ABSTRACT

Title of Thesis: UNDERSTANDING SPECIES AND FUNGICIDE RESISTANCE IN COLLETOTRICHUM POPULATIONS FROM THE MID-ATLANTIC STRAWBERRY FIELDS

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Anthracnose disease caused by *Colletotrichum* spp. is major concern in strawberry production nationwide. A total of 200 *Colletotrichum* spp. isolates were obtained from strawberry plants in the Mid-Atlantic region of U.S. Analysis of partial *g3pdh, tub2*, and/or ITS sequences showed that four *Colletotrichum* species, *C. nymphaeae, C. fioriniae*, *C. siamense,* and *C. lineola* were associated with strawberry anthracnose, with *C. nymphaeae* being predominant. All isolates were tested for resistance to quinone outside inhibitor (QoI) fungicide azoxystrobin. *C. siamense* isolates were additionally tested for resistance to benzimidazole carbamate (MBC) fungicide thiophanate-methyl. Overall frequency of resistance to QoIs and MBCs was 48 % and 67 %, respectively. G143A or F129L mutation in the cytochrome b gene (*cyt b*) and E198A mutation in the β-tubulin (*tub2*) gene were found to be linked with respective resistance phenotypes. Moreover, microsatellite primers were selected for determining genetic diversity within *C. nymphaeae* populations, followed by fragment sizing and preliminary down-stream analysis.

UNDERSTANDING SPECIES AND FUNGICIDE RESISTANCE IN COLLETOTRICHUM POPULATIONS FROM THE MID-ATLANTIC STRAWBERRY FIELDS

by

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Introduction

Strawberry is one of the top grossing fruit industries in the United States, valued at more than $2.6 billion and produced over 1.1 billion tons of berries in 2019 (USDA, 2019). The US produces almost 20% of the crop worldwide and leads in production per unit area (Samtani et al., 2019). In the Mid-Atlantic US, strawberry acreage had declined from its peak in the 1970s, but it remains relatively stable during the past decades (Samtani et al., 2019). Two production systems, plasticulture and matted-row strawberries are widely used by growers in the region. The strawberry plasticulture production system is an annual production system, where young strawberry plants are transplanted in late summer and early fall into raised soil beds that are covered with black plastic mulch. It has virtually replaced the traditional matted row system (perennial production) throughout the southeastern U.S. and warmer locations in the mid-Atlantic region due to higher yields, earlier harvests, better fruit quality, and reduced labor costs (E. B. Poling, 1993).

Among a variety of diseases affecting strawberries, anthracnose caused by species within the genus of *Colletotrichum* is one of the most devastating fungal diseases that can affect the plant at any stage (Henz et al., 1992). All parts of strawberry including fruits, crowns, leaves, petioles and runners are susceptible to anthracnose (Howard et al., 1992). The incidence can exceed 50% when extended wet and warm environmental conditions occur and effective control measures are not applied (Ellis and Grove, 1982; Turechek et al., 2006). When leaves, petioles,

or runners of the transplants are infected with the disease, symptoms may not develop for some time due to the biotrophic phase that typically occurs early in the infection process (Curry et al., 2002; N. A. Peres et al., 2005). Research and extension personnel have long cited clean transplants as critical to disease management (McInnes, 1992; Nichols et al., 2018; Poling, 2008; Rahman et al., 2019; Rahman and Louws, 2017; Samtani et al., 2019; Sjulin, 2008), but the cryptic nature of *Colletotrichum* combined with no cost-effective means for identifying disease presence in asymptomatic plants has resulted in issues persisting.

Three main species, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides* and *Colletotrichum fragariae*, were identified in earlier literature as the causal agents of strawberry anthracnose (Smith, 1998; Smith, 2008). *C. acutatum* is primarily known to cause a destructive fruit rot with some runner and petiole lesions. It is capable of sometimes causing anthracnose crown rot (ACR) (Howard et al., 1992; Smith, 1998), but it is largely limited to fruit rot. *C. gloeosporioides* and *C. fragariae* infections often result in crown necrosis that leads to plant wilting or death , but they sometimes can cause anthracnose fruit rot (AFR) (Smith, 1998). Identification of species based on morphology has been problematic, but with the development of molecular techniques, uncertainties and earlier misidentifications are being resolved. Both *C. acutatum* and *C. gloeosporioides* are now known to be species complexes. To date, 34 and 23 species are accepted within each complex respectively, including the species bearing the complex's names (Baroncelli et al., 2017; Bragança et al., 2016; Damm et al., 2012; Jayawardena et al., 2016; Weir et al., 2012). *C. fragariae* is now considered to be part of the *C. gloeosporioides* complex. The greatest economic losses due to anthracnose on strawberry are from fruit rot caused by *C. acutatum* complex pathogens and these pathogens can also infect many other vegetable and fruit crops, including apples, peaches, tomatoes, peppers, grapes,

blueberries and blackberries (Bernstein et al., 1995; Howard et al., 1992). Strawberry crown infection by *C. acutatum* often result in stunted plants rather than dead plants, and infected plants usually do not thrive and produce few berries at harvest (Smith, 2008). Characteristic symptoms of fruit rot on strawberry appears as dark brown, sunken necrotic lesions with orange-to-pink conidial masses produced on the fruit surface, and the typical symptoms of flower blight are brown lesions on petals (Howard et al., 1992; Peres et al., 2005). Crown rot is characterized by brown-to-black decayed roots and crown necrosis by reddish-to-brown necrotic crown tissues visible after cutting the infected crowns (Freeman & Katan, 1997; Howard et al., 1992; Peres et al., 2005). The distinctive morphological characteristic of *C. acutatum* complex species *in vitro* is the shape of the conidia, which have acute ends (Simmonds, 1966), and pink, orange or beige colonies with predominantly cream, pink reverse colony colors (Smith and Black, 1990). In contrast, the *C. gloeosporioides* complex species typically have cylindrical conidia with rounded ends (Wharton and Diéguez-Uribeondo, 2004), and develop grey or olive-gray colonies with dark to grey olive reverse colony colors (Smith and Black, 1990).

Recent work has focused on identifying species that are most frequently causing AFR and ACR via multilocus sequencing. *C. nymphaeae* and *C. fioriniae*, belonging to the *C. acutatum* complex, were found to comprise 98% and 2% of 217 isolates collected from five southern states and California, suggesting that *C. nymphaeae* was the dominant species of concern (Wang et al., 2019). In contrast, all 287 *Colletotrichum* isolates obtained from various vegetative tissues of strawberry in eastern China were identified to be species within *C. gloeosporioides* complex, including *C. fructicola*, *C. gloeosporioides*, *C. siamense*, and *C. aenigma* (X. Y. Chen et al., 2019; Zhang et al., 2020). Furthermore, Adhikari et al. (2019) reported that *C. siamense* caused both AFR and ACR in North Carolina. *C. gloeosporioides*

complex species (including *C. fragariae*) were most commonly found in the southeastern US causing ACR (Smith 2008; Rahman et al. 2015).

During 2018 and 2019 growing seasons, outbreaks of strawberry anthracnose were observed in many locations in the Mid-Atlantic US, with some individual growers reporting 50% fruit loss or more. While the annual plasticulture system plantings were affected the most due to use of plastic mulch which increases rainsplash, matted-row plantings were also affected to some extent, especially on susceptible cultivars. Information mentioned above, such as identity of *Colletotrichum* isolates, is lacking for Mid-Atlantic strawberry producers, making targeted control measures impossible. Thus, one of the main objectives was to identify *Colletotrichum* isolates from strawberry fields in the Mid-Atlantic region to the species level.

Materials and methods

Colletotrichum isolates collection

To process strawberry samples and obtain *Colletotrichum* cultures, a 25 mm^2 piece of symptomatic tissue from each of the fruit, crown, runner, or stem samples, was cut from the edge of the lesion and surface sterilized by 10% bleach (Clorox®, 6.15% sodium hypochlorite) for 1min. The sterilized tissue was washed twice with sterilized distilled water for one minute, air-dried and placed on potato dextrose agar (PDA) plates. Plates were then sealed with parafilm and placed in a clear storage box under room conditions. After three to five days of incubation, emerging colonies largely resembling *Colletotrichum* were transferred to fresh PDA plates to obtain pure fungal cultures. Constant fluorescent light conditions were provided to stimulate sporulation once the purified colonies reached the edge of plates. Upon the sporulation, single-

spore isolation was conducted for each colony as described previously (Hu et al., 2015), and no more than two single-spore isolates were obtained from each symptomatic plant sample. A mycelial plug from each isolate was transferred to a new PDA plate containing ten 25 mm^2 pieces of sterilized filter papers, and incubated for three to four days at 22 °C. Filter papers covered with fungal mycelia were collected and dried in a desiccator for two weeks, then placed in a 1.5 mL centrifuge tube containing silica gel and stored at -20 °C.

Genomic DNA extraction

Genomic DNA was extracted from all single-spore isolates of *Colletotrichum* spp. using a previous published protocol (Chi et al., 2009). Briefly, 10-20 mg fungal mycelia were scratched from the edge of colony of three to five days old on PDA and transferred to a 1.5 mL centrifuge tube filled with 0.5 mL extraction buffer (1M KCL, 100mM Tris-Hcl, 10mM EDTA). Subsequently, fungal mycelia were pulverized with a plastic pestle for 30s, and then cell lysates were centrifuged at 8,000 rpm for 5min. The supernatant was transferred with a pipette to a 1.5 mL centrifuge tube containing 0.3 mL iso-propanol. The supernatant and iso-propanol were then mixed and centrifuged, and the resulting DNA was dissolved with 50 µl sterilized distilled water and stored at -20 °C for future use.

Colletotrichum* spp. *identification

Isolates were first identified to *C. gloeosporioides* or *C. acutatum* complex level based on the presence and size of the internal transcribed spacer (ITS) region using a polymerase chain reaction (PCR) method developed previously (Mills et al., 1992; Sreenivasaprasad et al., 1996). Based on the identified species complex, appropriate primers were selected and used to amplify

fragments of the glyceraldehyde 3-phosphate dehydrogenase (*g3pdh*) gene and the beta tubulin (*tub2*) gene for species-level identification. A subset of the 200 isolates was selected for species identification based on their colony morphologies and geographical locations. Specifically, three to five isolates with the same colony morphology were selected from each sampling site in a given location. PCR reactions were performed using the T100 Bio-Rad thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA), according to the cycling protocols described previously (Hu et al., 2015). The total reaction volume was 25 µl, containing 12.5 µl 2 x Taq Master Mix (Apex™, Genesee Scientific Corporation, San Diego, CA), 9.5 µl water, 1 µl forward and reverse primer (10 µM), respectively, and 1 µl template DNA. The PCR products were purified from unincorporated primers and dNTPs using ExoSAP-IT™ PCR Product Cleanup (Affymetrix Inc., Santa Clara, CA) according to the manufacturer's instruction. Sanger sequencing was conducted at Genewiz® (South Plainfield, NJ) and Arizona State University Genomic Facility (Tempe, AZ). Primers used for PCR and Sanger sequencing are listed in Table 1. DNA sequences were analyzed and aligned using Benchling software (https://www.benchling.com/; San Francisco, CA, USA).

Table 1. Primers used for PCR amplification and sequencing.

Primer name	Primer sequences (5′-3′)	Descriptions	References
ITS-1F	TCCGTAGGTGAACCTGCGG	Forward primer for amplifying and sequencing ITS region	Gardes and Bruns, 1993
ITS-4	TCCTCCGCTTATTGATATGC	1) Reverse primer for amplifying and sequencing ITS region 2) Reverse primer to detect *C. acutatum* or *C. gloeosporioides* complex species	White et al., 1990
CaInt2	GGGGAAGCCTCTCGCGG	Forward primer to detect *C. acutatum* complex species	Sreenivasaprasad et al., 1996
CgInt	GGCCTCCCGCCTCCGGGCGG	Forward primer to detect *C. gloeosporioides* complex species	Mills et al., 1992
GDF1	ATGGCTCCCATCAAGGTCG	Forward primer for amplifying and sequencing *g3pdh*	Hu et al., 2015
GDR	GGGTGGAGTCGTACTTGAGCATGT	Reverse primer for amplifying and sequencing *g3pdh*	Templeton et al., 1992
T1	AACATGCGTGAGATTGTAAGT	Forward primer for amplifying and sequencing *tub2*	O'Donnell and Cigelnik, 1997
TubR1	TTCTGGACGTTGCGCATCTG	Reverse primer for amplifying and sequencing *tub2*	Hu et al., 2015
Cocytb-F1	CTTATAGATACATCACAACC	Forward primer for amplifying and sequencing *cytb* from *C. nymphaeae* and *C. fioriniae*	This study
Cocytb-R1	AGGTCTAAATTGGTAACC	Reverse primer for amplifying and sequencing *cytb* from *C. nymphaeae* and *C. fioriniae*	This study
Cocytb-F2	TTACTTGCAGTTTGTTTAGG	Forward primer for amplifying and sequencing *cytb* from *C. siamense* (fragment 1)	This study
Cocytb-R3	ACCAGTGACCCAGTCTGTA	Reverse primer for amplifying and sequencing *cytb* from *C. siamense* (fragment 1)	This study
Cocytb-F3	TCTATTACAGAAGCAAGTACAG	Forward primer for amplifying and sequencing *cytb* from *C. siamense* (fragment 2)	This study
Cocytb-R4	ACTCAACGATATCTTGTCCA	Reverse primer for amplifying and sequencing *cytb* from *C. siamense* (fragment 2)	This study

Phylogenetic analysis

The representative isolates used for phylogenetic tree construction were most closely related to *C. nymphaeae*, *C. fioriniae*, or *C. siamense*, compared with other species within the *C. acutatum* or *C. gloeosporioides* complex. Phylogenetic trees were constructed based on the combined sequences of *g3pdh* and *tub2*. The compared sequenced were selected from publications of Damm (2012) and Weir (2012) and their *g3pdh* and *tub2* sequences were retrieved from GenBank (Table 2). The length of combined sequences of representative isolates were cut based on length of combined selected sequences from GenBank. Maximum Likelihood (ML) method was used based on the Kimura 2-parameter model which was determined as the best fit for the sequences using MEGA version 10. A complete deletion option was used to treat gaps/missing data and the robustness of the tree was estimated with 1,000 bootstrap replications.

Table 2. GenBank accession numbers of the *g3pdh* and *tub2* sequences from *Colletotrichum spp*. isolates used for the phylogenetic analyses.

***Colletotrichum* species**	**Culture**	**GenBank accession number**		**Source**
		g3pdh	***tub2***	
C. acutatum complex				
C. nymphaeae	CBS 126504	JQ948595	JQ949916	GenBank
C. nymphaeae	CBS 100064	JQ948554	JQ949875	GenBank
C. fioriniae	CBS 125396	JQ948629	JQ949950	GenBank
C. fioriniae	CBS 119292	JQ948643	JQ949964	GenBank
C. scovillei	CBS 126529	JQ948597	JQ949918	GenBank
C. chrysanthemi	IMI 364540	JQ948603	JQ949924	GenBank
C. guajavae	IMI 350839	JQ948600	JQ949921	GenBank
C. walleri	CBS 125472	JQ948605	JQ949926	GenBank
C. simmondsii	CBS 294.67	JQ948607	JQ949928	GenBank
C. indonesiense	CBS 127551	JQ948618	JQ949939	GenBank
C. sloanei	IMI 364297	JQ948617	JQ949938	GenBank
C. brisbanense	CBS 292.67	JQ948621	JQ949942	GenBank
C. lupini	IMI 351261	JQ948507	JQ949828	GenBank
C. acutatum	IMI 223120	JQ948684	JQ950004	GenBank
C. salicis	CBS 129972	JQ948797	JQ950117	GenBank
C. australe	CBS 116478	JQ948786	JQ950106	GenBank

C. paxtonii	CBS 502.97	JQ948616	JQ949937	GenBank
C. orchidophilum	CBS 631.80	JQ948482	JQ949803	GenBank
C. gloeosporioides complex				
C. siamense	OD12-1	KJ769240	KM245089	GenBank
C. siamense	ICMP 17795	JX010051	JX010393	GenBank
C. siamense	ICMP 18642	JX010019	JX010410	GenBank
C. tropicale	ICMP 18653	JX010007	JX010407	GenBank
C. aeschynomenes	ICMP 17673	JX009930	JX010392	GenBank
C. fructicola	RR12-3	KJ769247	KM245092	GenBank
C. nupharicola	ICMP 18187	JX009972	JX010398	GenBank
C. alienum	ICMP 12071	JX010028	JX010411	GenBank
C. aenigma	ICMP 18608	JX010044	JX010389	GenBank
C. musae	ICMP 17817	JX010015	JX010395	GenBank
C. queenslandicum	ICMP 1778	JX009934	JX010414	GenBank
C. salsolae	ICMP 19051	JX009916	JX010403	GenBank
C. asianum	ICMP 18696	JX009915	JX010384	GenBank
C. gloeosporioides	ICMP 17821	JX010056	JX010445	GenBank

Results

Identification of *Colletotrichum* spp. causing strawberry anthracnose

In total, 200 *Colletotrichum* spp. isolates were collected from symptomatic strawberry plants of 13 or more cultivars from commercial and experimental strawberry fields in Maryland (129 isolates, 10 sites), Pennsylvania (42 isolates, 4 sites), Virginia (16 isolates, 2 sites) and North Carolina (13 isolates, 2 sites) during 2015-2019 seasons.

In conjunction with the ITS4 primer, *C. acutatum* complex specific primer CaInt2 and *C. gloeosporioides* complex specific primer CgInt amplified an expected 490 bp and 450 bp ITS fragment from all isolates except for one isolate, respectively (Figure 2). Of the 199 isolates, 187 were found to be *C. acutatum* complex species, while 12 were shown as *C. gloeosporioides* complex. The remainder isolate exhibited a distinct colony morphology, and its ITS region was

therefore amplified and sequenced, which showed 100% identity with *Colletotrichum lineola* (GenBank accession no. HQ239359).

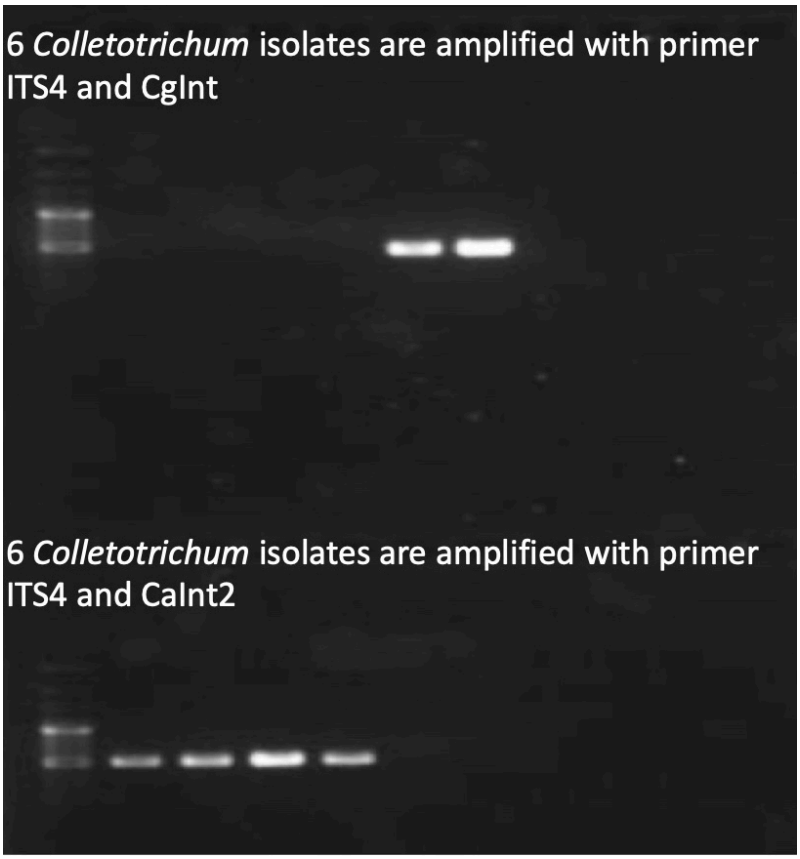
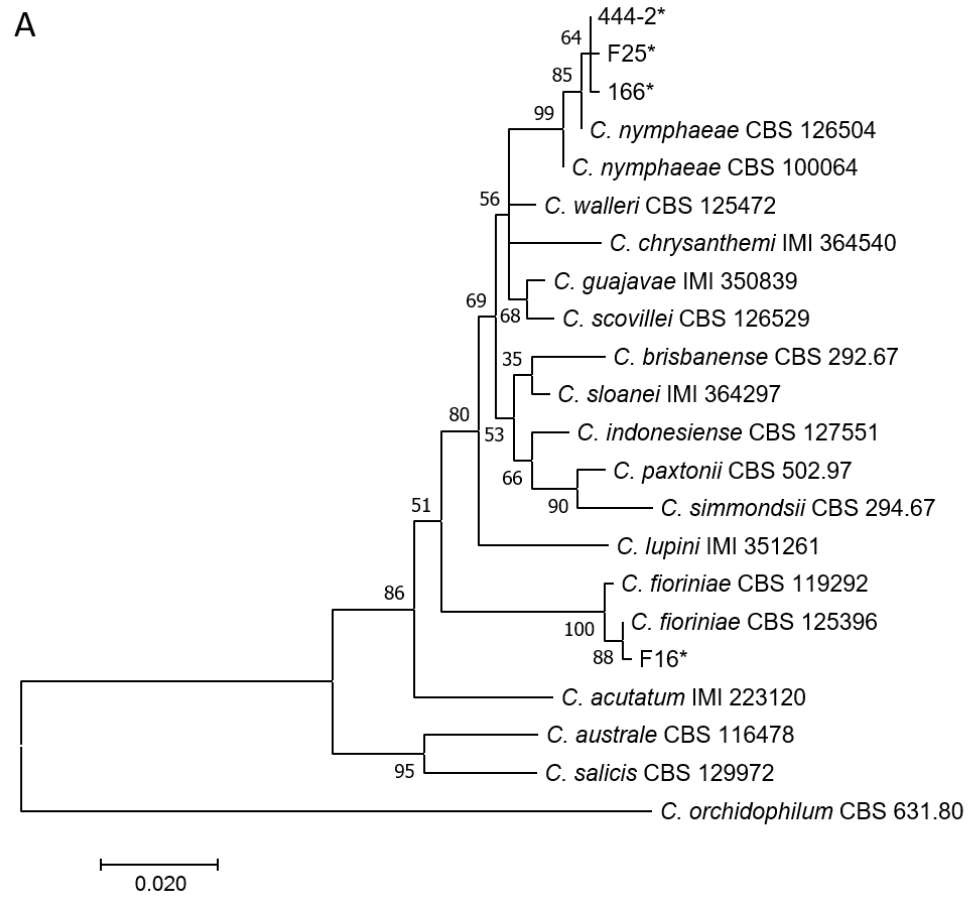


Figure 1: DNAs from six *Colletotrichum* isolates were amplified with two complex specific primer pairs ITS4/CgInt (C. gloeosporioides complex specific primer pair) and ITS4/CaInt2 (*C. acutatum* complex specific primer pair.

For the selected 101 isolates identified as *C. acutatum* or *C. gloeosporioides* complex species, *g3pdh* and *tub2* fragments were further amplified and sequenced for species-level identification. Approximately 200 bp and 1,300 bp of *g3pdh* and *tub2* were amplified from each of the selected isolates, respectively. Analysis of both *g3pdh* and *tub2* sequences from all selected 101 isolates revealed > 99% similarity with those of *C. nymphaeae* (GenBank accession nos. JQ949907 and JQ948588), *C. fioriniae* (JQ949969 and JQ948648), or *C. siamense* (JX010393 and JX009922), respectively (Damm et al., 2012; Weir et al., 2012).

The phylogenetic trees, based on the combined sequences of *g3pdh* and *tub2*, further indicated that the representative isolates used in our study were most closely related to *C. nymphaeae*, *C. fioriniae*, or *C. siamense*, compared with other species within the *C. acutatum* or *C. gloeosporioides* complex (Figure 2). The *g3pdh* sequences for *C. nymphaeae*, *C. fioriniae*, and *C. siamense* obtained in this study were deposited into GenBank under accession nos. MT740204, MT740203, and MT740202, respectively. The accession numbers MT740205 - MT740207, MT740208, and MT740209 were obtained via GenBank submission for the *tub2* sequences for representative isolates of *C. siamense*, *C. fioriniae*, and *C. nymphaeae*, respectively.



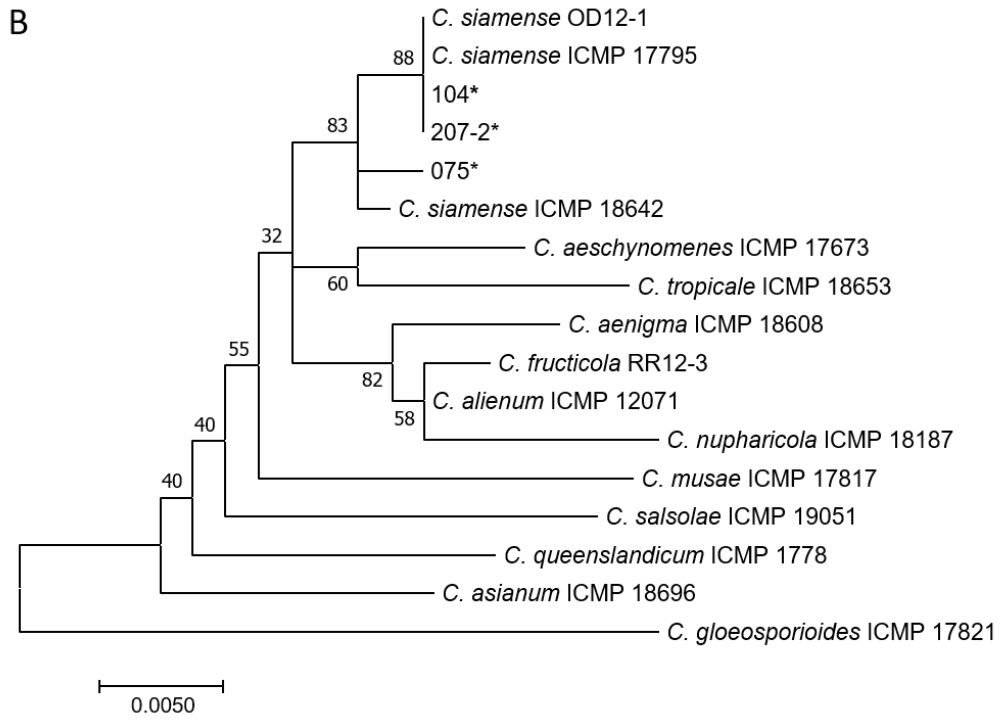


Figure 2. Phylogenetic tree of the combination of *g3pdh* and *tub2* sequences of *Colletotrichum acutatum* complex (A) and *C. gloeosporioides* complex (B), including isolates used in this study (marked with asterisks) and those previously reported along with GenBank accession numbers. Bootstrap frequencies were calculated with 1,000 replicates**.**

Characteristics of colony morphology

Distinct colony morphologies were observed between the four *Colletotrichum* species grown on PDA plates, whereas isolates within the same species showed largely identical colony morphologies. *C. nymphaeae* formed white to creamy mycelia that turned light gray as it aged, and a creamy light orange reverse. Colonies of *C. fioriniae* appeared gray to olivaceous outlined by a pinkish-red ring, the reverse was also pinkish-red. In contrast, colonies of *C. siamense* appeared white and relatively fluffy, with dense and dark-green mycelia in the center, and an olive speckled reverse. Compared to other species, colonies of *C. lineola* had a uniform gray color with a white rim, with somewhat concentric rings of mycelia in compact tufts, dark green from the reverse (Figure 3).

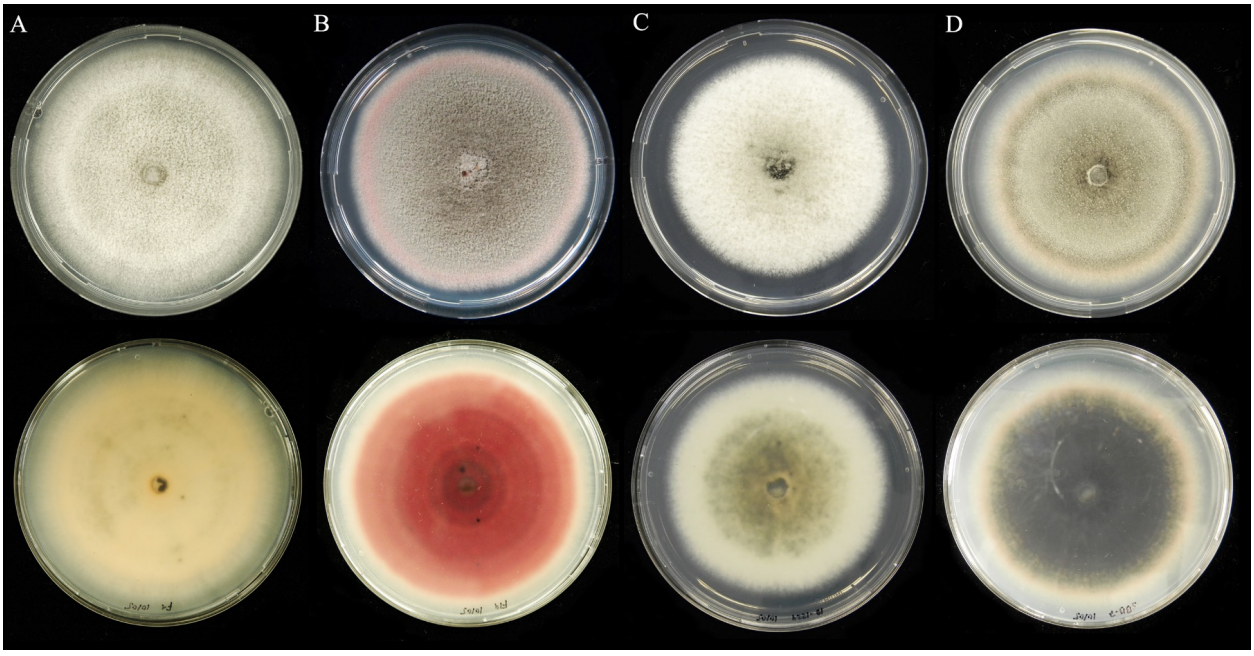


Figure 3. Colony morphology of *Colletotrichum nymphaeae* (A), *C. fioriniae* (B), *C. siamense* (C) and *C. lineola* (D) grown on the surface (upper plates) and reverse (lower plates) of potato dextrose agar medium.

Prevalence of *Colletotrichum* species recovered from strawberry cultivars, organs and production systems

C. nymphaeae under the *C. acutatum* complex was found to be predominant, comprising 179 of 200 isolates collected in the Mid-Atlantic strawberry fields. Twelve and eight isolates were found to be *C. siamense* within the *C. gloeosporioides* complex and *C. fioriniae* within the *C. acutatum* complex, respectively (Table 3). *C. nymphaeae* was recovered from all strawberry organs examined, accounting for 95%, 49%, 100%, and 100% of the isolates from the fruit, crowns, runners, and petioles, respectively (Figure 4). *C. siamense* was only recovered from crowns, representing 12 of the 25 crown isolates, and *C. nymphaeae* and *C. lineola* accounting

for the other 12 and one isolates, respectively. All *C. fioriniae* isolates were obtained from the fruit. The only *C. lineola* isolate was obtained from the crown (Figure 4).

Table 3. *Colletotrichum* spp. isolates from Mid-Atlantic strawberry fields used in this study.

	C. acutatum		***C. gloeosporioides***		
State	***C. nymphaeae***	***C. fioriniae***	***C. siamense***	**C. *lineola***	**Total**
Maryland	121	6	2	-	129
Pennsylvania	36	2	3	1	42
Virginia	9	-	7	-	16
North Carolina	13	-	-	-	13
Total	179	8	12	1	200

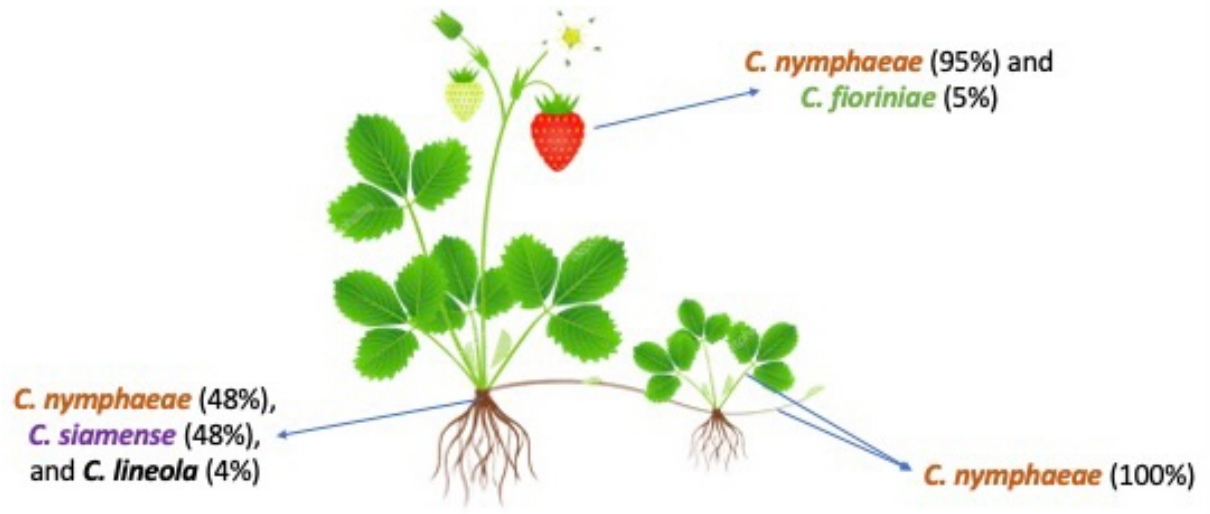


Figure 4. *Colletotrichum* species isolated from different organs of strawberry plant (the plant picture was obtained from Depositphotos.com)

In addition, the cultivar information is available for 197 of the 200 *Colletotrichum* spp. isolates in this study. Among these 197 isolates, 60% and 11% were collected from cultivar

Chandler and Camarosa, respectively. The remaining 29% isolates were collected from a variety of cultivars including Darselect, AC Valley, Earliglow, Albion, Honeoye, Seascape, San Andreas, Camino Real, Sweet Charlie, Ruby June, and Flavorfest. *C. nymphaeae* was isolated from 8 of 13 cultivars examined, followed by *C. siamense* which was isolated from 6 cultivars. Although equal number of *C. nymphaeae* and *C. siamense* isolates were obtained from the crown, *C. siamense* was found to be present in more cultivars. In addition, the majority of these isolates were collected from strawberry plants grown on plasticulture system (Table 4).

Further, 178 of the 200 isolates were collected from strawberry plants grown on plasticulture system, whereas 21 isolates were collected from matted-row production system. All isolates from matted-row system were collected in Pennsylvania. The only one isolates of *C. lineola* was collected from plasticulture system, whereas the other 3 *Colletotrichum* spp. species were obtained from both plasticulture and matted-row systems. *C. nymphaeae* accounted for 76.2% and 91.1% of matted-row and plasticulture strawberries, respectively. *C. fioriniae* composed 2% in matted-row and 3.4% in plasticulture system, whereas *C. siamense* made up 3% of matted-row system and 5% of plasticulture system, respectively (Table 4).

Table 4. Frequency (%) of resistant *Colletotrichum* spp. isolates from different organs of strawberry cultivars.

	Fruit		**Crown**			**Runner/petiole**
Cultivar[a]	***C. nymphaeae***	***C. fioriniae***	***C. nymphaeae***	***C. siamense***	***C. lineola***	***C. nymphaeae***
Chandler (P)	105 (35)[b]	-	2 (50)	4 (100; 50)[c]	-	9 (56)
Camarosa (P)	13 (100)	-	8 (100)	-	-	-
Darselect or AC Valley Sunset (M)	15 (0)	2 (100)	-	-	-	-
Sweet Charlie (P)	11 (9)	-	-	-	-	-
Earliglow (P/M)	1 (0)	6 (100)	-	1 (100; 0)	-	-
Albion (P)	2 (100)	-	2 (50%)	-	-	3 (0)
Seascape (P/M)	3 (100)	-	-	-	-	-
Ruby June (P)	-	-	-	3 (100; 67)	-	-
San Andreas (P)	2 (100)	-	-	-	-	-
Honeoye (M)	-	-	-	2 (100; 100)	-	-
Camino Real (P)	-	-	-	1 (100; 0)	-	-
Flavorfest (P)	-	-	-	-	1 (100; 100)	-
B2862 (P)	-	-	-	1 (100; 100)	-	-
Unknown (P)	3 (67)	-	-	-	-	-
Total	155 (39)	8 (100)	12 (83)	12 (100; 58)	1 (100; 100)	12 (42)

[a] 'P' or 'M' in the parenthesis indicates plasticulture or matted-row system from which the cultivar was sampled.
[b] The first number indicate the number of isolates, followed by the frequency (%) of resistant isolates (in parenthesis).
[c] The two numbers in parenthesis indicate the frequencies (%) of azoxystrobin- and thiophanate-methyl-resistant isolates, respectively.

Discussion

In agreement with a previous study (Wang et al., 2019), *C. nymphaeae* (within the *C. acutatum* complex) was found to be the primary cause of strawberry anthracnose in the Mid-Atlantic region. As noted, this species was recovered from all host tissues examined, whereas *C. siamense* and *C. fioriniae* were limited to crown and fruit tissues, respectively. However, prevalence and species of *Colletotrichum* isolates causing the disease seem to be more variable between countries. For example, 18% and 9% of 148 isolates collected in the United Kingdom were *C. fioriniae* and *C. godetiae* (Baroncelli et al., 2015), while four species within the *C. gloeosporioides* complex were exclusively found in Chinese strawberry fields (Chen et al., 2019; Zhang et al., 2020). Different cultivars, production practices, host tissue examined, and environmental conditions may have contributed to such increased diversity between countries. *C. gloeosporioides* complex species (including *C. fragariae*) have been considered the major pathogens responsible for ACR, which are believed to be primarily distributed in the southeastern United States (Smith, 2008).

Interestingly, a *C. gloeosporioides* species (i.e. *C. siamense*) was isolated from crown rot samples from Virginia, Maryland, and Pennsylvania, indicating it may have established in the Mid-Atlantic region. In addition, one isolate of *C. lineola* was obtained from crown tissue. This species has thought to be synonymous with *C. dematium* (Cannon et al., 2012), which has been reported to cause anthracnose lesions on strawberry fruit and vegetative tissues (Beraha and Wright, 1973; McLean and Sutton, 1992; Mertely and Legard, 2004; Smith, 1998) and may be a soil-borne pathogen (Mattner et al., 2008).

The reason why C. *acutatum* complex species have become the predominant anthracnose fruit rot pathogens is not well resolved. Previous publications illustrated that *C. acutatum* species can germinate and sporulate on symptomless strawberry leaves, indicating appressoria and secondary conidia of *C. acutatum* produced on symptomless strawberry foliage may be significant sources of inoculum for fruit infection (Leandro et al., 2003). As a rain-splashed pathogen as other *Colletotrichum* species, inoculum dispersal within strawberry fields may be limited in distance. Field studies have shown that fruit in plots 2 meters from introduced inoculum had very few infections initially (Debode et al., 2015; Madden et al., 1993; Madden et al., 1996), but with continued dispersal measured at 1.75 m per week from neighboring plants, spread could be much greater over time (Debode et al., 2015). Another study revealed that pathogenic *C. acutatum* population were unlikely to move from one host to another and cause an epidemic (MacKenzie et al., 2009), indicating the transmission of pathogen may only occur within the same host. These facts may have, to some extent, contributed to the less diverse sources of disease inoculum, thus a less diverse pool of *Colletotrichum* species.

Our studies showed that *C. nymphaeae* can cause infections on multiple strawberry organs including fruit, crown, runner, and petiole. While *C. fioriniae* was only isolated from strawberry fruit, this species has been associated with leaf curl, crown necrosis, petiole lesions and leaflets on celery (Pavel, 2016). Moreover, *C. nymphaeae* seems to be more aggressive than *C. fioriniae* when infecting strawberry roots and crowns (Wang et al., 2019). *C. siamense* was only isolated from strawberry crown, however, *C. gloeosporioides* complex species have been long associated with strawberry fruit in the southeast US. (Howard, 1984). While no preference of *Colletotrichum* species was observed on strawberry cultivars, 48% of our isolates were collected from “Chandler” which is considered highly susceptible to anthracnose (Table. 4).

Chapter 2: Fungicide sensitivity and molecular bases of resistance in *Colletotrichum* species to azoxystrobin and thiophanate-methyl

Introduction

Chemical control has been a major pillar in the integrated management of anthracnose. Quinone-outside inhibitors (QoI) are the most commonly used single-site fungicides for anthracnose control, but resistance has been an increasing concern. Forcelini et al. (2016) showed that all 133 isolates collected in Florida strawberry fields prior to 2013 were sensitive to the QoI fungicide azoxystrobin, with the EC_{50} values less than 0.6 µg/ml, whereas 43 out of 48 isolates collected in 2013 and 2014 were resistant to the same fungicide, with the EC_{50} values greater than 100 µg/ml. In addition to QoIs, methyl benzimidazole carbamates (MBC) fungicides are effective against *C. gloeosporioides* complex but not *C. acutatum* complex species (Horn et al., 1972; Howard, 1971; Lamondia, 1995). However, high frequencies of resistance to the MBC fungicides carbendazim or thiophanate-methyl have been detected in *C. siamense* and *C. fructicola* (within the *C. gloeosporioides* complex) isolates from strawberries and other crops (Han et al., 2018; Lin et al., 2016). Furthermore, both *C. acutatum* and C. *gloeosporioides* complex species were found to be naturally resistant to the majority of succinate dehydrogenase inhibitors (SDHI) fungicides, one of the most important chemical groups used for disease management for many crops, including strawberries (Hideo Ishii et al., 2016). Other fungicides labelled for strawberry are typically not recommend for anthracnose fruit rot or crown rot control, due to much less or unknown efficacy. Phenylpyrroles such as fludioxonil and the multi-site fungicide captan (FRAC M4) may provide some help but are not reliable under high disease pressure. A number of demethylation inhibitors (DMIs) fungicides have been developed and labelled for strawberry production, but they seem to

vary their efficacy against *Colletotrichum* species, and differential sensitivity to the same fungicide has been reported in *Colletotrichum* spp. (Chen et al., 2016).

QoI fungicides inhibit ATP production during mitochondrial respiration by binding to the Qo center on the cytochrome b complex (*cytb*) (Bartlett et al., 2002; Sauter et al., 1999; Ypema & Gold, 1999). Resistance to QoI fungicides has been mainly found to be associated with an amino acid mutation from glycine to alanine at codon 143 (G143A) of the *cytb* gene across fungal pathogens, which typically confers a high level of resistance to QoIs among resistant isolates (Gisi et al., 2002; Sierotzki et al., 2000). Two other mutations, a substitution of phenylalanine by leucine at position 129 (F129L) and a change of glycine by arginine at position 137 (G137R), have also been documented to be related with moderate resistance (Bartlett et al., 2002; Pasche et al., 2004; Sierotzki et al., 2007). Both G143A and F129L mutations in *cytb* have been found in strawberry *C. acutatum* complex isolates with high and moderate resistance to QoIs, respectively (Forcelini et al. 2016). The MBCs include benzimidazoles and thiophanates and both function by inhibiting nuclear division via mitosis by binding beta tublin (*tub2*) subunits (Davidse, 1986; Köller, 1999). Consequently, resistance to MBCs has been linked to missense point mutations in *tub2* gene. The substitution of glutamic acid by alanine at codon 198 (E198A) or the substitution of phenylalanine by tyrosine at codon 200 (F200Y) was found to confer resistance in various fungal pathogens including *C. gloeosporioides* complex species (Han et al., 2018; Ma & Michailides, 2005). Compared to F200Y, E198A usually leads to a higher level of resistance to MBCs (Chen et al., 2013; Chung et al., 2006, 2010).

Understanding resistance mechanisms allows for the development of molecular resistance detection methods (Luo et al., 2008; Ma et al., 2005). This would greatly facilitate large-scale and routine resistance monitoring, helping growers identify potential resistance issues and most

effective spray programs. In addition, resistance mechanisms provide a basis for further understanding resistance levels, risk, and fitness costs associated with a given resistance genotype. For example, G143A mutation often confers hundreds of folds increase in EC_{50} values (A. Leadbeater, 2012), whereas overexpression of sterol 14α-demethylase (cyp 51) (Cools et al., 2012) or enhanced drug efflux pump activity (Kretschmer et al., 2009) usually increases about 10-fold or less in EC_{50} values (Hu, 2019). Interestingly, *cytb* genes from some fungal species contain a self-splicing intron just downstream of the G143 codon, which mitigates the risk of resistance development to QoIs (Luo et al., 2010). Further, understanding fitness costs associated with fungicide resistance is becoming an emerging research topic, as it would directly inform resistance management strategies. Previous studies have shown certain resistance-conferring mutations may undergo fitness costs while others may be as competitive as wide genotypes (Cosseboom et al., 2020; Ren et al., 2016).

Collectively, the objectives under this chapter were to determine sensitivity of the 200 *Colletotrichum* spp. isolates from the Mid-Atlantic strawberry fields to QoI fungicide azoxystrobin and MBC fungicide thiophanate-methyl, and characterize molecular bases of resistance in different *Colletotrichum* species to the respective fungicides.

Materials and methods

Detection of resistance to azoxystrobin and thiophanate-methyl

All of the 200 *Colletotrichum* spp. isolates except for three without live cultures were screened for resistance to the QoI fungicide azoxystrobin *in vitro* using a single discriminatory dose assay (Forcelini et al., 2016a), and all *C. siamense* isolates identified in this study were additionally screened for resistance to the MBC fungicide thiophanate-methyl, as *C. acutatum* complex

isolates were confirmed to be inherently resistance to MBC fungicides (Han et al., 2018). The active ingredients of azoxystrobin and thiophanate-methyl were used as formulated products Abound (Syngenta Crop Protection, Basil, Switzerland) and Topsin M (United Phosphorous Inc., King of Prussia, PA), respectively. The fungicides were added to autoclaved PDA media cooled to 50 °C at a concentration of 100 µg/ml active ingredient, and 20 ml media were poured into 90 mm petri dishes (Greiner Bio-One, Monroe, NC) one day before the experiment. Isolates were recovered from filter paper stocks on PDA at 25 °C in the dark. Two mycelial plugs (5 mm in diameter) per isolate were punched out with a cork borer from the periphery of 5-day-old colonies, and placed upside down onto fungicide amended or unamended PDA plates. After incubation for three days at 25°C in the dark, colony diameters were measured in two perpendicular directions, and an average colony diameter was calculated. Inhibition rates were calculated according to the formula (Bekker et al., 2006): Inhibition rate = (C-T) / C * 100%. Where C = colony diameter (mm) of the control; T = colony diameter (mm) of the test plate. For all assays, isolates with inhibition rate ≤ 30% were considered resistant, isolates with inhibition rate ≥ 90% were considered sensitive, and isolates with inhibition rate between 30% and 90% were considered moderately resistant. The whole experiment was conducted twice.

Determination of EC_{50} values

The EC_{50} values, representing the concentration of the fungicide at which fungal development is inhibited by 50%, were calculated for a selection of sensitive and moderately resistant isolates using mycelial growth assays. Specifically, PDA plates amended with 0, 0.3, 2, 10, 30, 100 µg/ml, and 0, 0.01, 0.05, 0.3, 2, 10, 30, 100 µg/ml azoxystrobin were used to determine EC_{50} values of *C. fioriniae* and *C. nymphaeae* isolates with moderate resistance to azoxystrobin,

respectively. For sensitive *Colletotrichum* spp. isolates, the concentrations were adjusted as 0, 0.01, 0.05, 0.3, 2, 10 μg/ml for both azoxystrobin and thiophanate-methyl using the same medium. Fungicide dilution series were prepared in sterile deionized water. Fungicide amended or unamended PDA plates were prepared one day before the experiment with three replicates per fungicide concentration per isolate, and an active-growing mycelial plug (5-day-old) was placed in the center of each plate. After incubation for five days at 25 °C in the dark, colony diameters were measured and inhibition rates compared to growth on unamended PDA were calculated as described above. The EC_{50} values were calculated by regressing percentage of mycelial growth inhibition against the log of fungicide concentrations.

Amplification and sequencing analysis of fungicide-target genes

A selection of isolates from each *Colletotrichum* species with different resistance phenotypes to azoxystrobin and thiophanate-methyl were examined for mutations in fungicide resistance target genes. The beta-tubulin (*tub2*) gene was amplified and sequenced with the above primers (Table 1) and protocol from a total of eleven *C. siamense* isolates with or without resistance to thiophanate-methyl. The cytochrome b (*cytb*) gene was amplified and sequenced from eight *C. fioriniae*, twelve *C. siamense*, and five *C. nymphaeae* isolates with moderate or high resistance to azoxystrobin. In addition, *cytb* was amplified from six sensitive and 17 resistant *C. nymphaeae* isolates. Primers (Table 1) used to amplify and sequence *cytb* gene in *Colletotrichum* spp. were designed based on the *cytb* sequences of *C. acutatum* (MK118090) and *C. siamense* (KM885299) from GenBank. The thermal cycling protocol for amplification of the *cytb* gene included an initial denaturation at 94 °C for 3 min, followed by 34 cycles of 94 °C for 40 s, annealing at 55 °C for 40 s, and extension at 72 °C for 1 min, and a final elongation at 72 °C for

5 min. PCR reagent mixture, cleanup, sequencing, and sequence analysis were conducted as described in Chapter 1.

Results

Frequency of isolates with resistance to azoxystrobin and/or thiophanate-methyl

All the 197 *Colletotrichum* spp. isolates for which a live isolate was available were screened for fungicide resistance against azoxystrobin. For *C. nymphaeae* 39%, 3% and 58% of the 177 isolates were of a highly resistant (HR), moderately resistant (MR) and sensitive phenotypes (S), respectively (Table 5). All *C. fioriniae* isolates were MR, whereas nine out of 11 *C. siamense* isolates were HR. The other two *C. siamense* isolates were found to be moderately resistant to azoxystrobin, with inhibition rates of 32 and 33% at the discriminatory dose of 100 µg/ml (data not shown). The overall fungicide resistance frequency for azoxystrobin was 40%. In addition, *C. siamense* isolates were screened for resistance to thiophanate-methyl, with 64% and 36% of isolates being HR and S, respectively (Table 5). Interestingly, the one *C. lineola* isolate showed high and moderate resistance to azoxystrobin and thiophanate-methyl, respectively. Moreover, all isolates with resistance to thiophanate-methyl were also found to be resistant to azoxystrobin, despite the limited number of *C. siamense* isolates (Table 5).

Table 5. Resistance phenotype against azoxystrobin and thiophanate-methyl of *Colletotrichum* spp. isolates collected in this study.

	Azoxystrobin			**Thiophanate-methyl**		
Species	**S**	**MR**	**HR**	**S**	**MR**	**HR**
C. nymphaeae	103	5	69	-	-	-
C. fioriniae	0	8	0	-	-	-
C. siamense	0	2	9	4	0	7
C. lineola	0	0	1	0	1	0
Total	103	15	79	4	1	7

The resistance prevalence of *Colletotrichum* isolates recovered from different cultivars, organs and locations

Colletotrichum spp. isolates with resistance to azoxystrobin and/or thiophanate-methyl were obtained from all strawberry cultivars examined in this study (Table 4). However, resistant isolates were less frequently isolated from cultivars grown in matted-row system or from cultivars with higher tolerance to anthracnose disease. For example, all *C. nymphaeae* isolates from Darselect, AC Valley Sunset, or Earliglow were sensitive to azoxystrobin, and only one isolate out of 11 collected from Sweet Charlie was resistant. Additionally, resistant isolates were collected from all plant organs (Table 4). Interestingly, isolates from the fruit, runner, and petiole had a lower frequency of azoxystrobin-resistance, compared to those from the crown. Overall, resistance to these two fungicides was widely distributed in different strawberry cultivars and organs.

The frequency of *Colletotrichum* spp. isolates with resistance to azoxystrobin was indicated for each sampling site (by counties) in the Mid-Atlantic region, as shown in figure 5. Overall frequency of azoxystrobin -MR and -HR isolates, regardless of species, was 48% (Table 5). However, resistant isolates were not found in all locations. For example, no resistant isolates were found in both Carroll and Baltimore counties in Maryland, and only 5% of isolates with resistance were collected in Howard and Queen Anne's counties (Figure 5). In contrast, all isolates collected in Washington and Caroline counties, and 83% of isolates collected in Charles county were resistant. Similarly, widespread resistance to azoxystrobin on *Colletotrichum* spp. isolates was detected in Pennsylvania, with varying levels of frequency at different sites. Although the sampling sites were somewhat limited in Virginia and North Carolina, all isolates

from these two states were found to be highly resistant except for one isolate with moderate resistance (Figure 5).

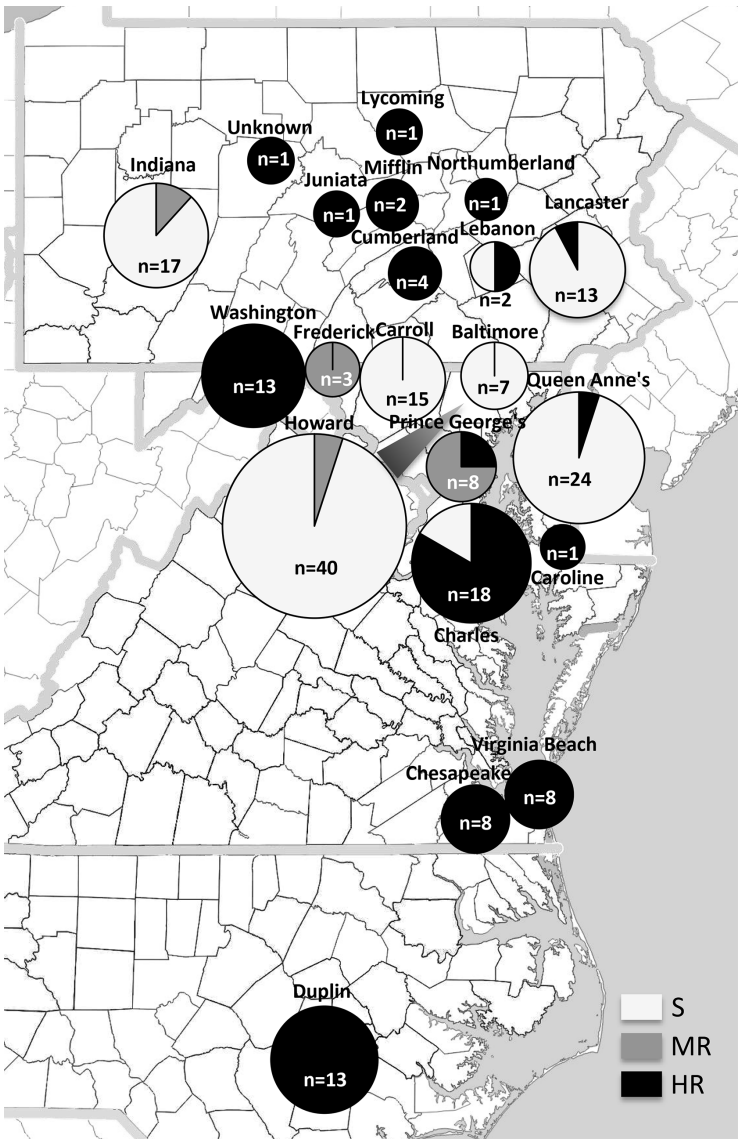


Figure 5. Frequency of *Colletotrichum* spp. isolates sensitive (S), moderately resistant (MR), and highly resistant (HR) to azoxystrobin, as indicated in circles with white, gray, and black color, respectively.

EC_{50} values of *Colletotrichum* spp. isolates with different resistance phenotypes

As mentioned above, all *C. fioriniae* isolates were found to be MR to azoxystrobin, with EC_{50} values ranging from 9.7 to 14.4 µg/ml. The EC_{50} of azoxystrobin -S, -MR and -HR *C. nymphaeae* isolates were 0.21 to 0.36, 2.6 to 7.8, and >100 µg/ml, respectively (Table 6). EC_{50} values of all *C. siamense* and *C. lineola* isolates, including both MR and HR phenotypes, were found to be > 100 µg/ml for azoxystrobin. With regard to thiophanate-methyl, sensitive *C. siamense* isolates had EC_{50} values of 1.1 to 1.3 µg/ml (Table 6).

Resistance-conferring mutations detected in the *cytb* and *tub2* genes

Partial sequences of *cytb* and *tub2* genes from *Colletotrichum* spp. isolates with or without resistance (including S, MR and HR), were sequenced and analyzed for known mutations conferring resistance to QoI and MBC fungicides. The primer pair Cocytb-F1/Cocytb-R1 amplified an approximately 895-bp fragment of *cytb* from both *C. nymphaeae* and *C. fioriniae*. Two sets of primers Cocytb-F2/Cocytb-R3 and Cocytb-F3/Cocytb-R4 were used to amplify an approximately 2,300-bp fragment from *C. siamense*, which possesses a 1912-bp intron. Both fragments contain all "hot spots" conferring resistance to QoIs, including 129, 137 and 143 amino acid positions. As mentioned above, the primer pair T1 and TubR1 were used to amplify a 1300 bp fragment of *tub2* from *C. siamense* isolates, containing amino acid positions of 198 and 120 that have been associated with resistance to MBCs

As a result, no mutation was detected in the *cytb* sequences from the moderately resistant *C. fioriniae* isolates compared with the reference *C. fioriniae* mitochondrial genome (GenBank accession number KU375885). In contrast, all *C. siamense* isolates, including nine HR and two MR phenotypes had glycine replaced by alanine at amino acid position 143 (G143A) when

compared with reference *C. siamense* mitochondrion genome (KX885103). The same mutation was found in all 16 HR *C. nymphaeae* isolates examined, but was not detected in any of the six S isolates examined. Moreover, no other amino acid variations at codon 129 and 137 were found in the *cytb* fragment from either HR or S isolates, comparing with the reference *C. acutatum* mitochondrial genome (NC_027280). With respect to the five *C. nymphaeae* MR isolates, two of them had phenylalanine replaced by leucine at codon 129 (F129L), while no mutations were found in the other three isolates (Table 6). The *cytb* sequences obtained in this study were deposited into GenBank under accession nos. MT740213 - MT740216, MT740212, and MT951627 - MT951628 for *C. nymphaeae*, *C. fioriniae*, and *C. siamense*, respectively. In addition, all seven resistant isolates examined exhibited a substitution of glutamic acid to alanine at codon 198 (E198A) compared with sensitive isolates (Table 6).

Table 6. Characterization of *Colletotrichum* isolates from strawberry with respect to fungicide sensitivity and resistance genotype.

Species/ isolate	Origin		Sensitivity (EC_{50}; µg/ml)[b]			Genotype	
	Location[a]	Year	Azoxystrobin	T-methyl	Phenotype[c]	*cytb*	*tub2*
C. fioriniae							
F14	Prince George's, MD	2019	-[d]	-	MR/-	G143	-
F15	Prince George's, MD	2019	-	-	MR/-	G143	-
F16	Prince George's, MD	2019	9.73	-	MR/-	G143	-
F17	Prince George's, MD	2019	-	-	MR/-	G143	-
F18	Prince George's, MD	2019	10.38	-	MR/-	G143	-
F19	Prince George's, MD	2019	-	-	MR/-	G143	-
F43	Indiana, PA	2019	-	-	MR/-	G143	-
F44	Indiana, PA	2019	14.43	-	MR/-	G143	-
C. siamense							
K792	Caroline, MD	2018	>100	1.27	HR/S	A143	E198
102	Virginia Beach, VA	2019	>100	>100	HR/HR	A143	A198
104	Virginia Beach, VA	2019	>100	>100	HR/HR	A143	A198
K840	Virginia Beach, VA	2018	>100	>100	HR/HR	A143	A198
19-1223	Lancaster, PA	2019	>100	>100	HR/HR	A143	A198
19-1224	Lancaster, PA	2019	>100	>100	MR/HR	A143	A198
19-1225	Lancaster, PA	2019	>100	-	HR/S	A143	-
K833	Virginia Beach, VA	2018	>100	1.29	MR/S	A143	E198
207-2	Virginia Beach, VA	2019	>100	>100	HR/HR	A143	A198
SB19-1P	Prince George's, MD	2019	>100	>100	HR/HR	A143	A198
075	Virginia Beach, VA	2019	>100	1.07	HR/S	A143	E198
K832[e]	Virginia Beach, VA	2018	-	-	-/-	A143	E198
C. nymphaeae							
F23	Charles, MD	2019	0.26	-	S/-	G143	-
SL1300	Queen Anne's, MD	2016	0.21	-	S/-	G143	-
SL515	Howard, MD	2016	0.36	-	S/-	G143	-
F39	Indiana, PA	2019	-	-	S/-	G143	-
SL683	Carroll, MD	2015	-	-	S/-	G143	-
SL510	Howard, MD	2015	-	-	S/-	G143	-
709-1	Fredrick, MD	2019	6.8	-	MR/-	G143	-
709-2	Fredrick, MD	2019	7.8	-	MR/-	G143, L129	-
709-3	Fredrick, MD	2019	2.6	-	MR/-	G143, L129	-
SL490	Howard, MD	2015	2.6	-	MR/-	G143	-

SL577	Howard, MD	2015	5.0	-	MR/-	G143	-
19-667	Cumberland, PA	2019	>100	-	HR/-	A143	-
19-1673	Lycoming, PA	2019	>100	-	HR/-	A143	-
186	Cumberland, PA	2019	>100	-	HR/-	A143	-
443-2	Lancaster, PA	2019	>100	-	HR/-	A143	-
444-4	Lancaster, PA	2019	>100	-	HR/-	A143	-
C4	Chesapeake, VA	2019	>100	-	HR/-	A143	-
F3	Duplin, NC	2019	>100	-	HR/-	A143	-
F7	Duplin, NC	2019	>100	-	HR/-	A143	-
F9	Duplin, NC	2019	>100	-	HR/-	A143	-
F11	Duplin, NC	2019	>100	-	HR/-	A143	-
F13	Duplin, NC	2019	>100	-	HR/-	A143	-
F27	Charles, MD	2019	>100	-	HR/-	A143	-
F31	Charles, MD	2019	>100	-	HR/-	A143	-
K757[e]	Virginia Beach, VA	2018	-	-	-/-	A143	-
SL1482	Washington, MD	2016	>100	-	HR/-	A143	-
SL1485	Washington, MD	2016	>100	-	HR/-	A143	-
SL1488	Washington, MD	2016	>100	-	HR/-	A143	-

[a] Locations are listed by counties in each state where isolates were collected

[b] EC_{50} = the concentration of the fungicide at which mycelia development is inhibited by 50%

[c] */* indicates isolates sensitive, moderately resistant or resistant, respectively, to azoxystrobin and thiophanate-methyl. S = sensitive, R = resistant, MR = moderately resistant

[d] Symbol '-' indicates data was not determined

[e] No live isolates available for K832 and K757, only DNA used for analysis.

Discussion

Both QoI and MBC fungicides are at high risk for resistance development (Hawkins & Fraaije, 2016; Andy Leadbeater, 2012). This fact, combined with heavy reliance on these two chemical classes for strawberry anthracnose management, may have led to a widespread and high frequency of resistance in *Colletotrichum* populations. It is noteworthy that only 15% of 91 fruit isolates collected in 2015 and 2016 were found to be resistant to azoxystrobin, whereas 72% of 64 fruit isolates collected in 2018 and 2019 were resistant (data not shown). Similar shift towards an increased frequency of resistance to QoIs was also observed in *Colletotrichum* populations in Florida strawberry fields (Forcelini et al., 2016b), emphasizing the need for resistance management. Moreover, the majority of our isolates were collected from plasticulture strawberry fields, which had a larger portion of resistant isolates than isolates from matted-row fields. Anthracnose affects annual plasticulture system plantings the most due to the use of plastic mulch, which increases rain splash, and of susceptible cultivars, such as Chandler and Camarosa (M. Rahman et al., 2013; Xiao et al., 2004). As a result, fungicides have been applied more frequently on plasticulture strawberry plants to cope with the high disease pressure, inevitably accelerating the selection for resistance.

While the frequency of resistance to azoxystrobin varied between counties and between states (Figure 5), *C. nymphaeae* isolates from the same field were found to be highly consistent in resistance phenotype in a given year (data not shown). Whether this phenomenon would apply to other species is unknown, due to the limited number of isolates and their scattered distributions. The most straightforward explanation is that isolates from the same field were the progenies of a single or a few isolates that had the same resistance phenotype. Nursery

transplants latently infected with *C. acutatum* species are considered the primary source of inoculum (Legard, 2000; N. A. Peres et al., 2005). When infected transplants are introduced, secondary conidiation can occur on the surface of vegetative tissues under favorable weather conditions shortly after planting, and this can augment inoculum levels (Leandro et al., 2003). As a rain-splashed pathogen, inoculum dispersal within strawberry fields is limited in distance (L. V. Madden et al., 1993b, 1996). These facts all indicate that *C. acutatum* isolates from the same field could have originated from just a few infected plants, which in turn may explain the consistency in their resistance phenotype. This would also imply the importance of managing resistance at nursery level, and that frequency of resistant isolates recovered from the production fields could vary dramatically between years and between sites especially for the plasticulture production system where new transplants are obtained from different sources and planted by growers every year. Nevertheless, analysis of genetic diversity within these isolates will further provide insights into origin and dispersal of strawberry anthracnose.

Interestingly, all *C. siamense* isolates with resistance to thiophanate-methyl exhibited resistance to azoxystrobin, but not all azoxystrobin-resistant *C. siamense* isolates were resistant to thiophanate-methyl. This may indicate that resistance to thiophanate-methyl in these isolates may have built on the backbone of resistance to azoxystrobin, despite the limited sample size. QoIs are highly effective against anthracnose (regardless of species) in the absence of resistant populations, leading to the extensive use of this chemical class in strawberry fields (Forcelini & Peres, 2018). Further, some succinate dehydrogenase inhibitors (SDHIs) labeled for use on strawberry are premixtures with QoIs such as Pristine (boscalid plus pyraclostrobin), Merivon (fluxapyroxad plus pyraclostrobin), and Luna Sensation (fluopyram plus trifloxystrobin). These fungicides have been commonly used for strawberry gray mold (caused by *Botrytis cinerea*)

control, which may indirectly select for resistance to QoIs in *Colletotrichum* species. It therefore stands to reason that resistance to azoxystrobin may have evolved earlier than to thiophanate-methyl in *C. siamense*. A statistical model showed that multi-fungicide resistance patterns did not evolve randomly in *Botrytis* populations from small fruits, instead, resistance to multiple fungicides were acquired through a stepwise accumulation of single resistance (Li et al., 2014).

G143A in *cytb* and E198A in *tub2* were found to be linked to isolates with high resistance to azoxystrobin and thiophanate-methyl, respectively. Both mutations have been commonly found in *Colletotrichum* and other fungal isolates with resistance to QoIs and MBCs (Forcelini et al., 2018; Han et al., 2018). Neither G143A nor F129L was found in the *cytb* from three MR *C. nymphaeae* isolates, suggesting other mechanism(s) such as mitochondrial heteroplasmy, alternative respiration, or efflux transporters may be responsible for the resistance (Fernández-Ortuño et al., 2008). Similarly, two *C. nymphaeae* isolates from a strawberry field in Ohio were resistant to azoxystrobin, but no mutation in *cytb* was detected (Chechi et al., 2020). Moreover, G143A or other mutations in *cytb* was either absent or not consistently found in QoI-resistant *Podosphaera fusca* (H. Ishii et al., 2001), *Venturia inaequalis* (Steinfeld et al., 2001), and *Monilinia* species (Hily et al., 2010). All *C. fioriniae* isolates collected were found to be moderately resistant azoxystrobin, and no mutations were detected in their *cytb* sequences. Due to the limited number of *C. fioriniae* isolates obtained, it is difficult to conclude whether the moderate resistance is a natural trait in the population. In a previous study, one *C. fioriniae* isolate from apple was shown to have much higher sensitivity to azoxystrobin (EC_{50} = 0.05 µg/ml), indicating the existence of a more sensitive phenotype (Chechi et al., 2019). However, two genetically distinct subgroups within *C. fioriniae* have been previously identified (Damm et al., 2012) and all *C. fioriniae* isolates used in this study belong to the subgroup 1 (data not

shown). Interestingly, the sensitivity of subgroup 1 and 2 to demethylation inhibitors, including difenoconazole, propiconazole, flutriafol, and fenbuconazole, were found to be significantly different (Chen et al., 2016). Similar differential fungicide sensitivity has also been documented for multiple species within the genus of *Colletotrichum* (Ishii et al., 2016). The one isolate of *C. lineola* exhibited resistance to azoxystrobin and thiophanate-methyl, analyses of phenotypes and genotypes in more isolates will reveal whether these are innate traits. Species within the *C. acutatum* complex are naturally insensitive to MBCs (Adaskaveg & Hartin, 1997; Peres et al., 2004) due to the enhanced expression of beta-tubulin 1 gene (Nakaune & Nakano, 2008). Sensitivity of those isolates to thiophanate-methyl was thus not determined (Table 6).

Frequent and widespread resistance detected in the local fungal population to primary fungicides poses a challenge for disease management. Studies have shown that fitness cost may not be associated with QoI resistance conferred by *cytb* G143A in *C. acutatum* isolates (Forcelini et al., 2018), further questioning the continued use of this chemical class. However, given the variation in resistance profiles between locations, resistance monitoring for specific locations will better aid in spray decisions. While use of molecular methods developed based on resistance-conferring mutations will facilitate resistance screening, it could underestimate resistance frequency due to the lack of *cytb* mutations in MR phenotypes revealed in our study. Future investigation on the efficacy of QoIs for the control of MR *Colletotrichum* isolates will be of interest. Although a few other fungicides such as captan are considered less effective against *Colletotrichum* species, they are less prone to resistance development and thus need to be integrated into the spray program. In addition, disease tolerant strawberry cultivars, non-chemical control measures, and cultural practices that reduce water-splash should be considered whenever possible, aiming to minimize the use of at-risk fungicides.

Chapter 3: Population genetic analysis on *C. nymphaeae* collection

Introduction

Population genetics consider the origin, maintenance and spatiotemporal distribution of genetic variation of species under the influence of mutation, gene flow, recombination, drift and selection (Zhan, 2016). The major focus of population genetics is to understand the evolutionary processes shaping and maintaining genetic variation within and among populations (Milgroom & Peever, 2003). Changes in genotype or allele frequencies in certain populations are considered evolutionary changes. The ultimate aims of a population genetics study should focus on understanding evolutionary history of pathogen, through which the evolutionary changes have occurred and predicting the potential evolution. Additionally, the aim should include the application of the acquired knowledge for sustainable plant disease management (Zhan, 2016). Since the 1980s, studies of genetic variation of plant pathogens have become very common, in part because of the availability of molecular genetic markers (Milgroom, 1997).

Molecular markers have been widely used to analyze the population dynamics of plant pathogens because they have a high level of precision and accuracy (Milgroom & Peever, 2003; Moges et al., 2016; Peixoto-Junior et al., 2014). DNA markers are rapid, highly specific, and have a low detection limit (M. G. Milgroom & Peever, 2003; Moges et al., 2016). Over the years, advances in population genetics research methodology have led to widespread use of codominant molecular markers (Vieira et al., 2016). Microsatellites or single sequence repeats (SSRs), and more recently, single nucleotide polymorphism (SNPs) are found in prokaryotes and eukaryotes. They are widely distributed throughout genomes (Pérez-Jiménez et al., 2013; Phumichai et al.,

2015), so they are usually developed from genomic DNA through the construction of SSR-enriched libraries (Moges et al., 2016). SNPs or SSRs require sequence information but they are locus specific, allowing the amplification of fungal DNA in a background of mixed DNA (De Backer et al., 2013). Most genotyping is performed with polymerase chain reaction (PCR) with oligonucleotide primer pairs (Schuelke, 2000). To analyze the length of the PCR products by electrophoresis or a laser detection system, fluorescent dye is added to one of the primer pair, which may be 6-carboxy-fluorescine (FAM) (Schuelke, 2000).

Population genetics analyses have been conducted on *Colletotrichum spp*. populations in the past with various goals. For example, a previous study conducted at two locations in Australia for three years revealed that sexual reproduction of *Colletotrichum tanaceti* was more prevalent in Tasmania than in Victoria based on evolutionary analysis using microsatellite primers. *C. tanaceti* most likely originated in Tasmania, which may have subsequently spread into Victoria, suggesting that *C. tanaceti* has a high evolutionary potential (Lelwala et al., 2019). The results from Rockenbach et al. (2016) suggested that isolates of *Colletotrichum fructicola* from Brazil capable of causing apple bitter rot and Glomerella leaf rot arose independently of those from Uruguay. Another study indicated that isolates of *Colletotrichum sublineola* on wild sorghum and Johnson grass were genotypically diverse in the southeastern U.S. using molecular markers. Further findings suggested that Johnson grass has the potential to serve as a refuge for *C. sublineola*, which can complicate efforts to develop and deploy resistant sorghum varieties in the region (Xavier et al., 2018).

Evolution history and relatedness among lineages of *Colletotrichum nymphaeae* isolates causing strawberry anthracnose can provide insights into the etiology of the disease and genetic polymorphism (Ureña-Padilla et al., 2002). The epidemiology of *Colletotrichum* spp. is unclear

on strawberry in the Mid-Atlantic region, and the detection of genetic polymorphism within a specific area can indicate the disease source, and/or gain insights into fungicide resistance evolution such as QoI resistance. As *C. nymphaeae* is the predominant species causing strawberry fruit anthracnose rot in the Mid-Atlantic region, efforts were then made to explore the genetic diversity within the *C. nymphaeae* populations.

Materials and methods

Selection of isolates and microsatellite primer

In total, 178 *C. nymphaeae* isolates were included in this experiment. Microsatellite primer candidates were selected from the primer pairs listed in Penet et al., 2017 and Ciampi et al., 2011. Primer candidates from the two publication had been evaluated with *C. acutatum* or *C. gloeosporioides* complex species, but not with *C. nymphaeae* (Table 7). Eight isolates of *C. nymphaeae* from four different states were selected to evaluate the suitability of those microsatellite primer candidates, using a touch-down thermal cycling protocol. PCR reactions were performed using the T100 Bio-Rad thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA). The total reaction volume was 25 µl, containing 12.5 µl 2 x Taq Master Mix (Apex™, Genesee Scientific Corporation, San Diego, CA), 9.5 µl water, 1 µl forward and reverse primer (10 µM), and 1 µl template DNA. The touch-down thermal cycling protocol for amplification included an initial denaturation at 95 °C for 5 min; and 5 cycles of 95 °C for 20 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min; followed by 20 cycles of 95 °C for 20 s, annealing temperature starting at 60 °C in the first cycle for 30 s with minus 0.5 °C per cycle, and extension at 72 °C for 1 min; then 12 cycles of 95 °C for 20 s, annealing at 50 °C for 30 s,

and extension at 72 °C for 1 min; and extension at 72 °C for 1 min; and a final elongation at 72 °C for 5 min.

Table 7. Microsatellite primer candidates selected from two publications

Primers from Ciampi et al., 2011			Primers from Penet et al., 2017				
CA01	CA13	CA23	CG12	CG90	CG109	CG136	CG163
CA02	CA14	CA24	CG14	CG91	CG115	CG137	CG164
CA03	CA15	CA25	CG16	CG92	CG116	CG144	
CA04	CA16	CA26	CG19	CG93	CG120	CG149	
CA08	CA17	CA27	CG37	CG95	CG122	CG150	
CA09	CA18		CG53	CG96	CG127	CG156	
CA10	CA19		CG68	CG97	CG129	CG159	
CA11	CA20		CG71	CG98	CG57	CG161	
CA12	CA22		CG83	CG110	CG131	CG162	

Table 8. Microsatellite primers used in the population genetics analysis

Primer name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Reference
CA03	GCACTGGCCGATTGATTTTC	CGGGTTGACGGACGGATGA	Ciampi et al., 2011
CA08	CTCTCACACACACACACACT	GAAGCTAGACAGAGTTACCC	Ciampi et al., 2011
CA12	GCATGCATACTTCTGGGTTG	CATTGTCTCTCCGTGCTGAC	Ciampi et al., 2011
CA13	GTAAACGAAAAGGCGGTCTG	CGGAGTATATGCAGCTACCAAC	Ciampi et al., 2011
CA22	CCGATTGGTGGCTTGC	TGGAGATGTCGGAGATGTTG	Ciampi et al., 2011
CA26	GTGCCAATAACGAGCCATC	CGTAAAGGAGGTTTGCCTTC	Ciampi et al., 2011
CG92	CATTTTCCACAGCCCACAC	GCAGCAGGTGTGAGAAGAGA	Penet et al., 2017

PCR amplification and genotyping

After the screening of microsatellite primers, selected microsatellite primer pairs were used for the remainder of 178 *C. nymphaeae* isolates on 96-well plates following the touch-down PCR protocol described above. The plates were then sent to Cornell Institute of Biotechnology (Ithaca, NY) for fragment analysis with size standard LIZ500. Fragment analysis raw data attained from Cornell were analyzed with software GeneMarker v3.0.1 (SoftGenetics LLC, PA).

Raw data were input into GeneMarker and were run with microsatellite dinucleotide template. The peak detection threshold was set at 10,000 as the minimum intensity and the analyzed data were exported in the allele list format. The abnormal fragment lengths were adjusted manually according to the electropherograms.

Data analysis

All down-stream population genetics analyses were conducted with the R package *poppr*. The genotype accumulation curve is constructed by randomly sampling x loci and counting the number of multilocus genotypes (MLGs). This repeated r time for 1 locus up to n-1 loci, creating n-1 distributions of observed MLGs (Kamvar et al., 2015). The genotypic richness, diversity, and evenness data were calculated with the function monpop_diversity. The Minimum spanning networks MSN (Paradis, 2018) are calculated via bruvo.msn and the function plot_poppr_msn was used to view the MSN in R. Principal coordinate analysis (PCoA) was drawn with function pcoa and neighbor-joining tree was computed with function nj in R.

Results

Selection of microsatellite primers

The primers were selected for the following population genetics analysis if clear fragment(s) were consistently amplified from all the isolates tested (Figure 6). For example, CA26 primer yielded a bright fragment across all the eight isolates, thus this primer was selected for the fragment analysis with all isolates (Figure 6). As a result, seven pairs of microsatellite primers

were selected and the forward primers were added with FAM fluorescent marker at the 5' end (Table 8).

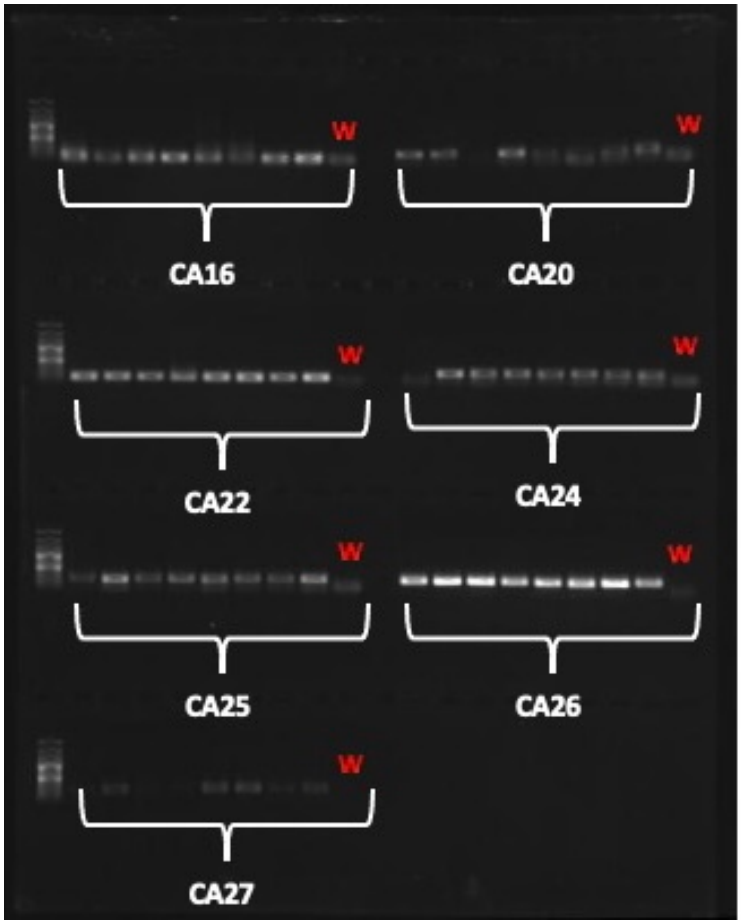


Figure 6. Example of gel electrophoresis picture showing DNA fragments amplified from eight *Colletotrichum* isolates with seven microsatellite primer pairs.

Genotype accumulation curve

A genotype accumulation curve can be useful in determining a threshold needed to distinguish between a given percentage of unique individuals given a random sample of n loci (Grünwald et al., 2017). Figure. 6 demonstrates the genotype accumulation curve for data from this research. The horizontal axis represented the number of loci randomly sampled without replacement up to 6 loci, whereas the vertical axis showed the number of multilocus genotypes observed, up to 47. The red dash line represented 100% of the total observed multilocus genotypes. The curve did

not seem to become plateaued with seven markers, indicating more markers may be necessary to ensure the complete resolution of all genotypes. However, the genotypes that have been resolved could still be useful for downstream analyses, offering a glimpse into the genetic diversity within *C. nymphaeae* population.

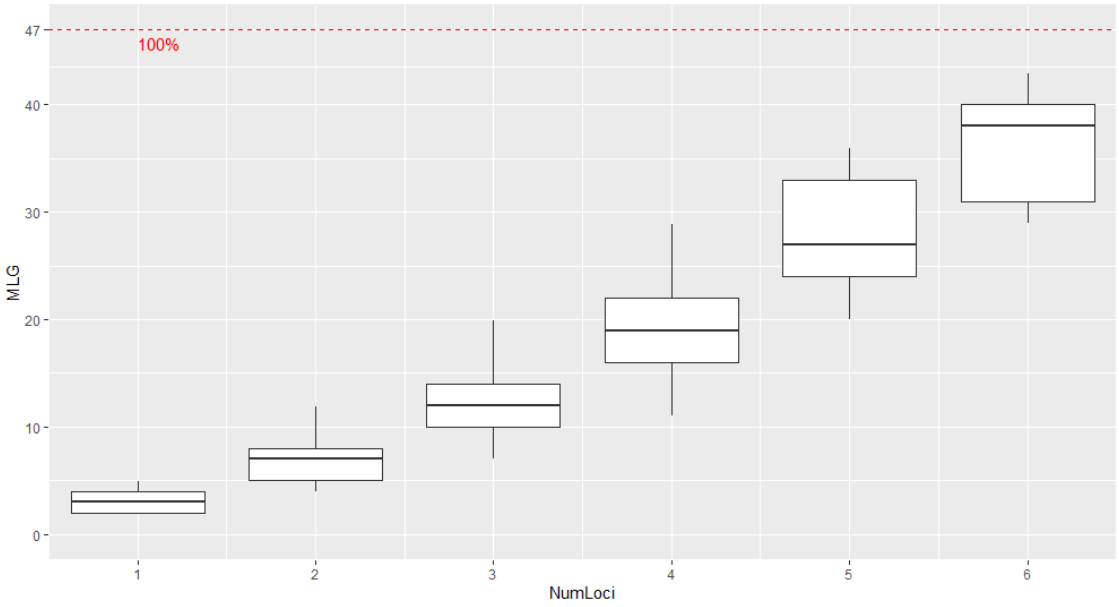


Figure 7. Number of multilocus genotypes resolved with increasing number of microsatellite loci in a population of 178 *C. nymphaeae* isolates from the strawberry fields in the Mid-Atlantic region.

Genotypic richness, diversity and evenness by locations

Basic diversity statistics, including genotypic diversity, evenness, and richness, were calculated (Grünwald et al., 2017). The number of observed MLGs is equivalent to genotypic richness. Isolates from Maryland had the largest richness, while isolates from Virginia and North Carolina were with lowest richness, presumably due to the uneven sample size (Table 9). Evenness is a measure of the distribution of genotype abundances, wherein a population with equally abundant genotypes yields a value equal to 1 and a population dominated by a single genotype is closer to zero (Grünwald et al., 2003). In our study, the MLGs observed in the Virginia population are the

closest to equal abundance (*E.5* = 0.840), followed by North Carolina, Pennsylvania and Maryland. Diversity measures incorporate both genotypic richness and abundance, and three measures of genotypic diversity were employed by *poppr*, the Shannon-Wiener index (H), Stoddart and Taylor's index (G), and Simpson's index (lambda). Similarly, based on these three calculated indexes, isolates from Maryland seemed to have the highest diversity, whereas isolates from North Carolina had the lowest. However, the number of isolates from VA, PA and NC are small, which may result in some bias in the analysis.

Table 9. The genotypic richness, diversity, and evenness of 178 *C. nymphaeae* isolates

Pop	N	MLG	eMLG	SE	H	G	Lamda	Corrected 1-D	E.5	Hexp	la	rBarD
MD	121	40	7.30	1.303	2.96	9.660	0.897	0.903	0.474	0.389	0.15	0.02
NC	12	7	6.15	0.657	1.70	4.24	0.764	0.833	0.724	0.365	0.50	0.17
PA	36	14	5.83	1.223	2.04	4.63	0.784	0.806	0.543	0.372	0.01	0.002
VA	9	7	7.00	0	1.83	5.40	0.815	0.916	0.840	0.359	0.474	0.12
Total	178	47	6.98	1.333	2.91	8.61	0.884	0.888	0.438	0.381	0.156	0.02

Pop: population
N: number of individuals observed
MLG: Number of multilocus genotypes (MLG) observed
eMLG: The number of expected MLG at the smallest sample size ≥ 10 based on rarefaction
SE: Standard error based on eMLG
H: Shannon-Wiener Index of MLG diversity (Shannon, 2001)
G: Stoddart and Taylor's Index of MLG diversity (Stoddart & Taylor, 1988)
lambda: Simpson's Index (Simpson, 1949)
E.5: Evenness, E_5
Hexp: Nei's unbiased gene diversity (Nei, 1978; Niklaus J. Grünwald et al., 2003)
la: The index of association, I_A (Brown et al., 1980; J. M. Smith et al., 1993)
rbarD: The standardized index of association

Minimum spanning networks with reticulation

Minimum spanning networks (MSN) are the method to visualize genetic relatedness among MLGs represented by size of the node and genetic distance among MLGs shown with connecting branches (Nei, 1978). Networks with reticulation provide the ability to show clusters of genetically like individuals. In the figures 7 and 8, each node represents a unique MLG, node shading (color) represent population membership, while edge widths and shading represent relatedness, edge length is arbitrary (Kamvar et al., 2014). From the 2 figures, no evident genetic relatedness neither between 4 different location populations nor 3 QoI fungicide resistance phenotype populations was observed.

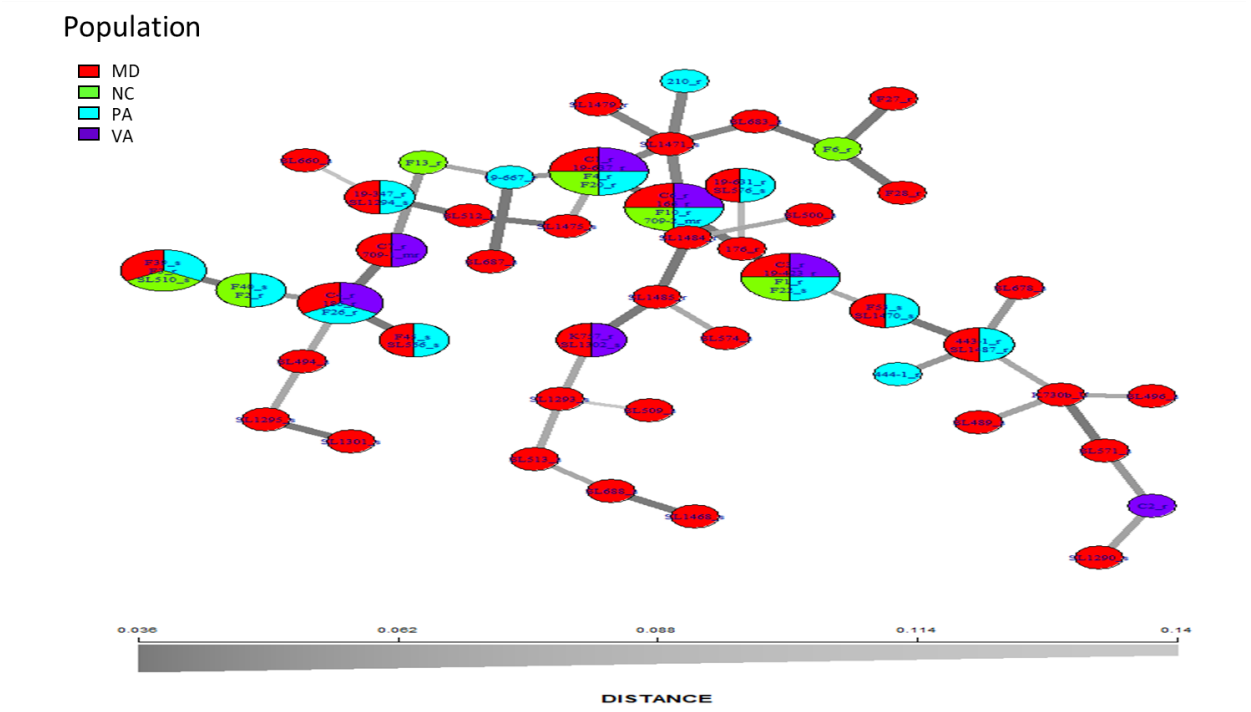


Figure 8. Minimum spanning networks showing the distribution of 178 *C. nymphaeae* isolates that grouped by locations.

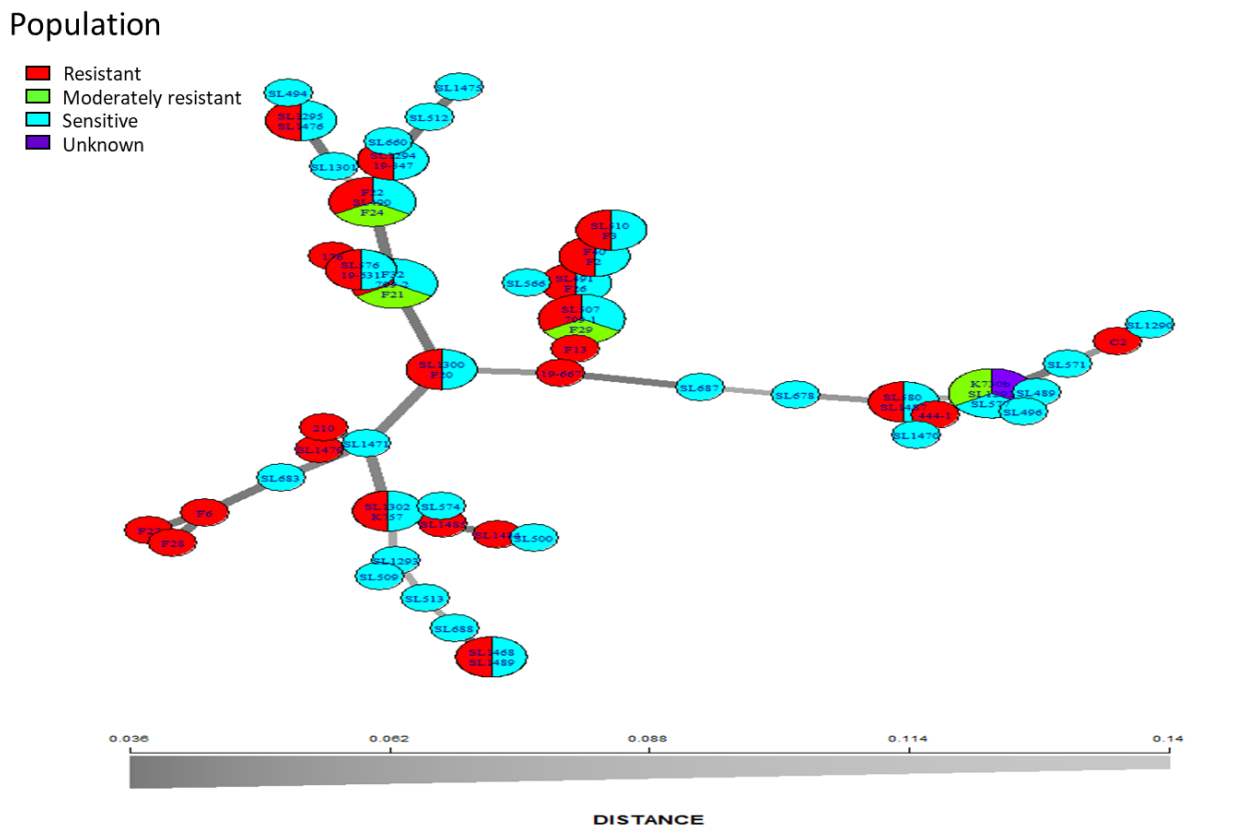


Figure 9. Minimum spanning networks showing the distribution of 178 *C. nymphaeae* isolates that grouped fungicide resistance phenotype

PCoA and NJ tree by location

Principal coordinate analysis (PCoA) is often used to show genetic similarity among populations, with populations clustered according to their geographical location and species identity (Muriira et al., 2018). Our PCoA results showed that the MD population is somewhat different in structure compared to the VA population, whereas PA and NC populations seem to largely overlap with the MD population. Neighbor-joining is one of the most widely used methods for constructing evolutionary trees, where distance matrices obtained from allele frequencies are used to produce a representation of population relationships (Kopelman et al., 2013). Similarly, the NJ tree suggested that VA population is less closely related to MD population, compared to the other two populations.

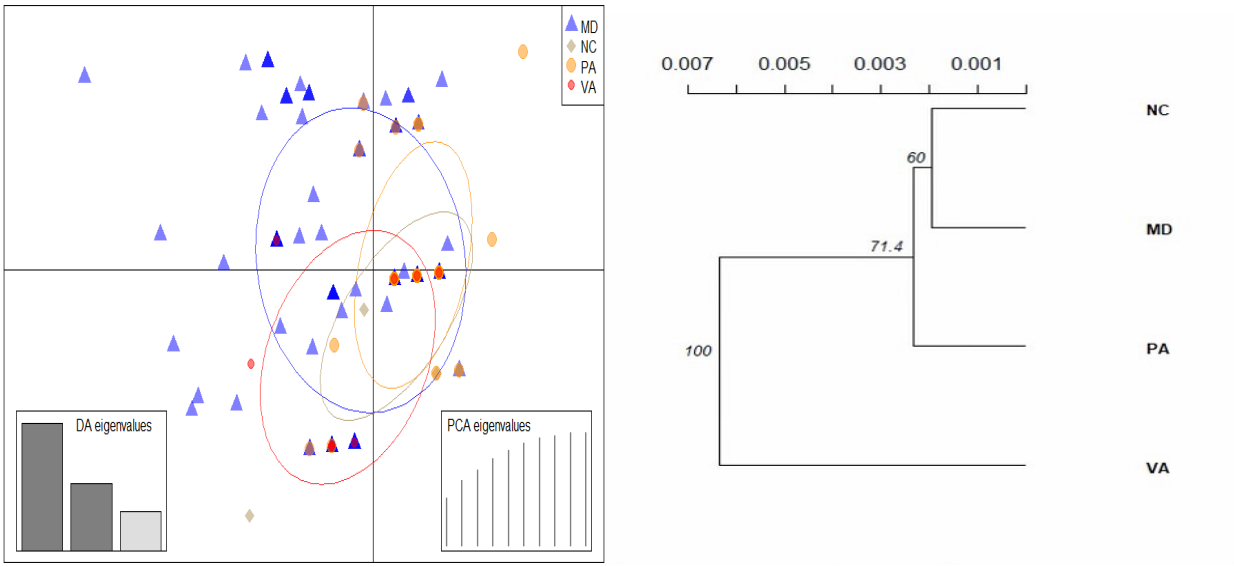


Figure 10. PcoA (left) and NJ tree (right) of 178 *C. nymohaeae* isolates in this study.

Discussion

Various factors can affect genetic diversity (Bennett et al., 2005). Among them, mutation, population gene flow, and sexual and asexual recombination are the main mechanism by which genetic diversity can be generated in populations of pathogenic microorganisms (Marulanda et al., 2014). Based on our preliminary results, it is not clear whether *C. nymphaeae* populations from different locations are genetically distinct. More genetic loci may be needed for better resolution of genotypes, as indicated by the accumulation curve (Figure 7).

In this study, no evident QoI fungicide resistance evolution history can be observed in the Mid-Atlantic *C. nymphaeae* populations (Figure 9). QoI fungicides represent one of the most widely used groups of fungicides to control agriculturally important fungal pathogens. Chen et al., (2007) conducted the first case study of the evolutionary process of QoI resistance in the

Plasmopara viticola population, and demonstrated that the G143A substitution had occurred several times in a plant-pathogen system and pathogen populations might be under strong directional selection for local adaption to fungicide pressure. Our results seem to also support that the emergence of alleles conferring resistance to QoI fungicides were on several occasions in *C. nymphaeae* populations from strawberry fields.

In conclusion, the microsatellite markers selected in this study were useful to comprehend the genetic diversity and population structure of *C. nymphaeae* isolates from strawberry growing in the Mid-Atlantic region. However, the sample size is small for the NC, PA and VA populations, which may have resulted in some bias in the downstream population genetic analyses. Further, more markers may be needed to further resolve genotypes of *C. nymphaeae* populations. Regardless, data generated from this study could be a foundation of future experiments.

Summary

In this study, a total of 200 *Colletotrichum* spp. isolates were obtained from various tissues of strawberry showing symptoms of anthracnose fruit rot or crown rot in the Mid-Atlantic fields. Based on morphological and phylogenetical characterizations, four *Colletotrichum* species including *C. nymphaeae*, *C. siamense*, *C. fioriniae*, and *C. lineola* were identified. While *C. nymphaeae* and *C. fioriniae* belong to *C. acutatum* species complex, *C. siamense* belongs to *C. gloeosporioides* species complex. *C. nymphaeae* was found to be predominant, compromising nearly 90% of the total isolates. Further, *C. nymphaeae* was isolated from all the host tissues examined. Analysis of sensitivity to QoI and MBC fungicides showed that resistance is widespread in *Colletotrichum* spp. isolates. The resistance-conferring mutation G143A was found in *C. nymphaeae* isolates with high levels of QoI-resistance, whereas F129L was detected in isolates moderately resistant to QoIs. With respect to molecular basis of resistance to MBCs, E198A mutation was found in all the MBC-resistant *C. siamense* isolates. These findings present a challenge for the sustainable production of strawberry in the Mid-Atlantic region, warranting the search for non-chemical control practices and breeding for resistant cultivars. In addition, a preliminary experiment was conducted to study genetic diversity within the *C. nymphaeae* populations. As a result, Maryland population seems to be somewhat different in the structure compared to Virginia population, whereas Pennsylvania and North Carolina populations seem to largely overlap with Maryland population. However, it is noteworthy that the sample size was quite limited for all states except Maryland, not allowing for drawing solid conclusions. No evident genetic relatedness was observed neither between four states nor three QoIs fungicide resistance phenotype populations.

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