple	1	Bourbon Co.
KY 553	Orchard 3	Apple	1	Bourbon Co.
KY 7007	Orchard 3	Apple	1	Bourbon Co.
KY 846	Orchard 3	Apple	1	Bourbon Co.
KY 849	Orchard 3	Apple	1	Bourbon Co.
KY 114	Orchard 3	Blueberry	1	Bourbon Co.
KY 116	Orchard 3	Blueberry	5	Bourbon Co.
KY 119	Orchard 3	Blueberry	1	Bourbon Co.
KY 120	Orchard 3	Blueberry	5	Bourbon Co.
KY 122	Orchard 3	Blueberry	1	Bourbon Co.
KY 655	Orchard 3	Blueberry	1	Bourbon Co.
KY 657	Orchard 3	Blueberry	5	Bourbon Co.
KY 668	Orchard 3	Blueberry	1	Bourbon Co.
KY 673	Orchard 3	Blueberry	1	Bourbon Co.
KY 760	Orchard 3	Blueberry	1	Bourbon Co.
KY 761	Orchard 3	Blueberry	5	Bourbon Co.
KY 809	Orchard 3	Blueberry	1	Bourbon Co.
KY 821	Orchard 3	Blueberry	1	Bourbon Co.

[y] Morphotype descriptions are as specified in Chapter 2 results.
[z] Any origin listed by county is from Kentucky

Table 3.2 Collection information for *Colletotrichum fioriniae* strains used for Southern blot telomere fingerprint analysis.

Strain	Group	Collection Date	Collection Year	Collection Details[z]
HC 296	Control	-	2013	Honey Crisp A9L3
HSP-11	Control	-	2013	Apple
KY 670	Control	7/26/16	2016	Apple
PA HC89	Control	-	-	Apple
RI 1.4.57	Control	-	2012	Apple
ERL 1379	Control	-	2005	Blueberry
KY 657	Control	7/5/16	2016	Blueberry
2.7.15	Control	-	1995	Strawberry
KY 615	Control	6/3/16	2016	Strawberry Earliglow
KY 547	Orchard 1	8/18/15	2015	Honey Crisp 1
KY 548	Orchard 1	8/18/15	2015	Honey Crisp 2
KY 549	Orchard 1	8/18/15	2015	Honey Crisp 3
KY 550	Orchard 1	8/18/15	2015	Jonagold 1
KY 551	Orchard 1	8/18/15	2015	Jonagold 2
KY 552	Orchard 1	8/18/15	2015	Jonagold 3
KY 561	Orchard 1	10/1/15	2015	Fuji
KY 646	Orchard 1	6/25/16	2016	Blueberry
KY 648	Orchard 1	6/25/16	2016	Blueberry
KY 763	Orchard 1	-	2017	Blueberry BB-1
KY 774	Orchard 1	7/8/17	2017	Blueberry BB-4
KY 832	Orchard 1	7/12/18	2018	Blueberry BB-3
KY 585	Orchard 1	-	2015	Strawberry
KY 41	Orchard 2	8/23/13	2013	Candy Crisp A1L2
KY 42	Orchard 2	8/23/13	2013	Candy Crisp A1L2
KY 44	Orchard 2	8/23/13	2013	Candy Crisp A2L1
KY 45	Orchard 2	8/23/13	2013	Candy Crisp A2L1
KY 46	Orchard 2	8/23/13	2013	Candy Crisp A2L1
KY 51	Orchard 2	8/23/13	2013	Idared A2L1
KY 52	Orchard 2	8/23/13	2013	Idared A2L1
KY 53	Orchard 2	8/23/13	2013	Idared A3L1
KY 54	Orchard 2	8/23/13	2013	Idared A3L1
KY 55	Orchard 2	8/23/13	2013	Idared A3L1
KY 558	Orchard 2	9/28/15	2015	Golden Delicious
KY 559	Orchard 2	9/28/15	2015	Gold Rush 1
KY 560	Orchard 2	9/28/15	2015	Gold Rush 2
KY 615	Orchard 2	6/3/16	2016	Strawberry Earliglow
KY 619	Orchard 2	6/3/16	2016	Strawberry

Table 3.2 (continued).

Strain	Group	Collection Date	Collection Year	Collection Details[z]
KY 17	Orchard 3	7/31/13	2013	Honey Crisp A1
KY 18	Orchard 3	7/31/13	2013	Honey Crisp A2L1
KY 124	Orchard 3	9/16/13	2013	Jonathan A1L1
KY 135	Orchard 3	9/16/13	2013	Mutsu A1L1
KY 553	Orchard 3	8/20/15	2015	Honey Crisp
KY 7007	Orchard 3	8/17/17	2017	Honey Crisp
KY 846	Orchard 3	8/25/18	2018	Honey Crisp A2
KY 849	Orchard 3	8/26/18	2018	Sweet Sixteen
KY 114	Orchard 3	9/5/13	2013	Blueberry A
KY 116	Orchard 3	9/5/13	2013	Blueberry C
KY 119	Orchard 3	9/5/13	2013	Blueberry F
KY 120	Orchard 3	9/5/13	2013	Blueberry
KY 122	Orchard 3	9/5/13	2013	Blueberry
KY 655	Orchard 3	7/5/16	2016	Blueberry
KY 657	Orchard 3	7/5/16	2016	Blueberry
KY 668	Orchard 3	7/21/16	2016	Blueberry
KY 673	Orchard 3	7/26/16	2016	Blueberry
KY 760	Orchard 3	6/19/17	2017	Blueberry BB-1
KY 761	Orchard 3	6/19/17	2017	Blueberry BB-2
KY 809	Orchard 3	6/16/18	2018	Blueberry BB-A
KY 821	Orchard 3	6/30/18	2018	Blueberry BB-C

[z] Apple strains are listed by cultivar if available; additional collection information is listed after the host; A#L# = apple number, lesion number.

Supplementary Table 3.1 Orchard 1 *C. fioriniae* Southern blot telomere fingerprint similarity matrix.

	KY547	KY548	KY549	KY550	KY551	KY552	KY561	KY646	KY648	KY763	KY774	KY832	KY585	PAHC89	KY41	KY17
KY547																
KY548	0.375															
KY549	0.515	0.484														
KY550	0.824	0.812	0.758													
KY551	0.429	0.515	0.529	0.829												
KY552	0.576	0.613	0.125	0.818	0.588											
KY561	0.706	0.687	0.636	0.765	0.657	0.636										
KY646	0.867	0.714	0.793	0.667	0.742	0.793	0.933									
KY648	0.862	0.704	0.786	0.655	0.733	0.786	0.913	0.040								
KY763	0.636	0.625	0.758	0.647	0.714	0.879	0.765	0.533	0.517							
KY774	0.647	0.625	0.758	0.588	0.657	0.879	0.765	0.533	0.586	0.059						
KY832	0.636	0.548	0.750	0.636	0.647	0.875	0.818	0.586	0.643	0.091	0.091					
KY585	0.371	0.515	0.529	0.771	0.389	0.647	0.543	0.742	0.733	0.657	0.714	0.706				
PAHC89	0.758	0.806	1.000	0.697	0.882	1.000	0.879	0.862	0.857	0.758	0.758	0.758	0.882			
KY41	0.882	0.947	0.939	0.824	0.943	1.000	0.824	0.667	0.655	0.824	0.824	0.879	0.886	0.697		
KY17	0.833	0.765	0.886	0.778	0.838	0.771	0.778	0.812	0.806	0.611	0.611	0.600	0.838	0.543	0.611	

Supplementary Table 3.2 Orchard 2 *C. fioriniae* Southern blot telomere fingerprint similarity matrix.

	KY41	KY42	KY44	KY45	KY46	KY51	KY52	KY53	KY54	KY55	KY558	KY559	KY560	KY615	KY619	PAHC89	KY547	KY17
KY41																		
KY42	0.086																	
KY44	0.926	0.929																
KY45	0.926	0.929	0.100															
KY46	0.926	0.929	0.000	0.100														
KY51	0.937	0.818	0.440	0.600	0.520													
KY52	0.879	0.824	0.462	0.615	0.538	0.032												
KY53	0.875	0.939	0.840	0.840	0.920	0.867	0.806											
KY54	0.875	0.939	0.840	0.840	0.920	0.867	0.806	0.000										
KY55	0.875	0.939	0.840	0.840	0.920	0.867	0.806	0.000	0.000									
KY558	0.886	0.889	0.786	0.786	0.714	0.333	0.176	0.636	0.636	0.636								
KY559	0.824	0.771	0.630	0.630	0.630	0.500	0.455	0.625	0.625	0.625	0.371							
KY560	0.771	0.889	0.786	0.929	0.929	0.697	0.706	0.818	0.818	0.818	0.444	0.771						
KY615	0.486	0.500	0.786	0.857	0.857	0.697	0.765	0.879	0.879	0.879	0.778	0.771	0.833					
KY619	0.471	0.543	0.778	0.852	0.852	0.687	0.758	0.875	0.875	0.875	0.771	0.824	0.829	0.029				
PAHC89	0.697	0.758	0.769	0.769	0.769	0.871	0.875	0.871	0.871	0.871	0.941	0.818	0.882	0.765	0.758			
KY547	0.824	0.824	0.704	0.704	0.704	0.812	0.818	0.625	0.625	0.625	0.486	0.471	0.771	0.771	0.765	0.758		
KY17	0.556	0.568	0.793	0.793	0.793	0.824	0.771	0.706	0.706	0.706	0.784	0.778	0.73	0.622	0.611	0.543	0.833	

Supplementary Table 3.3 Orchard 3 *C. fioriniae* Southern blot telomere fingerprint similarity matrix.

	KY17	KY18	KY124	KY135	KY553	KY7007	KY846	KY849	KY114	KY116	KY119	KY120	KY122	KY655	KY657	KY668	KY673	KY760	KY761	KY809	KY821	PAHC89	KY547	KY41
KY17																								
KY18	0.421																							
KY124	0.697	0.576																						
KY135	0.824	0.824	0.724																					
KY553	0.778	0.833	0.677	0.687																				
KY7007	0.676	0.784	0.875	0.879	0.943																			
KY846	0.405	0.189	0.437	0.818	0.771	0.667																		
KY849	0.647	0.706	0.517	0.667	0.937	0.818	0.636																	
KY114	0.789	0.737	0.818	1.000	0.889	0.676	0.730	0.882																
KY116	0.500	0.778	0.935	0.812	0.882	0.829	0.714	0.812	0.833															
KY119	0.842	0.789	0.939	1.000	0.889	0.676	0.730	0.882	0.000	0.778														
KY120	0.500	0.778	0.935	0.750	0.882	0.829	0.714	0.812	0.833	0.000	0.722													
KY122	0.789	0.789	1.000	0.941	0.889	0.622	0.730	0.882	0.053	0.722	0.053	0.778												
KY655	0.789	0.789	0.939	0.941	0.889	0.676	0.730	0.882	0.105	0.667	0.105	0.722	0.105											
KY657	0.543	0.829	0.933	0.806	0.879	0.824	0.706	0.871	0.886	0.333	0.886	0.333	0.829	0.829										
KY668	0.818	0.879	0.857	0.724	0.806	0.875	0.812	0.724	0.758	0.677	0.818	0.742	0.818	0.697	0.733									
KY673	0.714	0.829	0.867	1.000	0.939	0.706	0.824	0.806	0.429	0.758	0.371	0.758	0.371	0.371	0.625	0.800								
KY760	0.676	0.784	0.687	0.879	0.771	0.889	0.611	0.758	0.676	0.829	0.568	0.714	0.568	0.514	0.824	0.937	0.353							
KY761	0.632	0.895	0.879	0.824	0.944	0.892	0.784	0.941	0.789	0.111	0.737	0.111	0.737	0.684	0.371	0.819	0.829	0.676						
KY809	0.778	0.833	0.935	0.812	0.765	0.771	0.829	0.937	0.667	0.882	0.543	0.765	0.611	0.500	0.818	0.871	0.394	0.486	0.778					
KY821	0.737	0.842	0.879	0.941	0.833	0.784	0.568	0.765	0.526	0.833	0.526	0.722	0.684	0.579	0.829	0.879	0.371	0.027	0.737	0.556				
PAHC89	0.514	0.714	0.667	0.806	0.879	0.765	0.647	0.613	0.829	0.697	0.829	0.697	0.829	0.829	0.875	0.933	0.875	0.647	0.714	0.758	0.657			
KY547	0.833	0.611	0.871	0.875	0.824	0.886	0.714	0.814	0.833	0.882	0.833	0.882	0.833	0.833	0.818	1.000	0.697	0.771	0.833	0.882	0.778	0.758		
KY41	0.611	0.556	0.677	0.812	0.882	0.829	0.600	0.812	0.778	0.882	0.778	0.882	0.778	0.778	0.939	0.871	0.758	0.771	0.889	0.765	0.778	0.697	0.882	

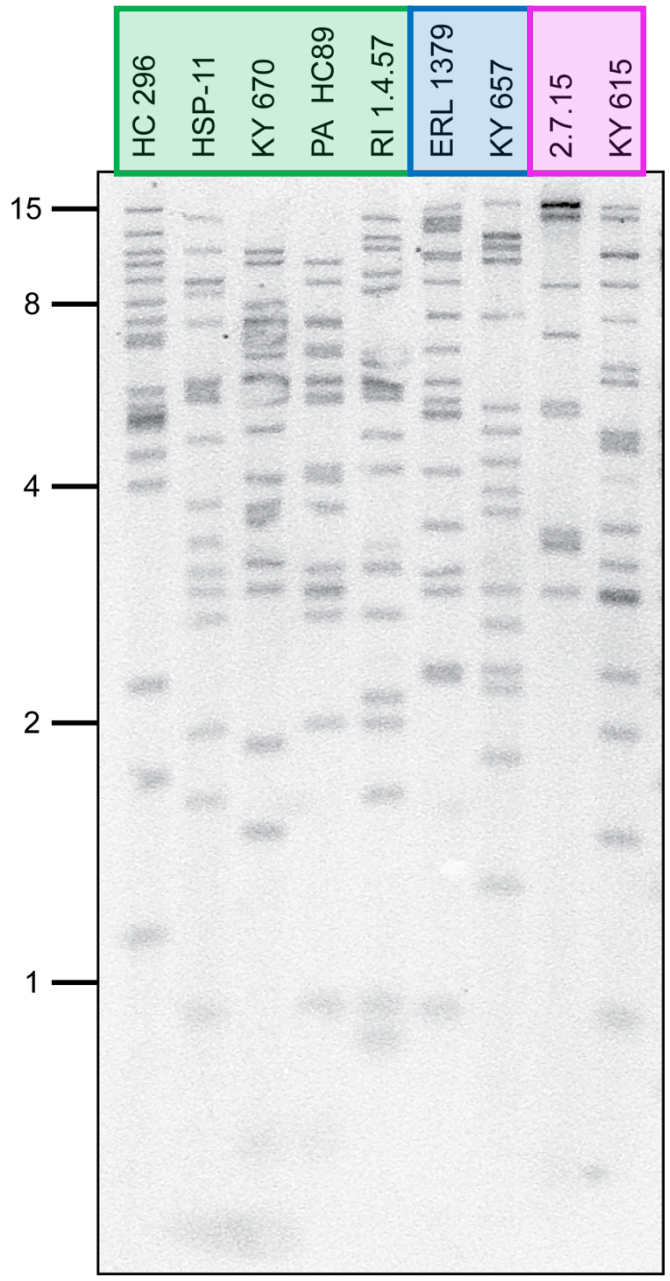


Figure 3.1 Southern blot using a telomere probe of the control group of *C. fioriniae* strains. Strains highlighted in green are from apple, blue from blueberry, and pink from strawberry. Numbers along the left side are fragment sizes in kilobases.

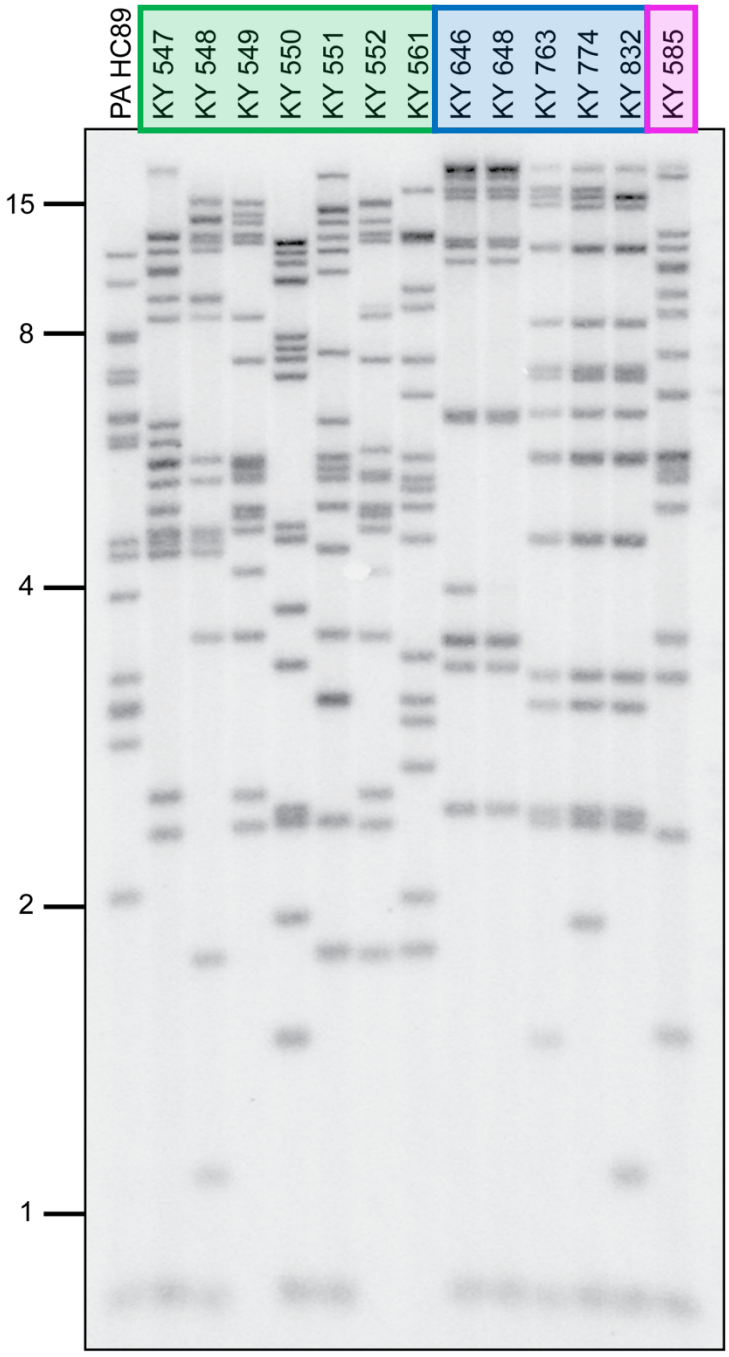


Figure 3.2 Southern blot using a telomere probe of the Orchard 1 *C. fioriniae* group. PA HC89 is a *C. fioriniae* strain from Pennsylvania used as a standard. Strains highlighted in green are from apple, blue from blueberry, and pink from strawberry. Numbers along the left side are fragment sizes in kilobases.

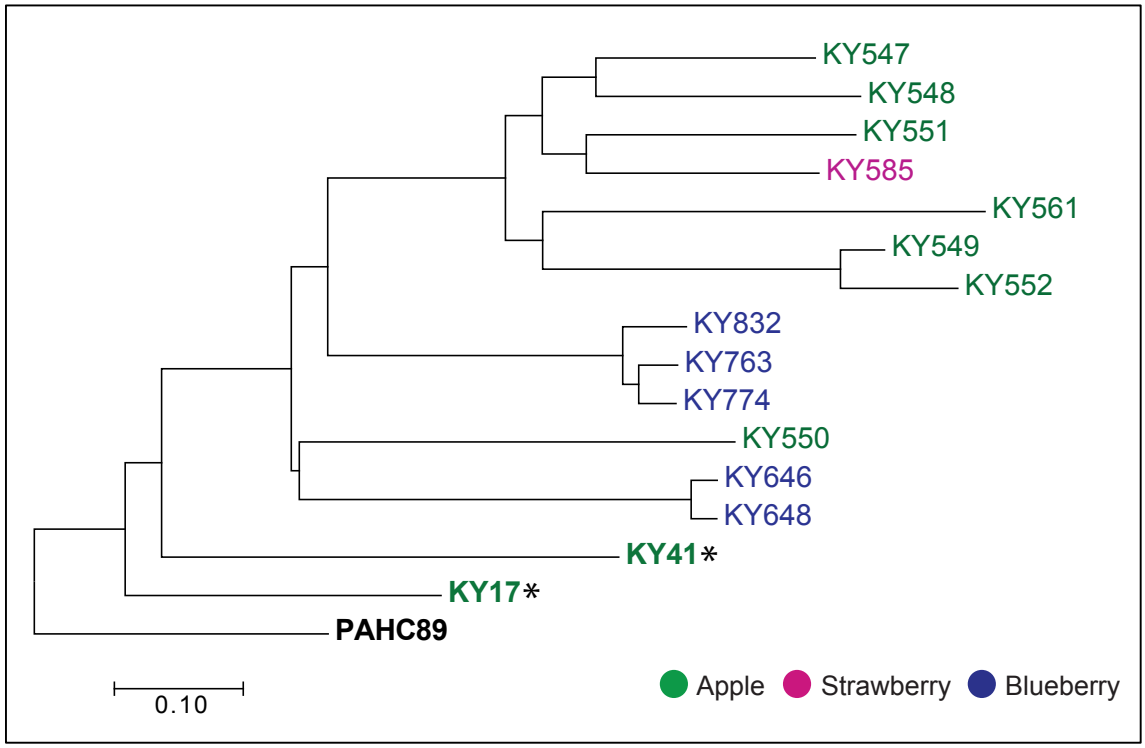


Figure 3.3 Phylogram of the Orchard 1 *C. fioriniae* group based on the Southern blot telomere fingerprint similarity matrix (Suppl. Table 3.1). The tree was built in MEGA7 using the Neighbor-joining tree function. The tree is rooted to the outgroup PAHC89; strains in bold are not from Orchard 1. Strains with an asterisk are from other Kentucky orchards (KY41, Orchard 2; KY17, Orchard 3).

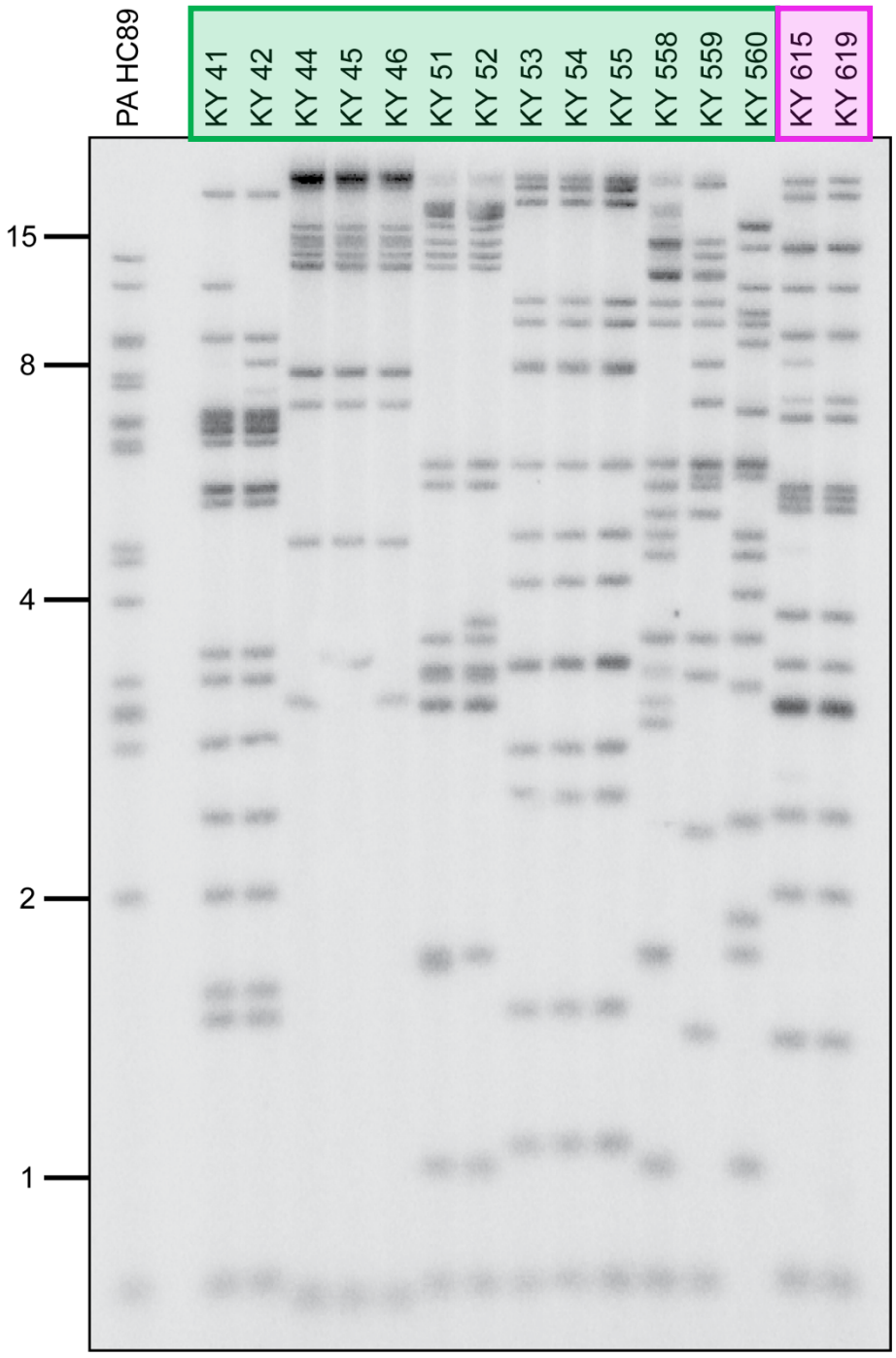


Figure 3.4 Southern blot using a telomere probe of the Orchard 2 *C. fioriniae* group. PA HC89 is a *C. fioriniae* strain from Pennsylvania used as a standard. Strains highlighted in green are from apple and pink strains are from strawberry. Numbers along the left side are fragment sizes in kilobases.

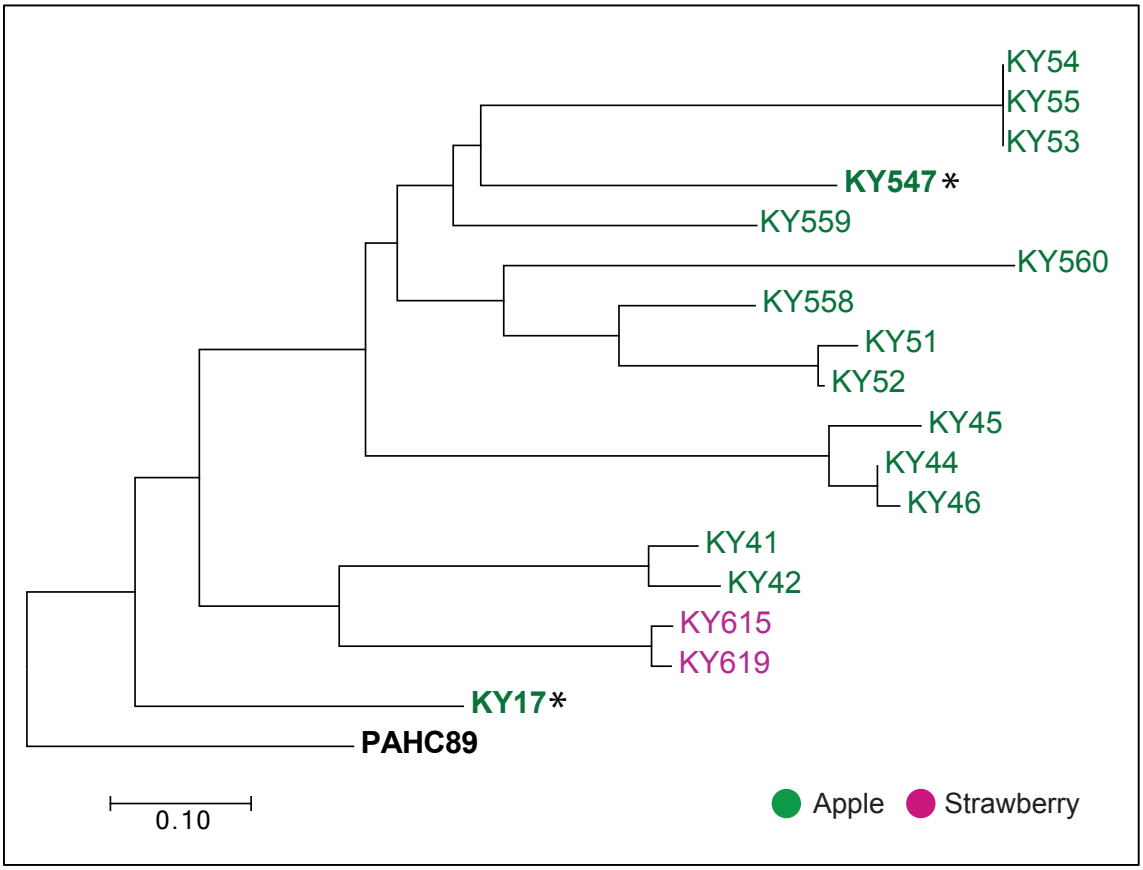


Figure 3.5 Phylogram of the Orchard 2 *C. fioriniae* group based on the Southern blot telomere fingerprint similarity matrix (Suppl. Table 3.2). The tree was built in MEGA7 using the Neighbor-joining tree function. The tree is rooted to the outgroup PAHC89; strains in bold are not from Orchard 1. Strains with an asterisk are from other Kentucky orchards (KY547, Orchard 1; KY17, Orchard 3).

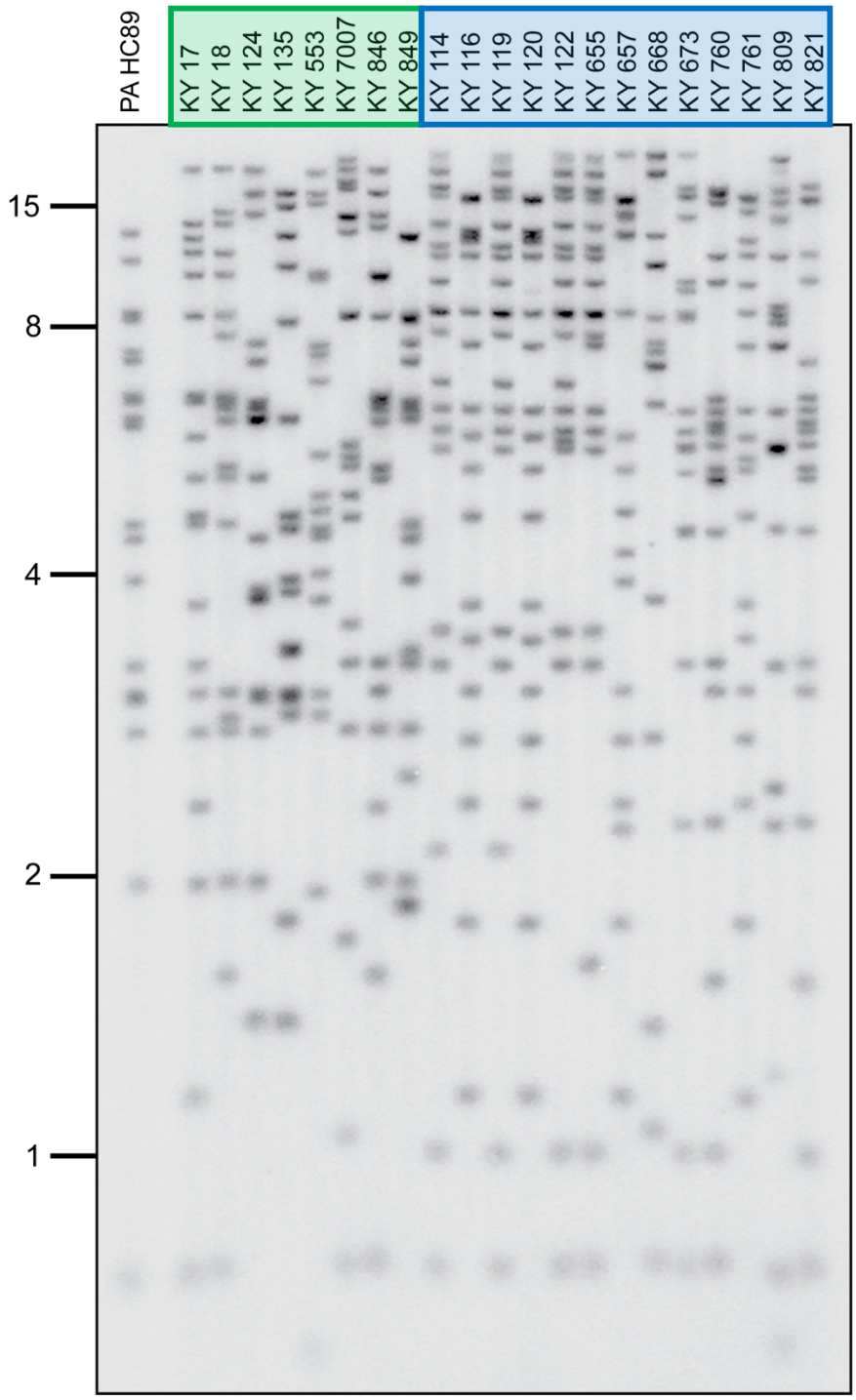


Figure 3.6 Southern blot using a telomere probe of the Orchard 3 *C. fioriniae* group. PA HC89 is a *C. fioriniae* strain from Pennsylvania used as a standard. Strains highlighted in green are from apple and blue are from blueberry. Numbers along the left side are fragment sizes in kilobases.

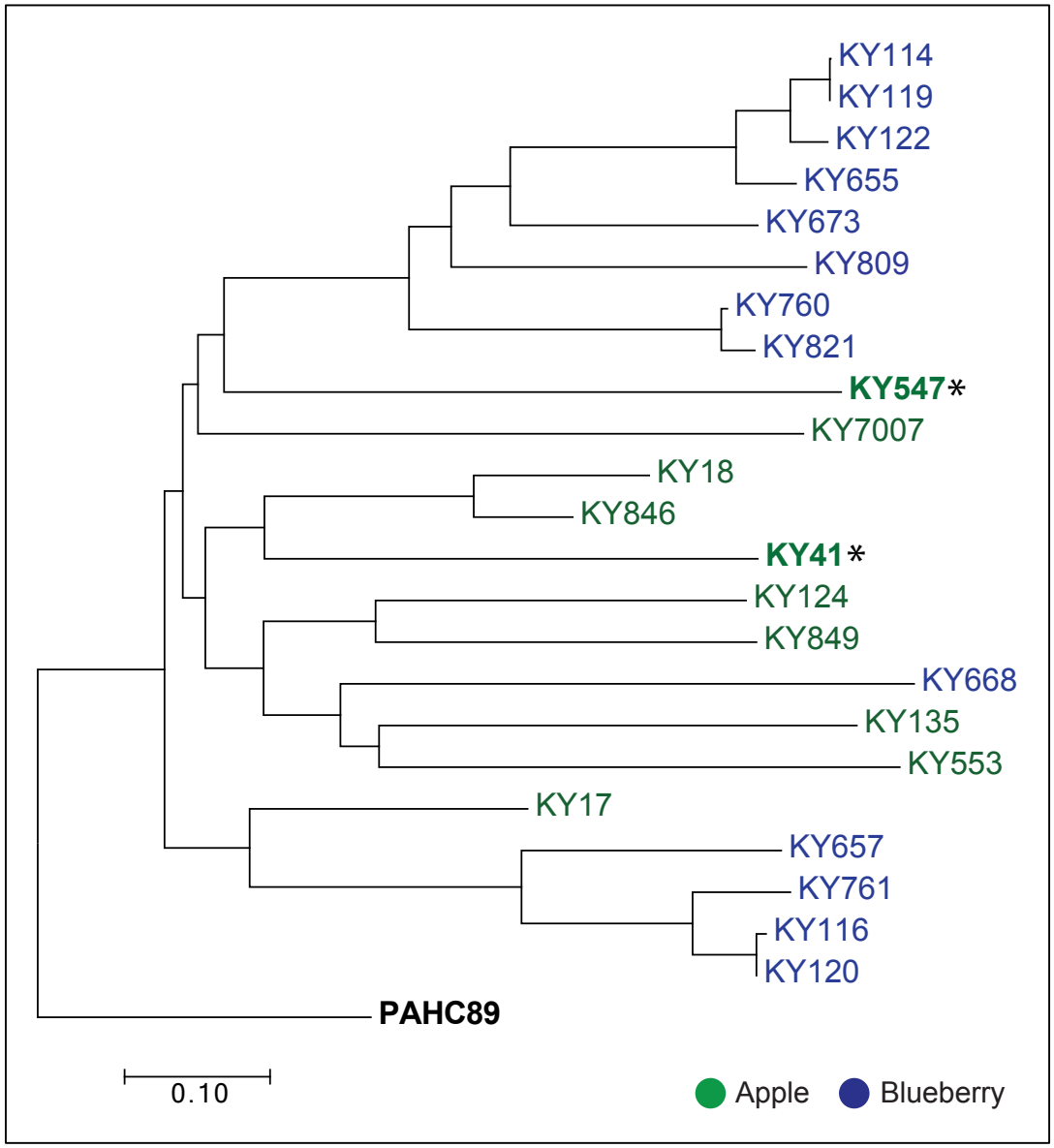
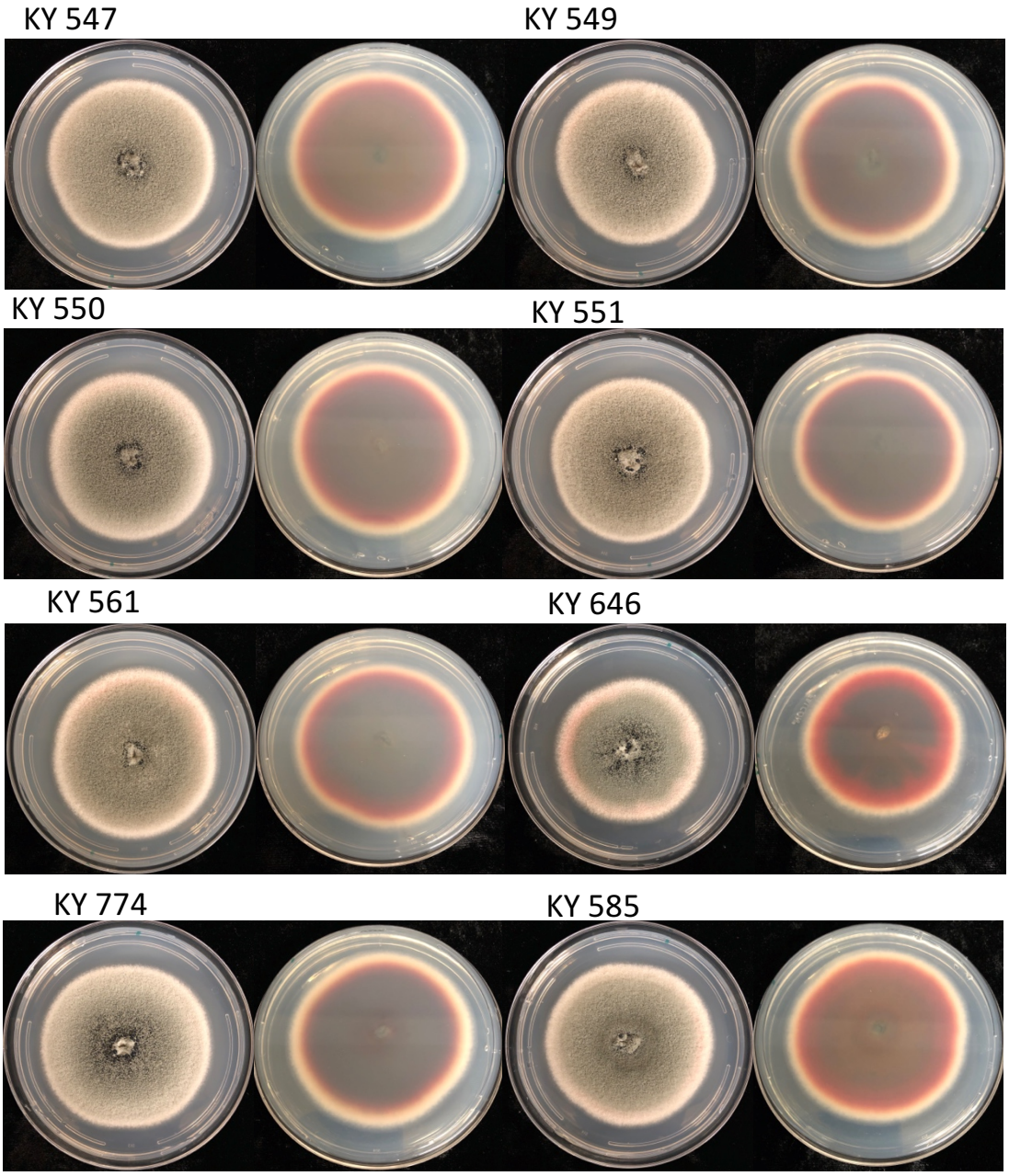
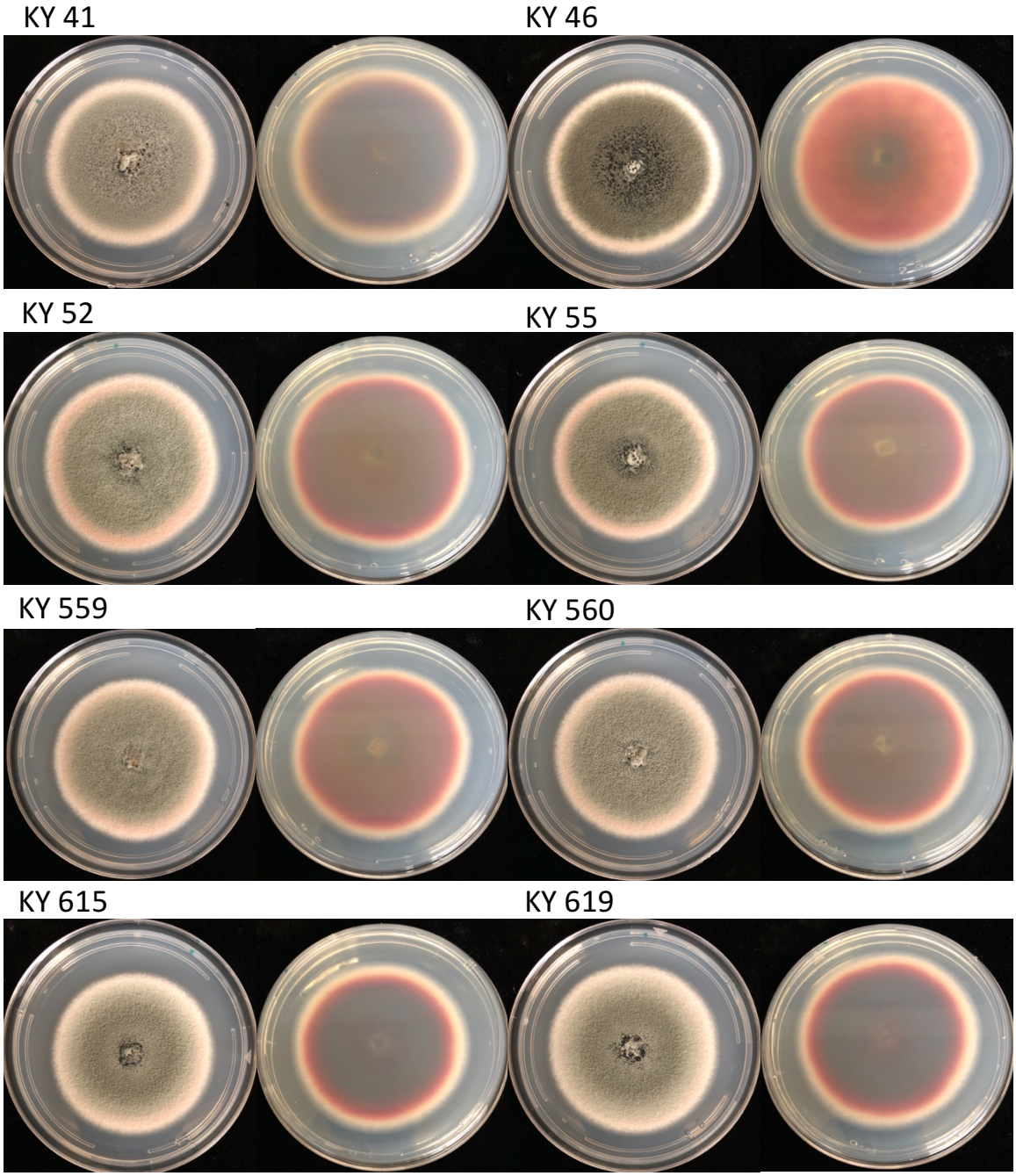


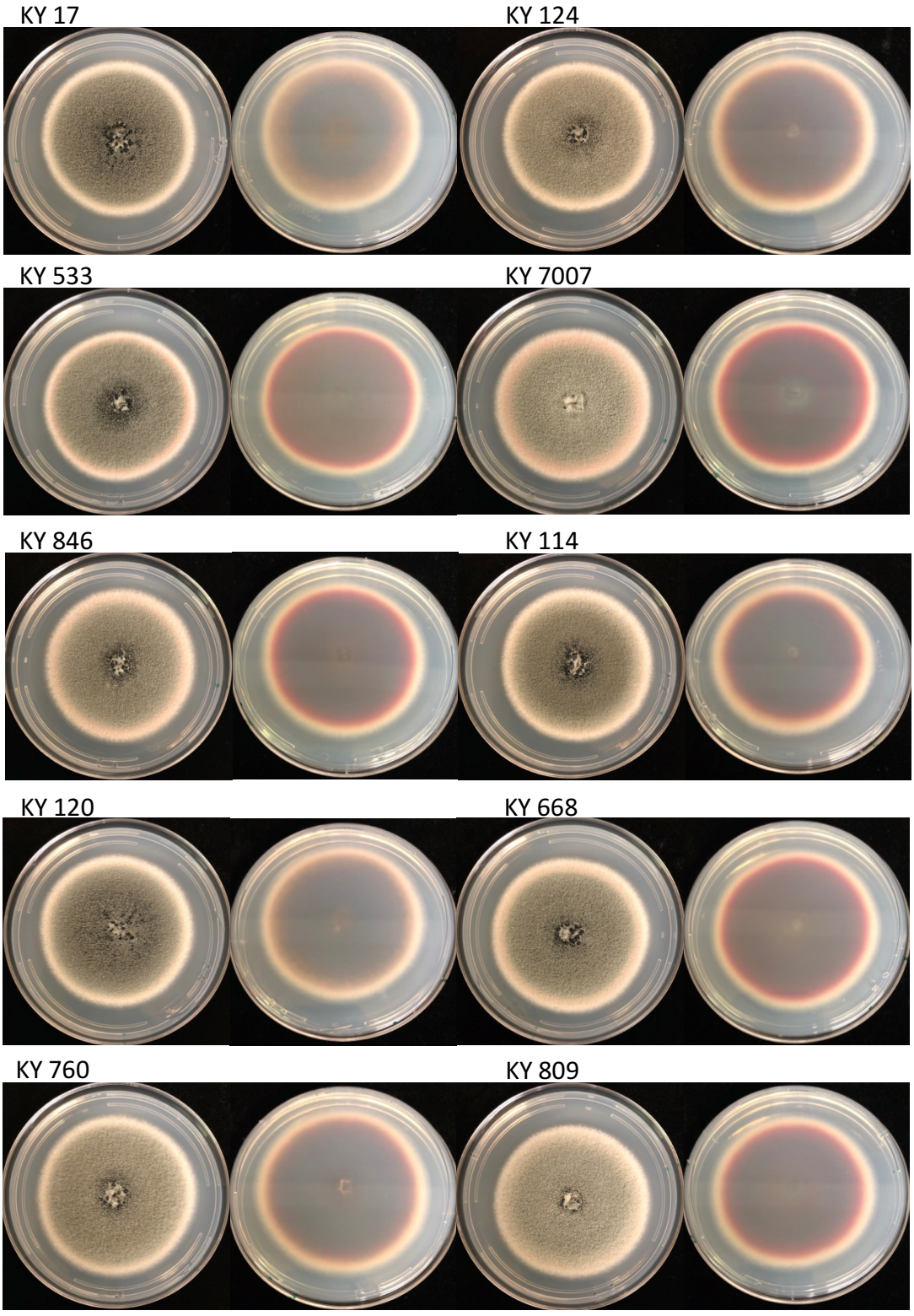
Figure 3.7 Phylogram of the Orchard 3 *C. fioriniae* group based on the Southern blot telomere fingerprint similarity matrix (Suppl. Table 3.3). The tree was built in MEGA7 using the Neighbor-joining tree function. The tree is rooted to the outgroup PAHC89; strains in bold are not from Orchard 1. Strains with an asterisk are from other Kentucky orchards (KY547, Orchard 1; KY41, Orchard 2).



Supplementary Figure 3.1 Representative images of *C. fioriniae* culture morphology for Orchard 1. KY 646 is Morphotype 6; all other strains are Morphotype 1.



Supplementary Figure 3.2 Representative images of *C. fioriniae* culture morphology for Orchard 2. KY 41 is Morphotype 5; all other strains are Morphotype 1.



Supplementary Figure 3.3 Representative images of *C. fioriniae* culture morphology for Orchard 3. KY 17 and KY 120 are Morphotype 5; all other strains are Morphotype 1.

CHAPTER 4. SUMMARY AND SIGNIFICANCE OF THE STUDY

Identification of *Colletotrichum* to the level of phylogenetic species is important for anthracnose disease management because species have been shown to differ in their fungicide sensitivity, pathogenicity, responses to host defense and environmental factors, and ability to cross-infect different hosts (Chechi et al. 2019; Chen et al. 2016; He et al. 2019; Lakshmi et al. 2011; Lu et al. 2018; Munir et al. 2016; Phoulivong et al. 2012). These factors impact disease management decisions, including the selection of fungicides and plant cultivars, resistance breeding, crop rotation, and even biosecurity quarantines (Cai et al. 2011; Talhinhas et al. 2018). When I began my study, there was limited knowledge regarding any of these factors in relation to *Colletotrichum* causing anthracnose diseases of small fruits in mixed-fruit orchards in Kentucky. I addressed this lack of information by thoroughly characterizing 117 small fruit *Colletotrichum* isolates collected from across the state. I used various methods to describe the strains, including morphotyping, species identification by molecular techniques, cross-inoculation potential, genome sequencing, and telomere fingerprinting.

Many publications have indicated the need for an improved taxonomic framework for the *Colletotrichum* genus. It has also been noted that morphological characters are generally unreliable for species identification due to their limited number and variability (Adaskaveg 1997; Afanador-Kafuri et al. 2003; Cai et al. 2009; Damm et al. 2012a; Du et al. 2005; Hyde et al. 2009; Phoulivong et al. 2010; Weir et al. 2012). My observations both corroborated and challenged this widely-held view. When I tested the effects of different growth conditions on culture morphology of *Colletotrichum*, I observed that morphology can change in response to growth medium, light, and temperature. Inconsistent conditions result in challenges regarding comparison of culture morphology descriptions across studies or to type specimens for identification because no standard growth conditions were established. However, when environmental conditions were controlled, the 117 isolates I studied consistently

grouped into seven different morphotypes: M1-M4 matched those described by Munir et al. (2016) on apples, while M5-M7 were novel morphotypes unique to small fruits.

I applied the standard recommended multigene analysis based on *GAPDH* and *TUB2* for identification of phylogenetic species within the *C. acutatum* and *C. gloeosporioides* complexes. I found that some species were represented by more than one morphotype, but that each morphotype included only a single species. All blueberry isolates (including M1, M5, and M6) were identified as *C. fioriniae*, while the vast majority of strawberry isolates (including M2 and M7) were identified as *C. nymphaeae*. Both of these species are within the *C. acutatum* species complex, which was accurately indicated by a species complex-specific PCR test based on the *ITS* sequence. A few other species were identified more rarely on strawberry, including *C. fioriniae* (M1), *C. siamense* (M3), and *C. fructicola* (M4). Again, the species complex-specific PCR assay was accurate and consistent with the multigene analysis for identification of *C. siamense* and *C. fructicola* as belonging to the *C. gloeosporioides* complex. These results showed that the morphotypes were consistent with molecular identifications, and that they could be used to predict phylogenetic species if cultures were grown under carefully controlled conditions.

For diagnostic purposes, it may be useful to have a single sequence that can accurately identify all of the relevant phylogenetic species within the *C. acutatum* and *C. gloeosporioides* clades. I addressed this question by comparing nine individual single gene trees to a tree based on SNP comparisons across whole genomes. The nine single genes were chosen because they had previously been applied to multigene phylogenetic analysis of *Colletotrichum*. Twenty-eight representative Kentucky fruit strains and several reference strains were included in each of the trees. Groups in the whole genome tree were generally consistent with morphology and *GAPDH* and *TUB2* sequences. However, I noted that the whole genome analysis provided better discrimination, as it clearly differentiated two of the novel morphotype groups (M6 and M7) from other morphotypes (M1 and M2, respectively) within the same phylogenetic species, thereby confirming that they are genetically distinct. Furthermore, the whole

genome tree revealed that the single isolate from strawberry identified by *GAPDH* sequence as *C. fructicola* did not group with other *C. fructicola* strains, but instead was similar to a reference isolate (GC 23, JGI, used with permission) belonging to an unknown species in the *C. gloeosporioides* complex. I observed that *CHS* for the *C. acutatum* complex and *ApMat* for *C. gloeosporioides* provided more accurate identifications, recapitulating the genome tree including the differentiation of variant morphotypes and novel species. Determining which single gene is the most accurate for species identification is likely to depend on the diversity of the *Colletotrichum* species tested. Genes suggested to be the most informative in the current literature may not be the best for a specific collection or geographic area. My study suggested that the use of single gene phylogenetic identification can be optimized for an isolate collection or location on a case-by-case basis. The single genes I identified here can be used to develop a PCR-based tool that is independent of sequencing, making it more suitable for diagnostic purposes.

The whole-genome analysis further reinforced the value of morphotype groups designated under controlled conditions. The *C. fioriniae* and *C. nymphaeae* subgroups in the genome tree were distinguished by their morphology. Further, across the whole tree at the species level, there were clear morphological distinctions that separated all species. Two other studies have also determined that morphological groups generally corresponded to phylogenetic species identifications (Munir et al. 2016; Prihastuti et al. 2009). The new variant morphotypes that I observed within the *C. fioriniae* and C*. nymphaeae* species should be further investigated to determine if they relate to variations in pathogenicity, fungicide sensitivity, or host preferences within the species. My study shows that the utility of *Colletotrichum* morphology should not be disregarded. Under controlled conditions and with prior phylogenetic knowledge, morphology can be employed as part of an accurate species identification tool to allow for simpler and quicker diagnosis. Taken together, my morphological, whole genome, and single gene phylogenetic results could be used to develop a simple yet robust *Colletotrichum* identification protocol that combines controlled and phylogenetically

informed morphotyping with a single gene-based PCR test for quick, accurate identification that can be used in applied fields.

Another important question that I addressed in my research was whether cross-infection occurs between small fruits and apples in Kentucky mixed-fruit orchards. The same four species that I identified on small fruits were also present on apple in Kentucky, and distribution comparison revealed that *C. fioriniae* was most common on apple and blueberry, whereas *C. nymphaeae* was the most frequent on strawberry. Cross-inoculation assays on apple, strawberry, and blueberry fruits revealed that all isolates were pathogenic on all three fruits, regardless of original host, but there were differences in aggressiveness in some cases. On apple, *C. gloeosporioides* was the most aggressive complex, and *C. siamense* was the most aggressive species. On strawberry, the difference between complexes was generally insignificant, and *C. siamense* and *C. nymphaeae* were both aggressive species. On blueberry, *C. acutatum* and *C. fioriniae* were most aggressive. Isolate host preference was more dependent upon species identification than on the original fruit host, suggesting a lack of individual isolate adaptation. Cross-infection may be more likely to occur between apple and blueberry because *C. fioriniae* is the most common species on both fruits. In contrast, even though apple and strawberry shared all the same species, the distributions were distinctly different, suggesting that cross-infection is less likely to occur between the fruits. My cross-inoculation studies also confirmed that *Colletotrichum* isolates have variable pathogenicity and cross-infection potential at a species level, supporting the need for correct species identification for management and increased epidemiological understanding.

I found that the species distributions I observed on apple, blueberry, and strawberry in the field did not predict the detached fruit cross-inoculation results. This indicates that there are additional environmental or biological factors involved in determining natural host range. Detached fruit inoculation is a common method used for testing cross-infection potential, host range, and isolate pathogenicity, but there are many limitations to this method. Fruits are usually wounded and inoculated under

controlled conditions. Under these optimal conditions, where potential environmental barriers are removed, certain species that do not cause disease under natural field conditions may be pathogenic, skewing host range concepts. For example, *C. fioriniae* was the only species found on blueberry from the field, but in my fruit inoculations, I found that all species tested caused disease on blueberry. *In vitro* fruit inoculations do not take into account environmental and pathogen behavior-related determinants of pathogenicity, like optimal temperature and overwintering site, which can affect success in the field and need to be considered when investigating cross-infection (Bernstein et al. 1995b; Peres et al. 2005). Continued research is needed in this area in order to address the issue of cross-infection more directly by expanding inoculation experiments to the field.

To further explore the incidence of cross-infection, I tested Southern blot telomere fingerprinting as a method for clone identification, using *C. fioriniae* as my experimental subject because it was the most common species identified. Four groups of *C. fioriniae* strains were analyzed by using telomere fingerprinting. The control group consisted of geographically diverse *C. fioriniae* strains from a wide range of hosts. Each strain had a distinct telomere fingerprint, confirming that the telomere fingerprinting method had sufficient intraspecies resolution capability for use in clone detection. The other three groups of strains were each collected from diseased apple, blueberry, and strawberry fruits from different mixed-fruit orchards near Lexington, KY, between 2013 and 2018. The Orchard 1 group included apple, blueberry, and strawberry *C. fioriniae* strains. Putative clones with identical or nearly-identical telomere fingerprints were identified on apple and blueberry. The Orchard 2 group included apple and strawberry strains, and clones were identified on both fruits. The Orchard 3 group included apple and blueberry strains, and clones were also identified on both fruits. Importantly, though clones were detected within the same fruit type in all three orchards, no clones were present on different fruit types in any of the orchards. This suggests a lack of cross-infection of *C. fioriniae* strains among these fruits. Even though cross-infection was not detected, the analysis was informative because it showed differences in strain diversity

among the apple, blueberry, and strawberry *C. fioriniae* populations. Thus, the apple population was more diverse, whereas the blueberry population included more clones. This may suggest that differential disease management has an effect on pathogen populations. Another interesting observation was that clones of a single strain were recovered across multiple years, indicating that strains persist within an orchard from year to year. It is important to point out that only a small number of strains were tested within each orchard, so additional experiments should be performed with an expanded strain collection to strengthen the conclusion that cross-infection does not occur. Considering my cross-inoculation and telomere fingerprinting results together, cross-infection among fruits is a complex disease issue that warrants further study.

Future work on *Colletotrichum* from small fruit and mixed-fruit orchards should prioritize increasing the small fruit strain collection and developing a consolidated species identification protocol. Additional sampling of small fruit anthracnose diseases across the state should be conducted, including collection data such as geographic coordinates, date, and specified fruit lesion. The basic morphotyping protocol I established should become standard practice, and continued morphological observation of the growing strain collection can be used to reinforce and refine the morphotypic scheme. As suggested previously, morphotypes should also be further connected with phylogenetic species identifications. Starting with the single genes I recommended, it may also be highly beneficial to develop primer sets and PCR protocols that do not require sequencing for more rapid species identifications. Combining improved morphotyping with PCR-based identification will create a powerful tool for anthracnose fruit rot diagnosis and management. One possibility for an interesting future experiment would be the release of a known clone in an orchard in order to track its movement based on telomere fingerprint identities of recovered strains.

In conclusion, my thesis research has significantly advanced our knowledge of the *Colletotrichum* spp. that cause anthracnose fruit rot diseases in Kentucky. It also established a starting point for the development of a morphological and sequence-based species identification tool to be used in a diagnostic setting. With this knowledge

and potential diagnostic tool, anthracnose disease management strategies can be improved for the benefit of Kentucky fruit growers.

APPENDICES

Appendix 1. First report of bitter rot of apple caused by a *Colletotrichum* sp. in the *C. kahawae* clade in Kentucky[1]

Apple bitter rot causes average annual yield losses of 30% in Kentucky, with individual losses as high as 100% in some orchards (Gauthier et al. 2017). Five *Colletotrichum* spp. were previously identified as causal agents of bitter rot in KY: *C. fioriniae* and *C. nymphaeae* in the *C. acutatum* species complex; and *C. siamense*, *C. fructicola*, and *C. theobromicola* in the *C. gloeosporioides* complex (Munir et al. 2016). Three of these species, *C. fioriniae, C. siamense*, and *C. fructicola*, have also been reported causing bitter rot in other states (Chechi et al. 2019; Kou et al. 2014). It is important to know which species are present in an orchard because they vary in pathogenicity and fungicide sensitivity (Munir et al. 2016; Chechi et al. 2019). A sixth *Colletotrichum* species was isolated in 2013 from typical bitter rot lesions on 'Honeycrisp' apples in a commercial orchard in Bourbon County, KY. Six isolates were collected from two apples on the same tree and single-spored for further study. Colonies were smooth and light to dark gray on top with a light orange border, and dark brown to black with an orange border on the reverse when grown on potato dextrose agar (PDA) at 23^{o}C with constant light. Conidia of two representative isolates were harvested from ten-day-old PDA plates. Conidia were hyaline and cylindrical with rounded ends, with some narrowing slightly at the base or center. Spore sizes for the two strains were (15.1-) 17.3 to 22.3 (-28.9) by (4.8-) 5.1 to 6.5 (-6.8) µm, and (14.7-) 15.9 to 20.9 (-21.4) by (4.9-) 5.3 to 7.1 (-7.5) µm. Hyphopodia on potato-carrot agar varied from rounded and smooth to oval with small midpoint lobes. Pathogenicity of

[1] Previously published

McCulloch, M. J., Gauthier, N. W., and Vaillancourt, L. J. 2020. First report of bitter rot of apple caused by a *Colletotrichum* sp. in the *C. kahawae* clade in Kentucky. Plant Dis 104:289.

two representative isolates was confirmed in detached fruit assays. 'Fuji' apples were surface sterilized, wound-inoculated with a spore suspension (1 x 10^5 spores/mL), and placed in humidity chambers for two weeks. Typical bitter rot lesions resulted from inoculation with the two apple isolates, but not from negative control treatments that consisted of mock-inoculated fruit, or fruit inoculated with *C. graminicola*, which is pathogenic to maize but not apples. The morphology of the fungus recovered from the inoculated apples matched the original strains, fulfilling Koch's postulates. Sequences of seven genes were used for species identification: actin (*ACT*); partial mating type protein 1-2-1 gene and Apn2-Mat1-2 intergenic spacer (*ApMat*); calmodulin (*CAL*); glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*); glutamine synthetase (*GS*), internal transcribed spacer region (*ITS*); and beta-tubulin 2 (*TUB2*) (Liu et al. 2015; Weir et al. 2012) (see Figure A1.2 for accession numbers). Individual NCBI and Q-bank blast reports indicated conflicting species identities, but all were in the *C. kahawae* clade of the *C. gloeosporioides* species complex. Phylogenetic trees were generated from concatenated multigene sequences using the method of Liu et al. (2015) and Weir et al. (2012). Trees using all sequences except *ApMat*, or only *ApMat* and *GS* (Liu et al. 2015), confirmed a close affinity of the unknown apple isolates with *C. kahawae*, but could not assign them to an identified species within the clade. *Colletotrichum kahawae* is the only member of this clade that has previously been reported to cause bitter rot, in a single study from Belgium (Grammen et al. 2019). That strain differed in pathogenicity and fungicide sensitivity from other bitter rot strains in the same study. Given the strong support for distinction within the trees, the isolates from KY may represent a new species, but more research is necessary to determine if that status is warranted. Meanwhile, it is important to publish this report because all previously identified bitter rot pathogens in the U.S. are only distantly related to members of the *C. kahawae* clade. Thus, the response of these strains to current bitter rot management regimes is unpredictable, and requires further study.

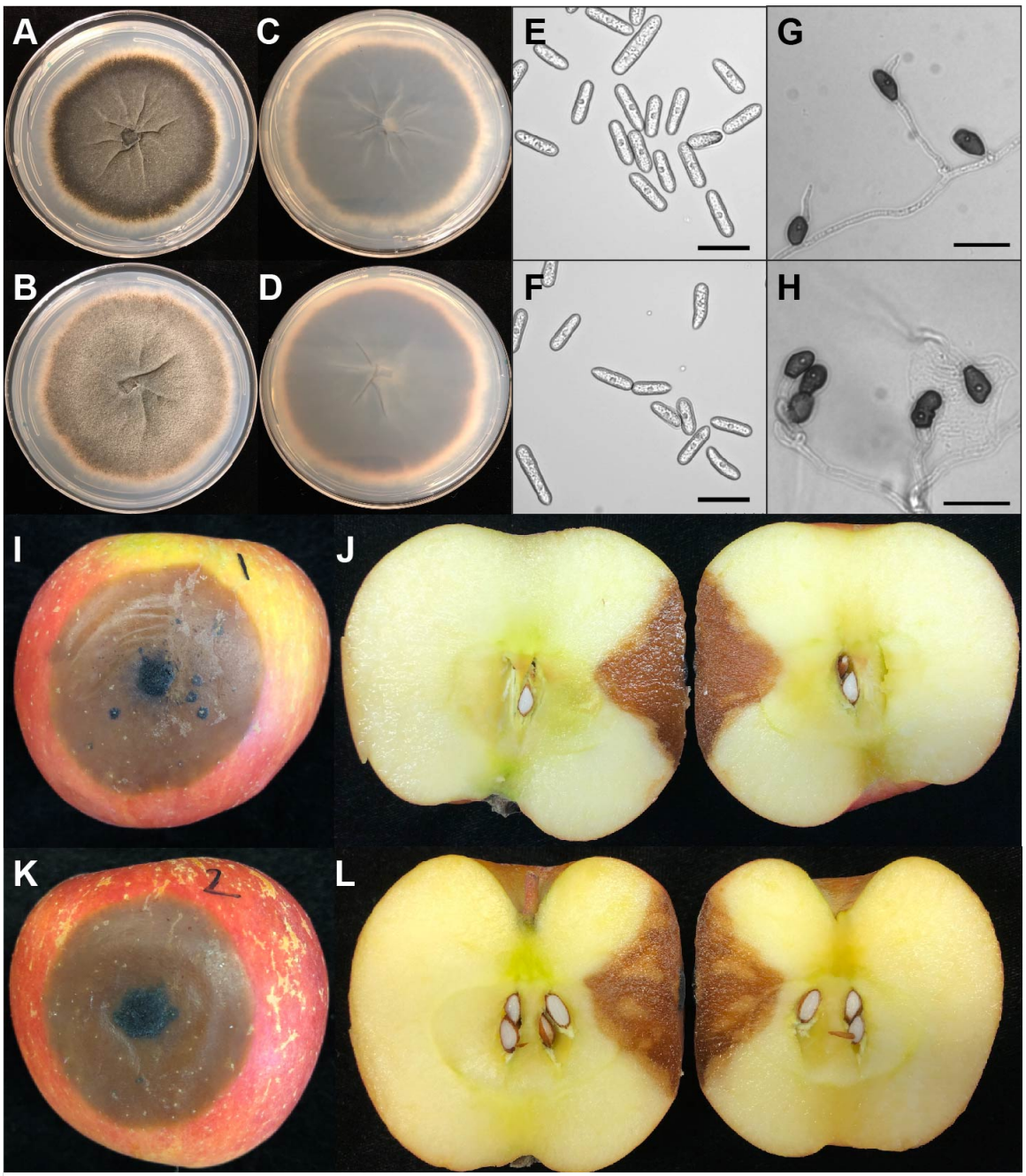


Figure A1.1 Colony, conidium, hyphopodium, and lesion morphology of two unknown *Colletotrichum kahawae* clade isolates from apple. (A, B) Upper colony surface on potato dextrose agar (PDA); (C, D) colony reverse on PDA; (E, F) morphology of conidia recovered from PDA; (G, H) hyphopodia produced on potato-carrot agar. (A, C, E, G) Isolate HC278; (B, D, F, H) isolate HC292. Scale bar is 20 µm. (I-L) Typical bitter rot lesions produced on two-week-old 'Fuji' apples inoculated with isolates HC278 (I, J) and HC292 (K, L).

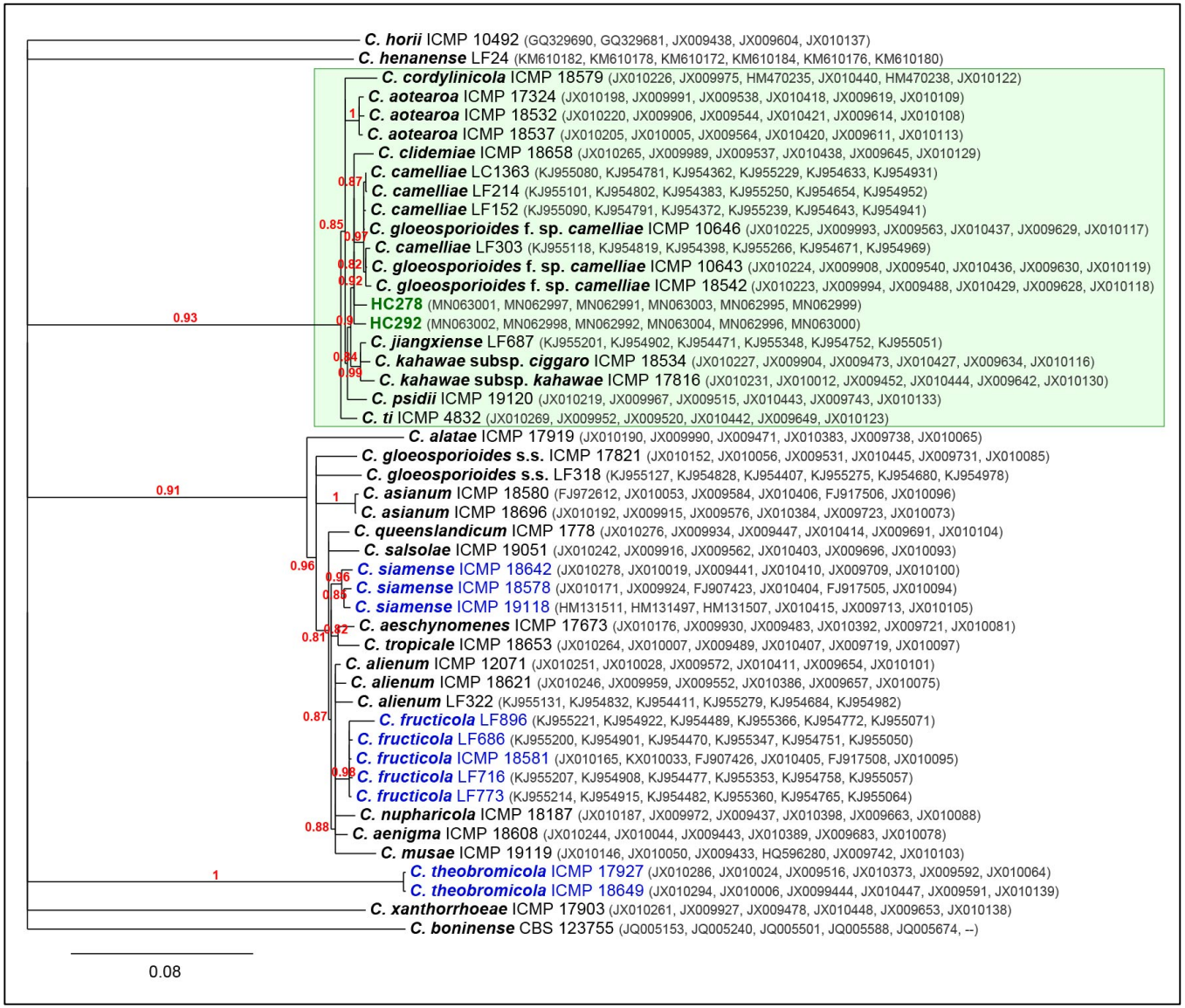

C. horii ICMP 10492 (GQ329690, GQ329681, JX009438, JX009604, JX010137)
C. henanense LF24 (KM610182, KM610178, KM610172, KM610184, KM610176, KM610180)
C. cordylinicola ICMP 18579 (JX010226, JX009975, HM470235, JX010440, HM470238, JX010122)
C. aotearoa ICMP 17324 (JX010198, JX009991, JX009538, JX010418, JX009619, JX010109)
C. aotearoa ICMP 18532 (JX010220, JX009906, JX009544, JX010421, JX009614, JX010108)
C. aotearoa ICMP 18537 (JX010205, JX010005, JX009564, JX010420, JX009611, JX010113)
C. clidemiae ICMP 18658 (JX010265, JX009989, JX009537, JX010438, JX009645, JX010129)
C. camelliae LC1363 (KJ955080, KJ954781, KJ954362, KJ955229, KJ954633, KJ954931)
C. camelliae LF214 (KJ955101, KJ954802, KJ954383, KJ955250, KJ954654, KJ954952)
C. camelliae LF152 (KJ955090, KJ954791, KJ954372, KJ955239, KJ954643, KJ954941)
C. gloeosporioides f. sp. camelliae ICMP 10646 (JX010225, JX009993, JX009563, JX010437, JX009629, JX010117)
C. camelliae LF303 (KJ955118, KJ954819, KJ954398, KJ955266, KJ954671, KJ954969)
C. gloeosporioides f. sp. camelliae ICMP 10643 (JX010224, JX009908, JX009540, JX010436, JX009630, JX010119)
C. gloeosporioides f. sp. camelliae ICMP 18542 (JX010223, JX009994, JX009488, JX010429, JX009628, JX010118)
HC278 (MN063001, MN062997, MN062991, MN063003, MN062995, MN062999)
HC292 (MN063002, MN062998, MN062992, MN063004, MN062996, MN063000)
C. jiangxiense LF687 (KJ955201, KJ954902, KJ954471, KJ955348, KJ954752, KJ955051)
C. kahawae subsp. ciggaro ICMP 18534 (JX010227, JX009904, JX009473, JX010427, JX009634, JX010116)
C. kahawae subsp. kahawae ICMP 17816 (JX010231, JX010012, JX009452, JX010444, JX009642, JX010130)
C. psidii ICMP 19120 (JX010219, JX009967, JX009515, JX010443, JX009743, JX010133)
C. ti ICMP 4832 (JX010269, JX009952, JX009520, JX010442, JX009649, JX010123)
C. alatae ICMP 17919 (JX010190, JX009990, JX009471, JX010383, JX009738, JX010065)
C. gloeosporioides s.s. ICMP 17821 (JX010152, JX010056, JX009531, JX010445, JX009731, JX010085)
C. gloeosporioides s.s. LF318 (KJ955127, KJ954828, KJ954407, KJ955275, KJ954680, KJ954978)
C. asianum ICMP 18580 (FJ972612, JX010053, JX009584, JX010406, FJ917506, JX010096)
C. asianum ICMP 18696 (JX010192, JX009915, JX009576, JX010384, JX009723, JX010073)
C. queenslandicum ICMP 1778 (JX010276, JX009934, JX009447, JX010414, JX009691, JX010104)
C. salsolae ICMP 19051 (JX010242, JX009916, JX009562, JX010403, JX009696, JX010093)
C. siamense ICMP 18642 (JX010278, JX010019, JX009441, JX010410, JX009709, JX010100)
C. siamense ICMP 18578 (JX010171, JX009924, FJ907423, JX010404, FJ917505, JX010094)
C. siamense ICMP 19118 (HM131511, HM131497, HM131507, JX010415, JX009713, JX010105)
C. aeschynomenes ICMP 17673 (JX010176, JX009930, JX009483, JX010392, JX009721, JX010081)
C. tropicale ICMP 18653 (JX010264, JX010007, JX009489, JX010407, JX009719, JX010097)
C. alienum ICMP 12071 (JX010251, JX010028, JX009572, JX010411, JX009654, JX010101)
C. alienum ICMP 18621 (JX010246, JX009959, JX009552, JX010386, JX009657, JX010075)
C. alienum LF322 (KJ955131, KJ954832, KJ954411, KJ955279, KJ954684, KJ954982)
C. fructicola LF896 (KJ955221, KJ954922, KJ954489, KJ955366, KJ954772, KJ955071)
C. fructicola LF686 (KJ955200, KJ954901, KJ954470, KJ955347, KJ954751, KJ955050)
C. fructicola ICMP 18581 (JX010165, KX010033, FJ907426, JX010405, FJ917508, JX010095)
C. fructicola LF716 (KJ955207, KJ954908, KJ954477, KJ955353, KJ954758, KJ955057)
C. fructicola LF773 (KJ955214, KJ954915, KJ954482, KJ955360, KJ954765, KJ955064)
C. nupharicola ICMP 18187 (JX010187, JX009972, JX009437, JX010398, JX009663, JX010088)
C. aenigma ICMP 18608 (JX010244, JX010044, JX009443, JX010389, JX009683, JX010078)
C. musae ICMP 19119 (JX010146, JX010050, JX009433, HQ596280, JX009742, JX010103)
C. theobromicola ICMP 17927 (JX010286, JX010024, JX009516, JX010373, JX009592, JX010064)
C. theobromicola ICMP 18649 (JX010294, JX010006, JX0099444, JX010447, JX009591, JX010139)
C. xanthorrhoeae ICMP 17903 (JX010261, JX009927, JX009478, JX010448, JX009653, JX010138)
C. boninense CBS 123755 (JQ005153, JQ005240, JQ005501, JQ005588, JQ005674, --)
0.08

Figure A1.2 PhyML Approximate Likelihood-Ratio Test tree of concatenated *ITS, GAPDH, ACT, TUB2, CAL,* and *GS* alignments indicating phylogenetic affinities for reference *Colletotrichum* spp. and the unknown apple *Colletotrichum* isolates HC278 and HC 292 (green font). Accession numbers are in gray in the gene order *ITS, GAPDH, ACT, TUB2, CAL, GS*. Missing genes are denoted by "—". *Colletotrichum boninense* was used as the outgroup. Bootstrap values are shown in red font. Previously reported *Colletotrichum* spp. on apple in KY are indicated in blue font. The *C. kahawae* clade is highlighted by the green box. The scale bar indicates 0.08 expected changes per site.

Appendix 2. Some notes on the development of the detached fruit inoculation assays

Apple fruit inoculations

The protocol for the apple fruit inoculations was worked out previously (Munir et al. 2016) and had already been optimized and extensively tested. Either organic store-bought, non-waxed apples (best results were obtained with apples purchased from Trader Joes), or apples from the University of Kentucky Horticulture Research Farm (South Farm), were used. One issue with apples from the UK Horticulture Research Farm was the presence of natural infection: sometimes lesions formed on the apples in addition to the ones that were produced by inoculation. Under those circumstances, measurements were still possible, as long as the lesions did not coalesce. The cultivars that typically gave the best results were 'Honeycrisp', 'Golden Delicious', and 'Fuji'. The cultivar 'Gala' can also be used, but it was less susceptible to infection. A cultivar called 'Jazz' was nearly immune and should not be used for the assays. It is best to use apples in season, and avoid the use of under-ripe fruit to get the best lesion formation (Shi et al. 1995). Thick rubber bands can be placed around the bases of apples to keep them from rolling or shifting; this is particularly useful during and after inoculation, until they can be placed upright the following day.

Strawberry fruit inoculations

The major difficulty with the strawberry inoculations was contamination with other fungi. Store-bought berries purchased in season held up best. Berries that were slightly under-ripe were more resilient and had fewer secondary infections. I always tried to purchase the most unripe berries available (white or green colored), but unfortunately most of the berries for sale were already ripe. I tried both organic and conventional berries, and I found that the contamination was equally prevalent for both; thus, I utilized conventional berries in my experiments because they were more cost-effective. I tried to avoid berries with dark, diffuse, slightly sunken spots that appeared to be related to *Botrytis* infection. Excess berries were needed because decay fungi were common in the moist chambers, and I wanted to ensure that there were

enough intact ones at the end of the assays. In general, approximately three times more berries than needed were inoculated. However, even when these precautions were taken, and even though strawberries were surface-disinfested with bleach, there was a high level (70%) of *Botrytis* and *Rhizopus* contamination. Contaminated berries were discarded immediately, but sometimes I still failed to get enough replications for statistical analysis. UV-C light treatment has been reported to reduce contamination in strawberry fruit (Janisiewicz et al. 2016). However, when store-bought berries were treated with UV light prior to inoculation in one trial, they did not show a decrease in the level of contamination. UV treatment may only be effective when applied to whole plants, or the source of UV light we have in the lab may not be sufficient for the purpose. For future strawberry assays, I suggest growing the berries in the greenhouse, regularly treating them with contact fungicides to keep them as pathogen-free as possible, and then harvesting and inoculating them before they are fully ripe.

Blueberry fruit inoculations

A standard inoculation protocol for blueberries had not been developed for the lab, so I developed one based on a published report (Miles et al. 2012). I tested two different conidia concentrations, and inoculation with and without wounding. Based on my observations, wound-inoculating with the lower spore concentration (5×10^5 spores per mL) seemed to give a higher disease incidence (Figure A2.1). Source of berries should also be considered. For most of my experiments, blueberries purchased in-season from a local U-Pick orchard were used. However, levels of natural infection were often high (40%). In Kentucky, ripe rot of blueberry, a storage rot, is not a disease of management concern. Berries are sold and consumed fresh, and long-term storage is not a factor. Therefore, growers do not manage this disease with fungicides or cultural practices. The high levels of natural infection skewed the control ratings and made statistical analysis complicated. Aging of blueberries prior to inoculation was also evaluated with U-Pick berries. The first trial was conducted a few days after harvesting, and the second trial was conducted after three weeks of storage in a 4°C cold room. The

results from the second aged-berry trial were better than the first trial because there was a higher level of disease incidence and the control ratings were lower. This was probably because, after three weeks of aging, I was able to identify and discard most berries with natural infection. I did one test to evaluate the performance of organic or conventionally-grown blueberries obtained from the local grocery store. In my test, the organic store-bought blueberries worked best. The control berries were clean, and disease incidence level was high for the *C. fioriniae* inoculated berries (Figure A2.2). However, in other trials that had previously been done in the lab with store-bought berries, infection levels varied, and sometimes the berries didn't become infected at all. It is possible the berries were sprayed with fungicides, pre-treated for storage, or were a resistant cultivar. More trials are needed to decide which type of store-bought blueberries yields the best inoculation results.

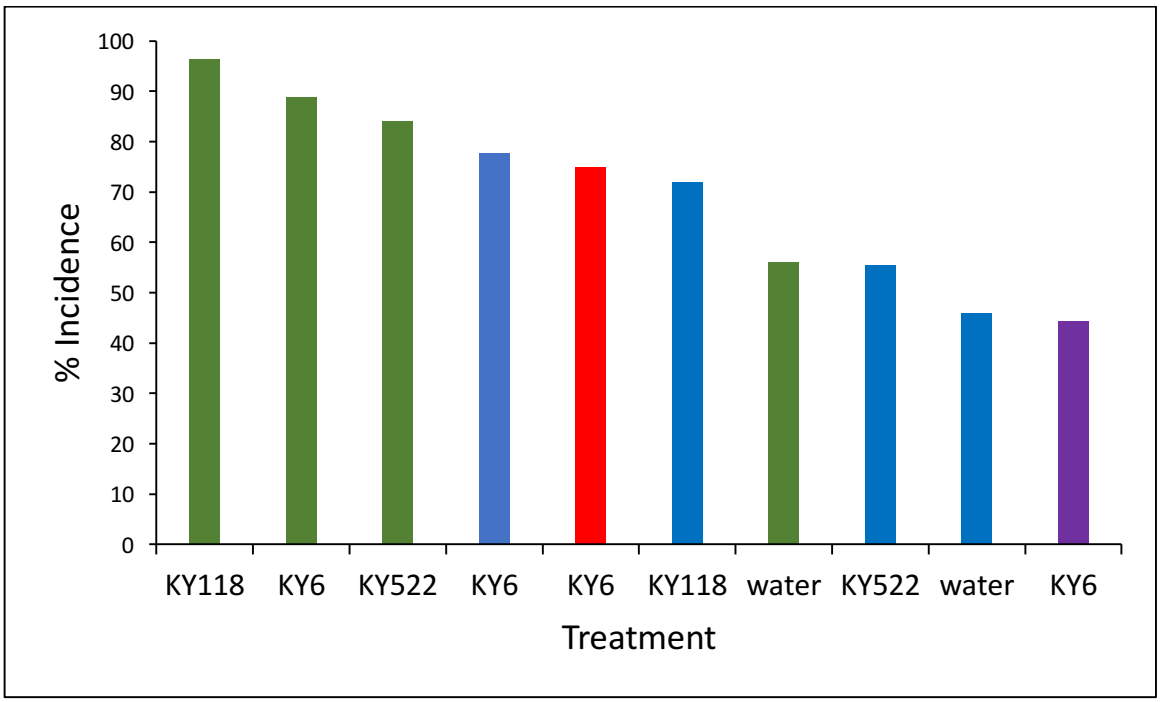


Figure A2.1 Ripe rot disease incidence at 10 dpi for select treatments from Trial 1 *Colletotrichum* inoculation of U-Pick blueberries. KY118 and KY6 are *C. fioriniae*; KY522 is *C. nymphaeae*. Green = wound inoculated with 5 x 10^5 spores/mL; blue = non-wound inoculated with 5 x 10^5 spores/mL; red = wound inoculated with 5 x 10^6 spores/mL; purple = non-wound inoculated with 5 x 10^6 spores/mL.

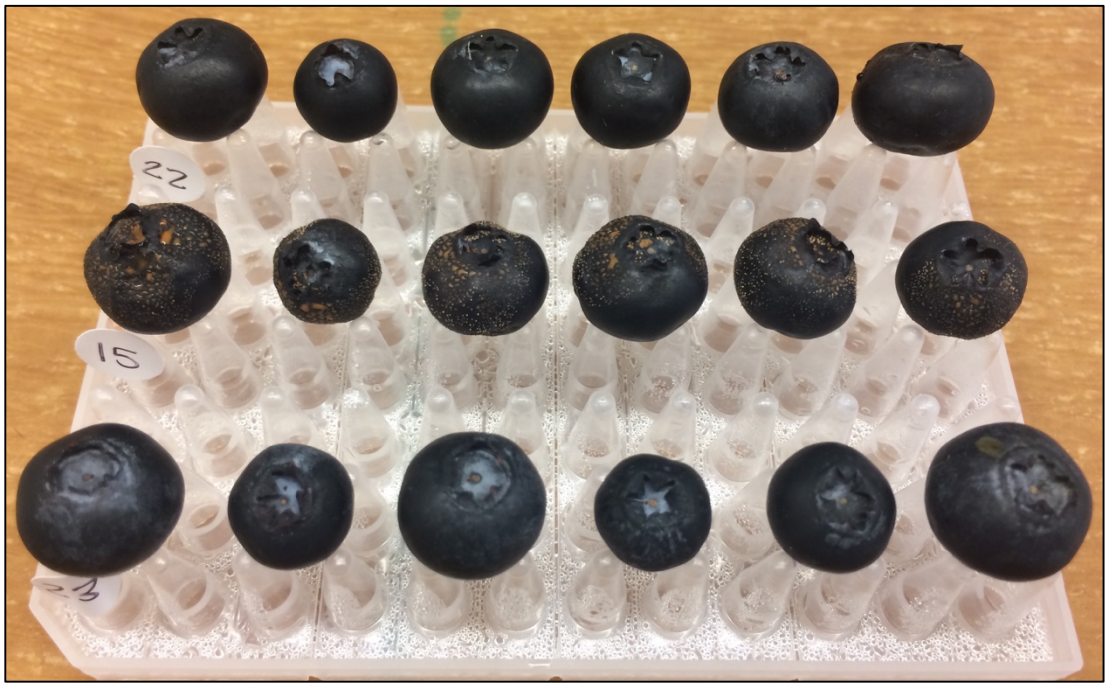


Figure A2.2 Representative image of the *Colletotrichum* inoculation trial using organic store-bought blueberries. The front row is the water control, the back row is the *Colletotrichum graminicola* negative control, and the middle row is inoculated with *Colletotrichum fioriniae*.

Appendix 3. Morphological types of representative isolates

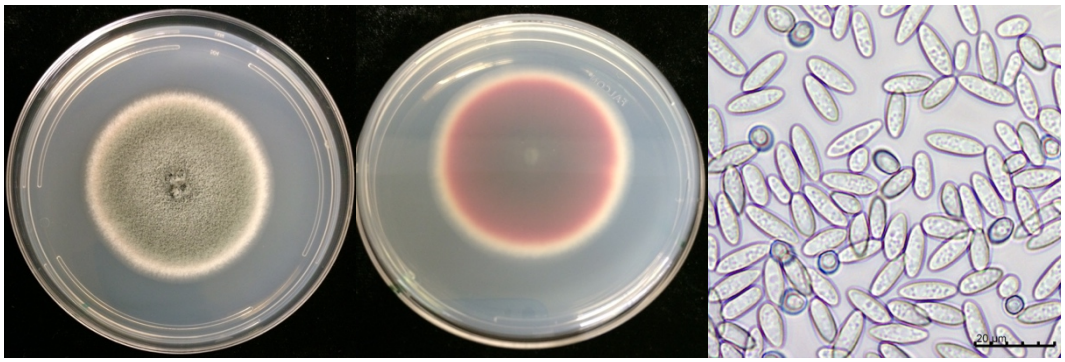
Table A3.1 Morphotypes and species identifications of representative Kentucky *Colletotrichum* isolates

Isolate	Host	Morphotype	Species
KY 6	Apple	1	*C. fioriniae*
KY 118	Blueberry	1	*C. fioriniae*
KY 640	Blueberry	1	*C. fioriniae*
KY 655	Blueberry	1	*C. fioriniae*
KY 615	Strawberry	1	*C. fioriniae*
HC 646	Apple	2	*C. nymphaeae*
KY 522	Strawberry	2	*C. nymphaeae*
KY 563	Strawberry	2	*C. nymphaeae*
KY 567	Strawberry	2	*C. nymphaeae*
KY 8	Apple	3	*C. siamense*
KY 254	Apple	3	*C. siamense*
KY 687	Strawberry	3	*C. siamense*
KY 748	Strawberry	3	*C. siamense*
KY 153	Apple	3	*C. theobromicola*
HC 540	Apple	4	*C. fructicola*
KY 332	Strawberry	4	*C. fructicola*[z]
KY 116	Blueberry	5	*C. fioriniae*
KY 657	Blueberry	5	*C. fioriniae*
KY 648	Blueberry	6	*C. fioriniae*
KY 646	Blueberry	6	*C. fioriniae*
KY 613	Strawberry	7	*C. nymphaeae*
KY 745	Strawberry	7	*C. nymphaeae*
HC 278	Apple	8	*C. kahawae* clade
HC 292	Apple	8	*C. kahawae clade*

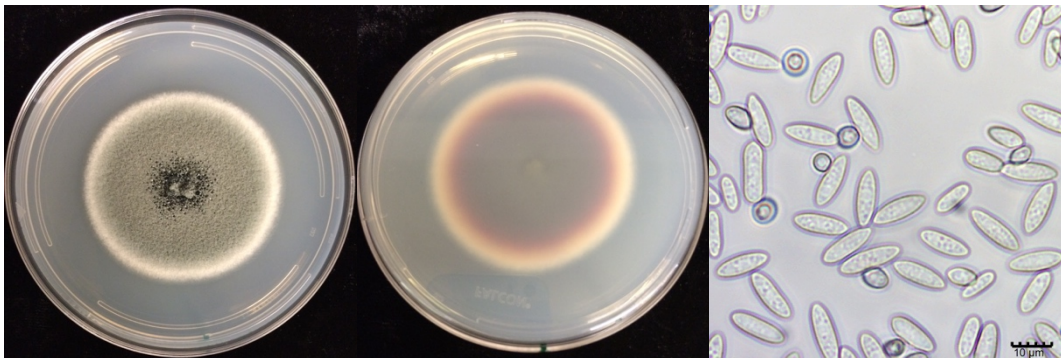
[z] Identified as *C. fructicola* based on *GAPDH* sequence alone and *C. gloeosporioides* unknown species by genome sequencing.

Morphotype 1

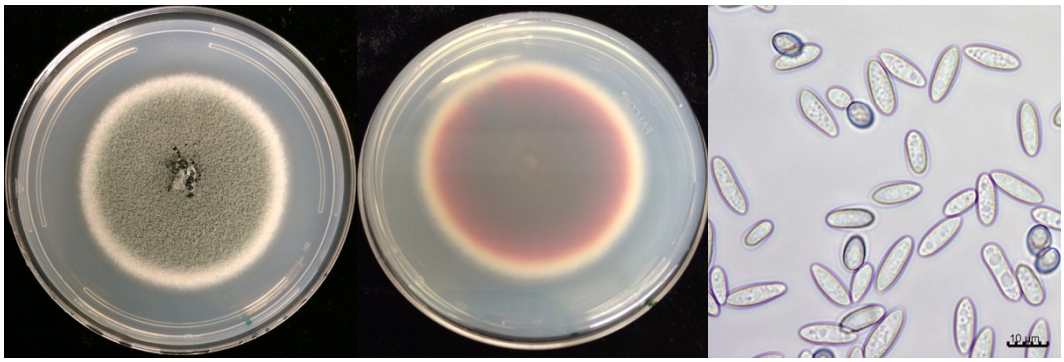
KY 6



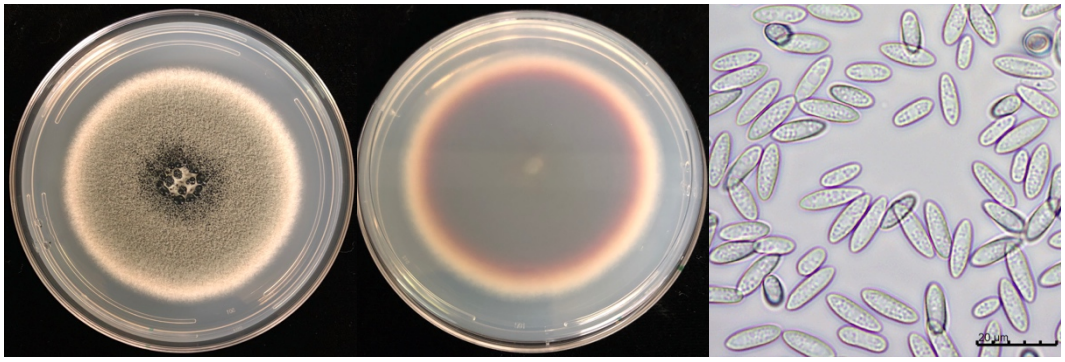
KY 118



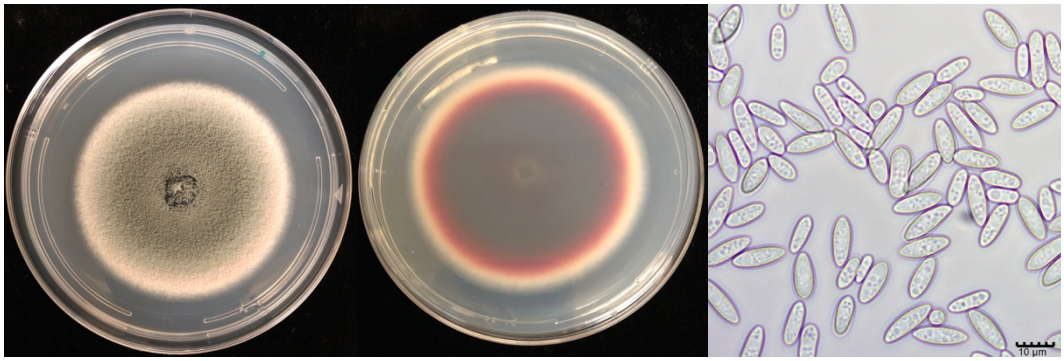
KY 640



KY 655

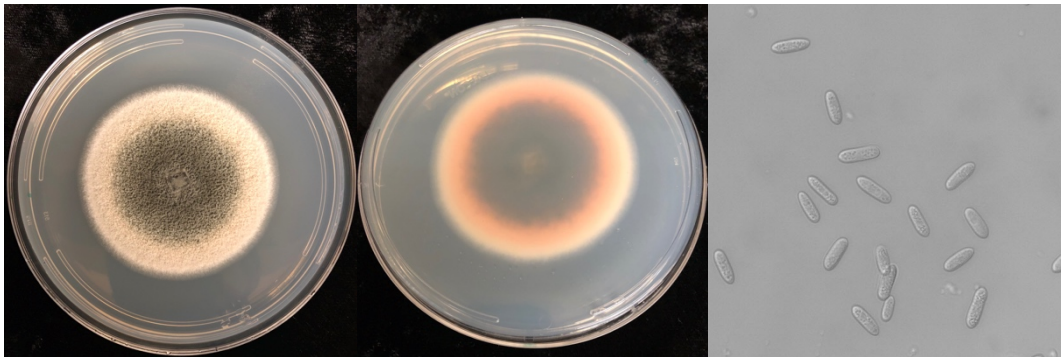


KY 615

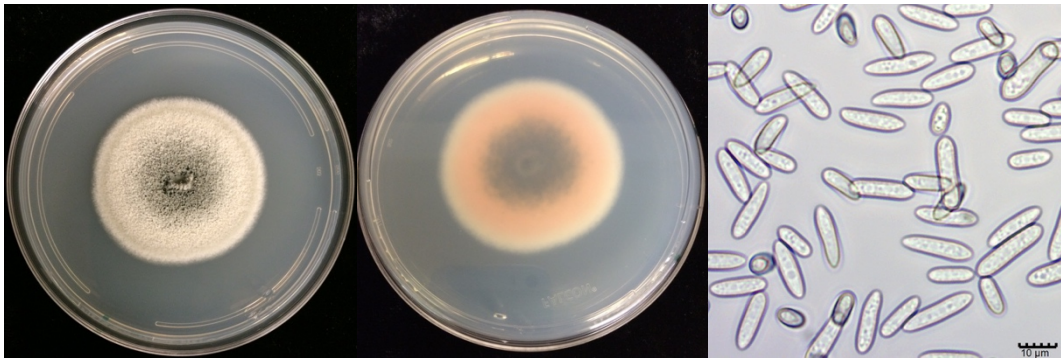


Morphotype 2

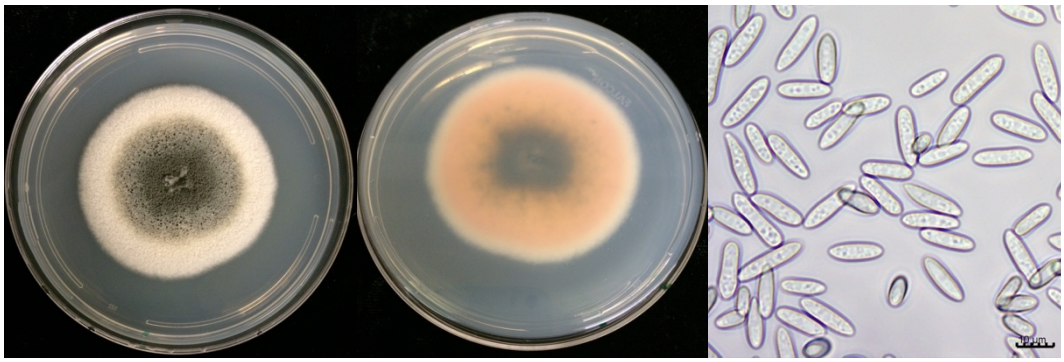
HC 646



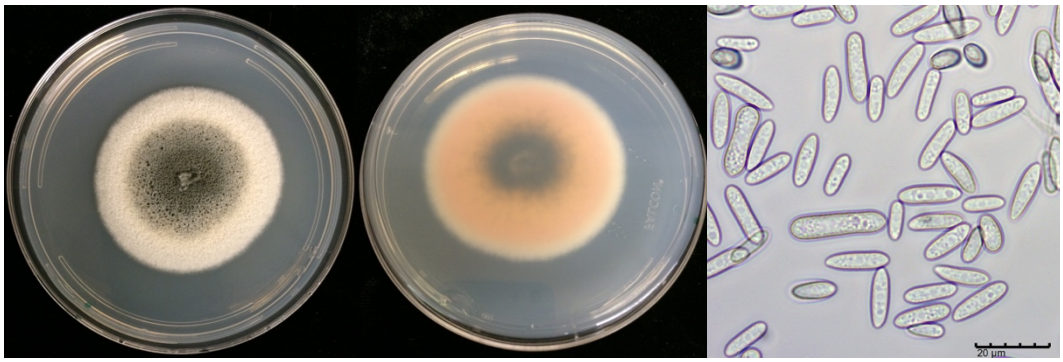
KY 522



KY 563

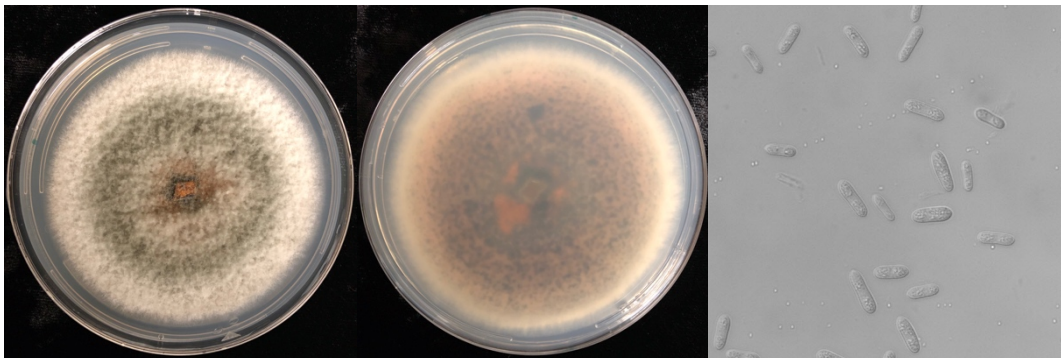


KY 567



Morphotype 3

KY 8



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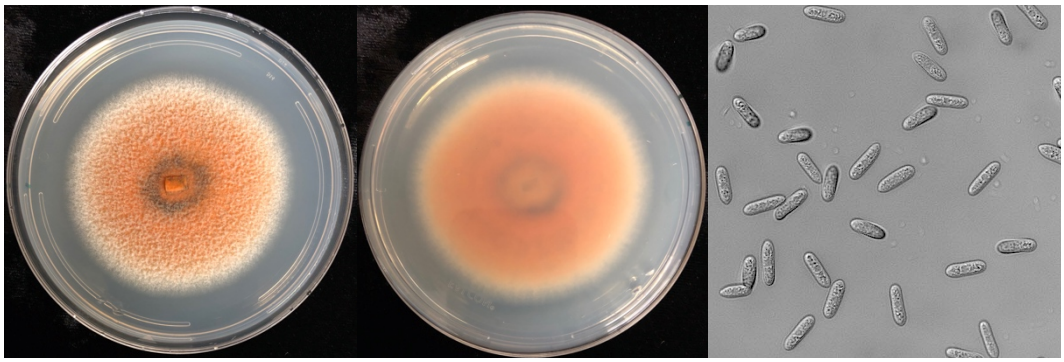
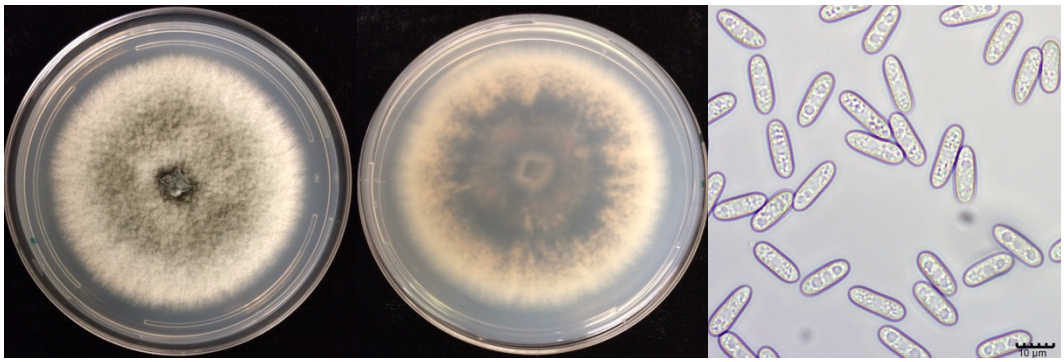
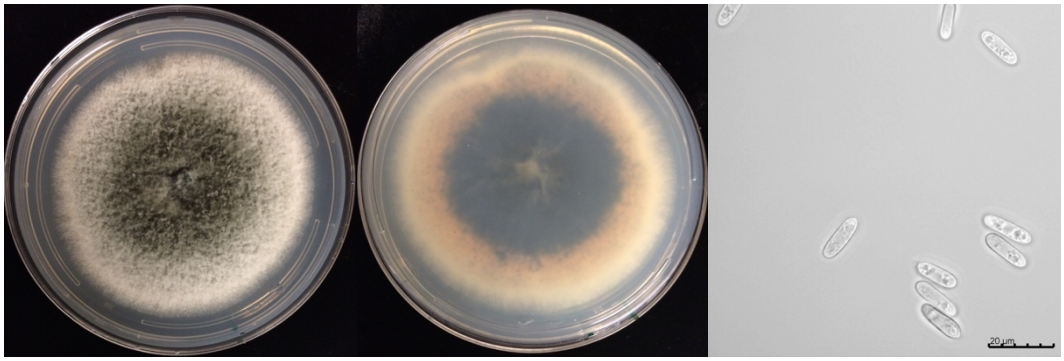


KY 687



KY 748



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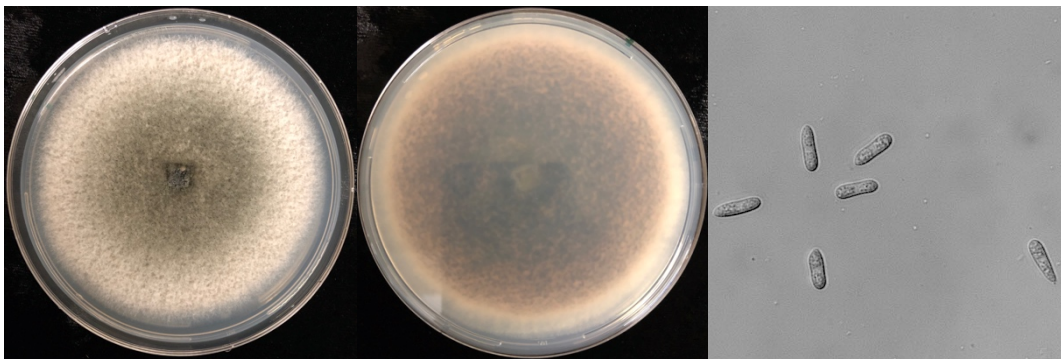
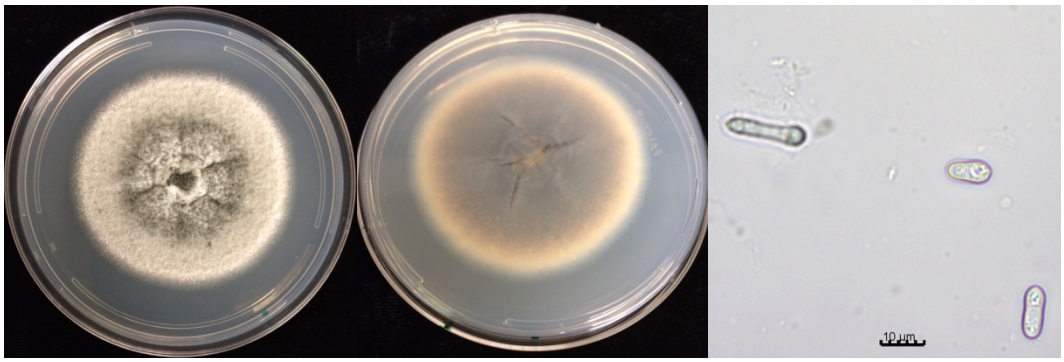
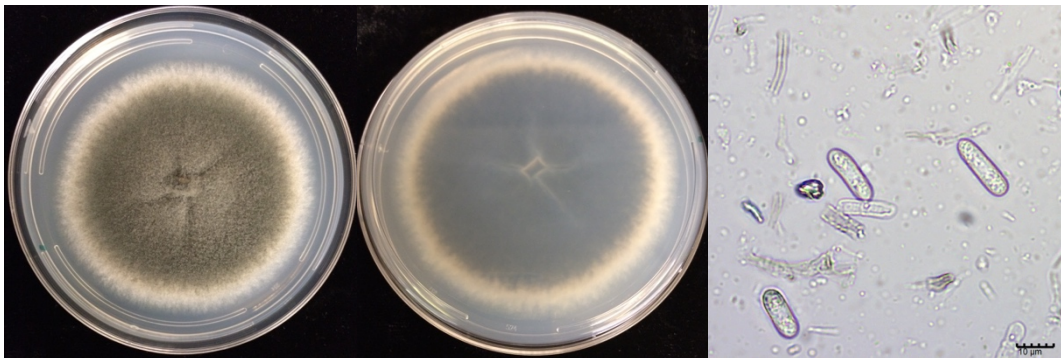


Morphotype 4

HC 540

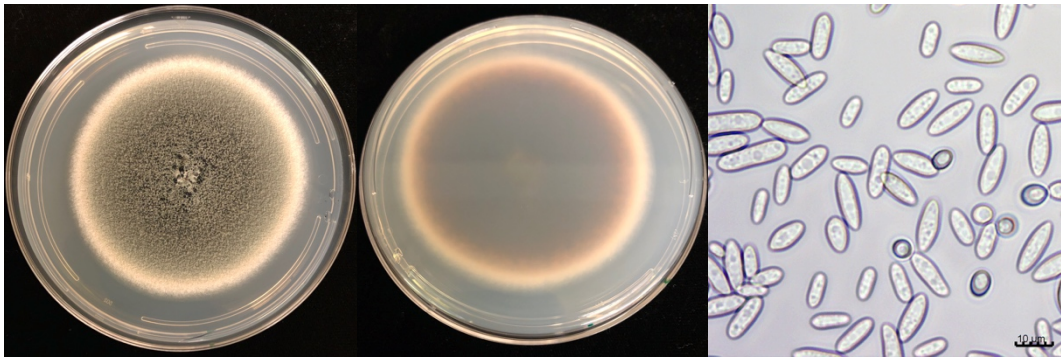


KY 332

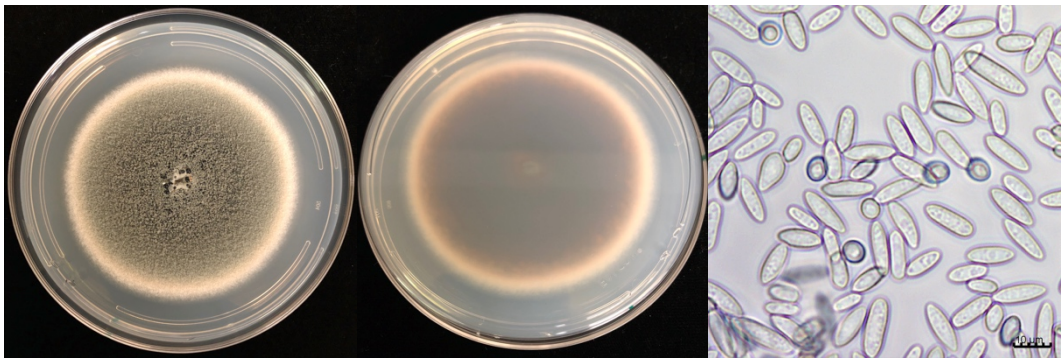


Morphotype 5

KY 116

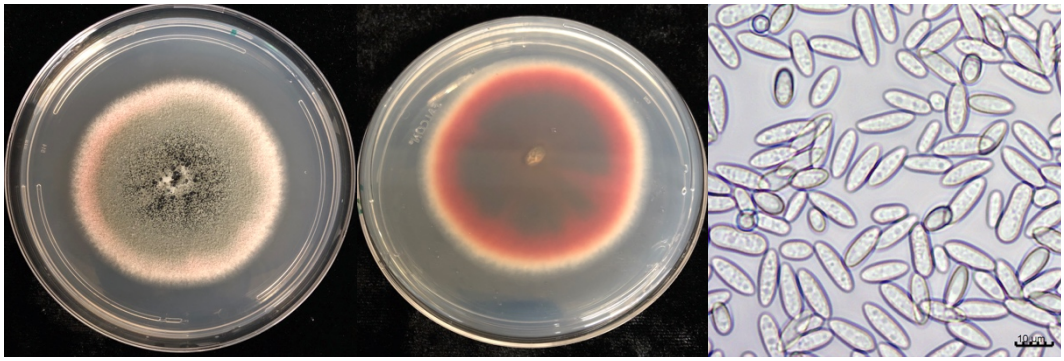


KY 657

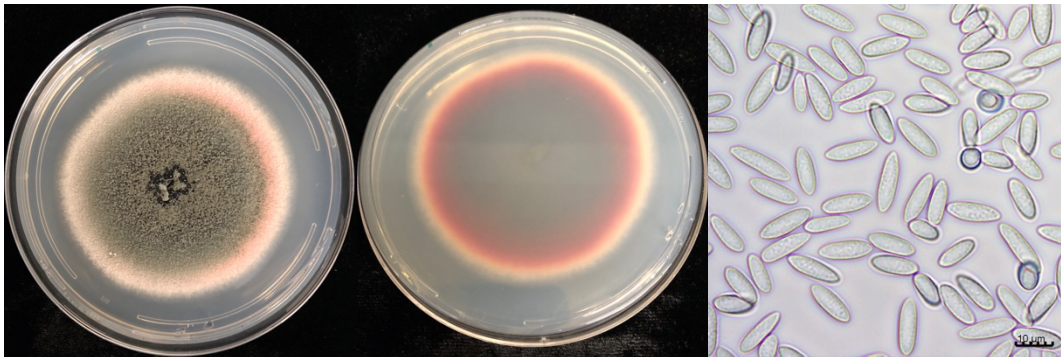


Morphotype 6

KY 646

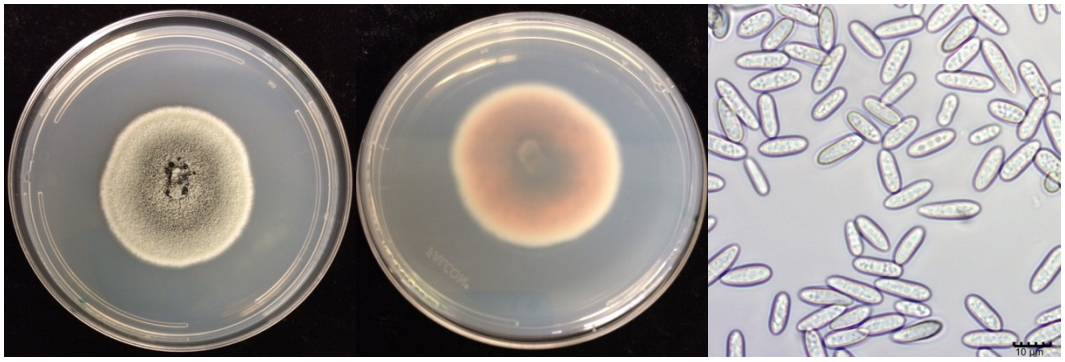


KY 648

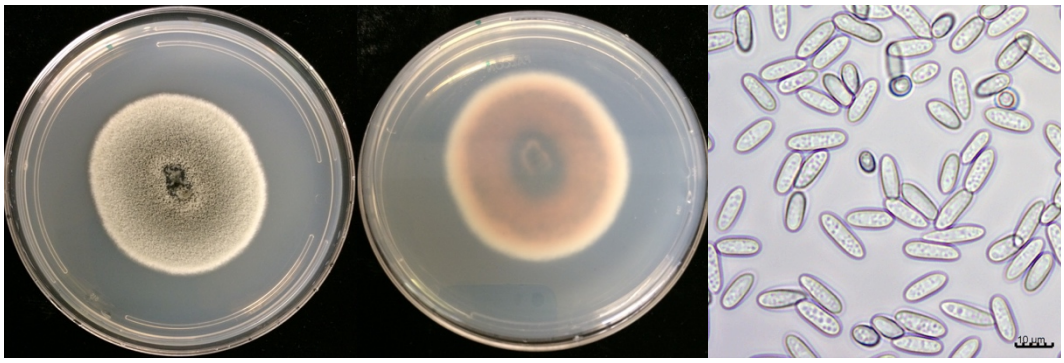


Morphotype 7

KY 613

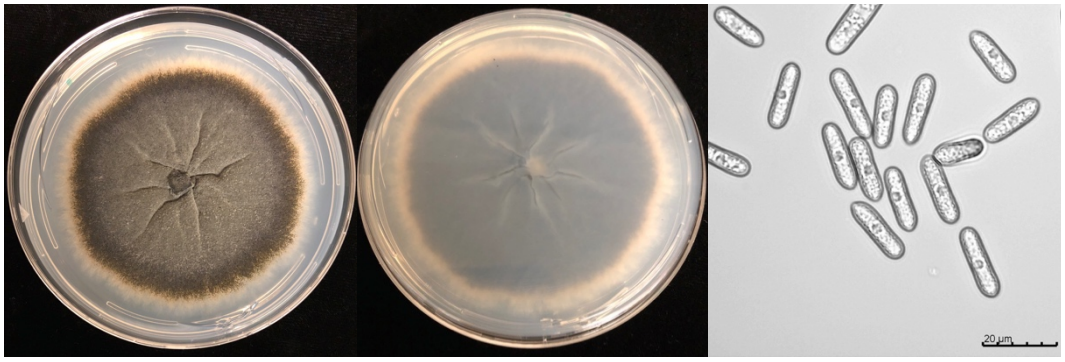


KY 745

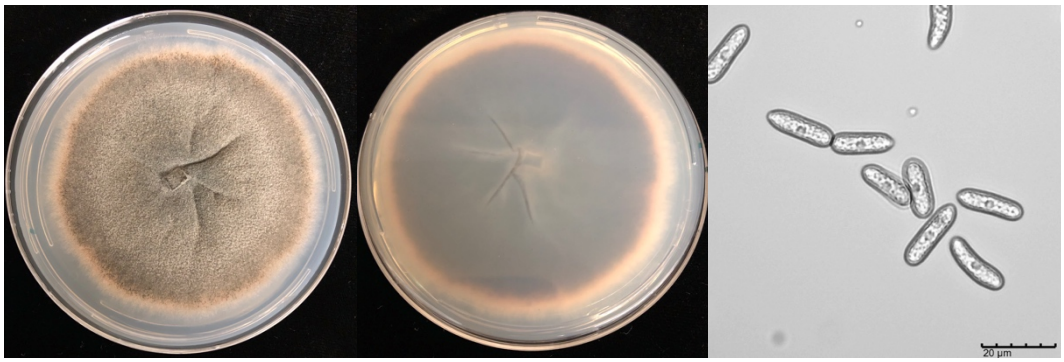


Morphotype 8

HC 278



HC 292



Appendix 4. *Colletotrichum* lesion characteristics on apple, strawberry, and blueberry

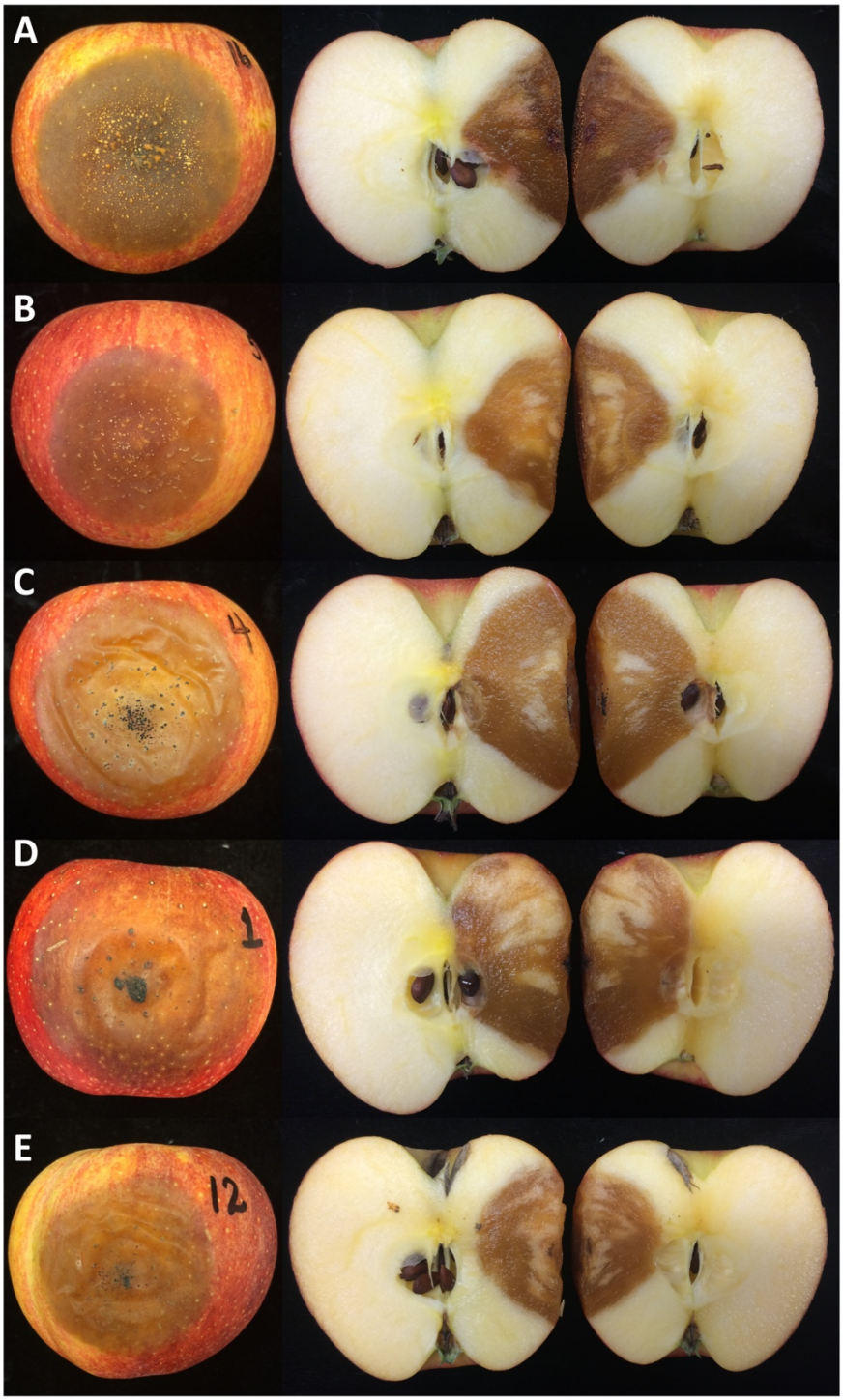


Figure A4.1 Exterior and interior *Colletotrichum* lesion characteristics on 'Gala' apples at 14 dpi. (A) *C. fioriniae*; (B) *C. nymphaeae*; (C) *C. siamense*; (D) *C. fructicola*; (E) *C. kahawae* clade.

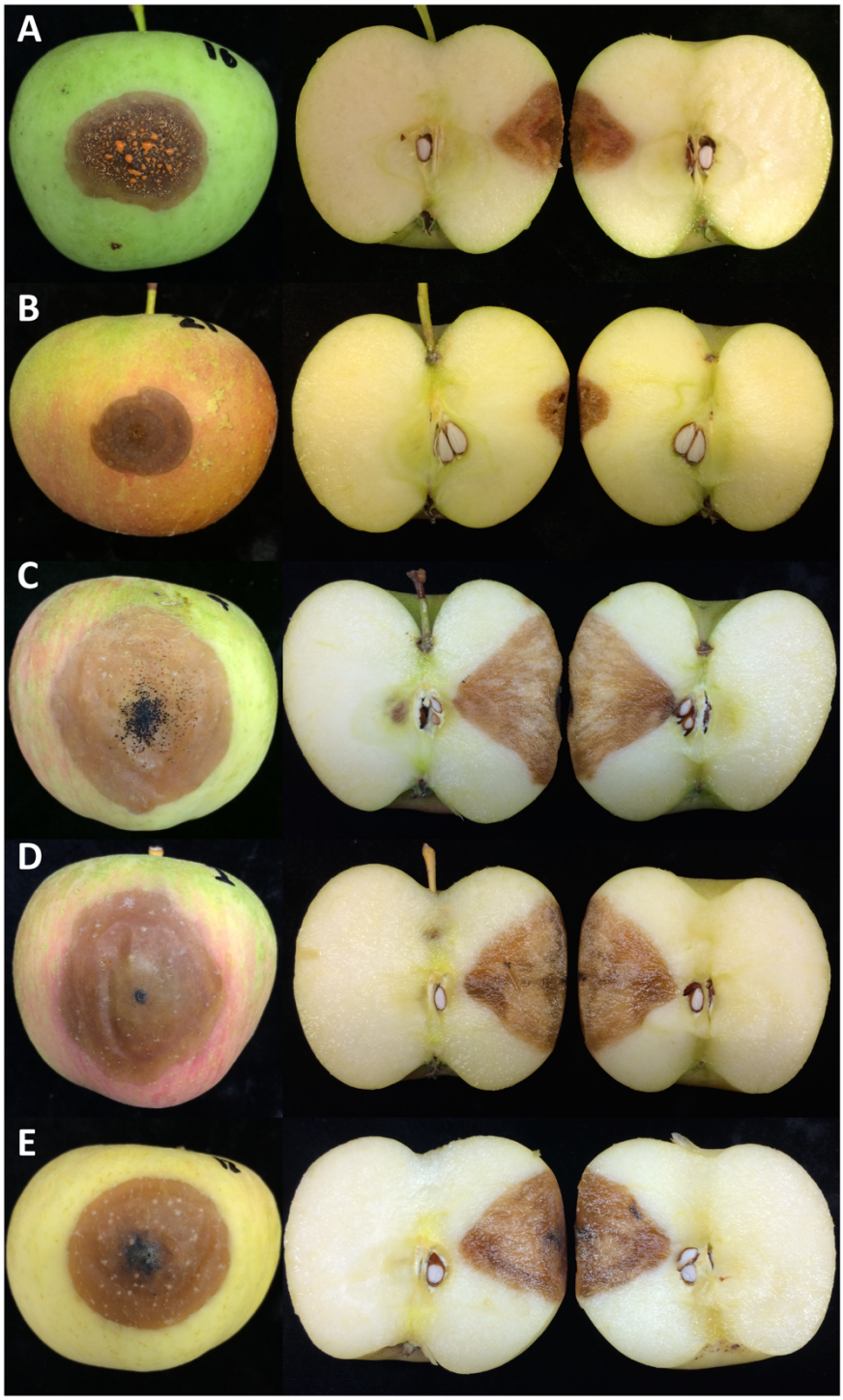


Figure A4.2 Exterior and interior *Colletotrichum* lesion characteristics on 'Fuji' apples at 14 dpi. (A) *C. fioriniae*; (B) *C. nymphaeae*; (C) *C. siamense*; (D) *C. fructicola*; (E) *C. kahawae* clade.

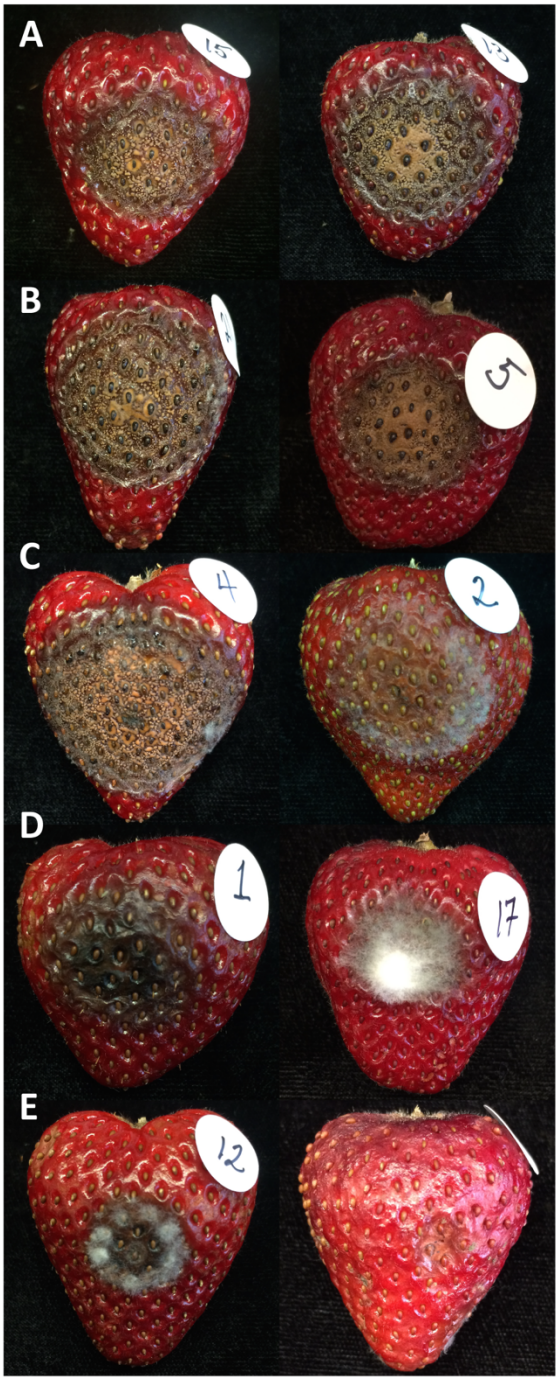


Figure A4.3 Exterior *Colletotrichum* lesion characteristics on strawberries at 7 dpi. (A) *C. fioriniae*; (B) *C. nymphaeae*; (C) *C. siamense*; (D) *C. fructicola*; (E) *C. kahawae* clade.

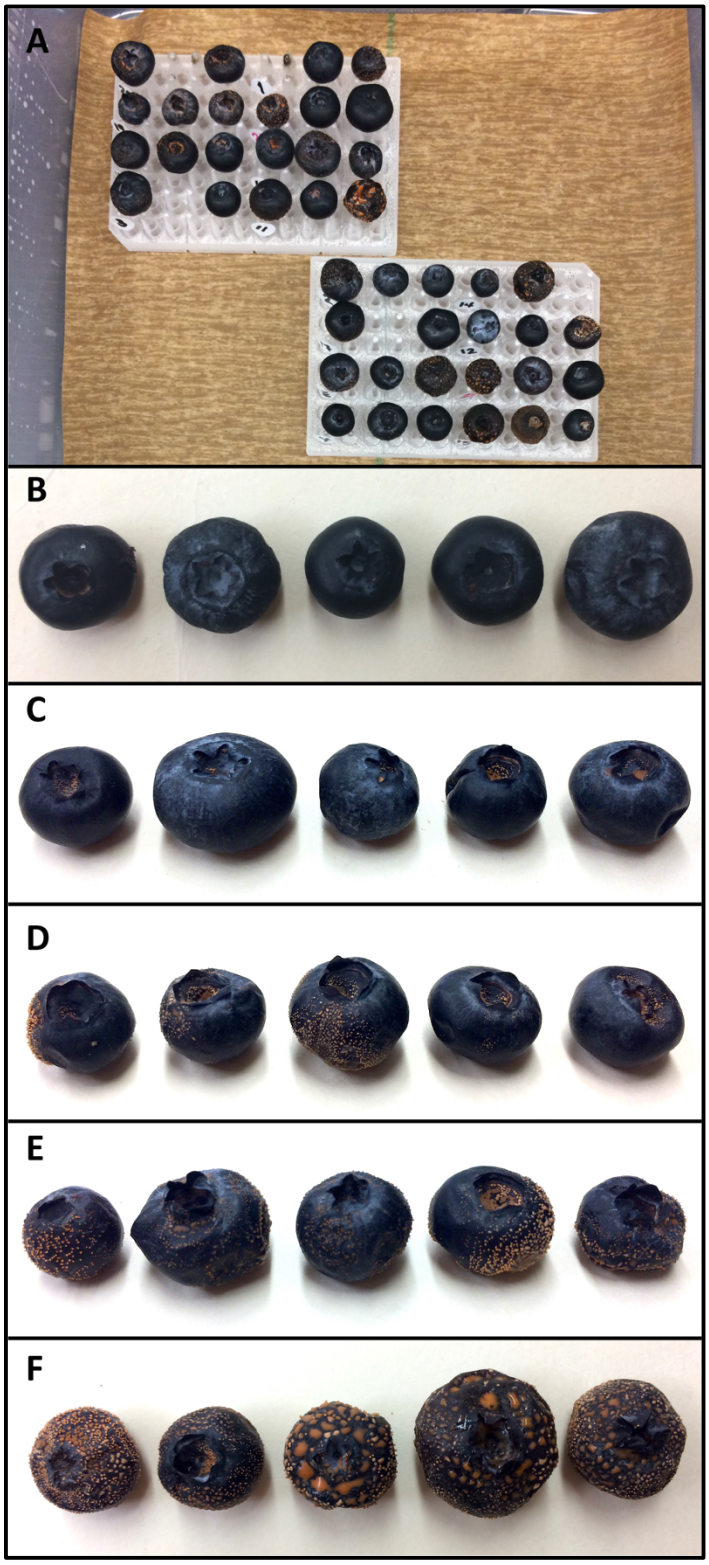


Figure A4.4 Symptoms and signs of blueberry *Colletotrichum* infection. (A) Experimental setup with germination paper and PCR multiplates in a humidity chamber. (B-F) Blueberry disease severity rating scale from 0 to 4 at 10 dpi: (B) 0 = 0% fungal spore coverage; (C) 1 = 1-10%; (D) 2 = 11-49%; (E) 3 = 50-99%; (F) 4 = 100% fungal spore coverage.

Appendix 5. Location of additional resources

All additional data not directly presented as a part of this thesis is stored on an external hard drive kept in the laboratory of Dr. Vaillancourt for reference. This includes raw gene sequences and alignments, fruit inoculation and spore measurements, various photographs, strain collection databases, and protocols. All lab notebooks with further details and protocols are also available for reference in the laboratory of Dr. Vaillancourt.

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In progress

Eaton, M. J., Edwards, S., Inocencio, H. A., Machado, F. J., Nuckles, E. M., Farman, M., Gauthier, N. A., Vaillancourt, L. J. 2020. Diversity and cross-infection potential of *Colletotrichum* causing fruit rots in Kentucky mixed-fruit orchards. Plant Disease.

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