Resistance in *Colletotrichum siamense* From Peach and Blueberry to Thiophanate-Methyl and Azoxystrobin

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Abstract

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Anthraxnose fruit rot was observed in some late-season peach cultivars in South Carolina in the 2012 and 2013 production seasons as well as increased anthracnose leaf spot of blueberry in a commercial operation of the same state in 2012. Single-spore isolates of *Colletotrichum siamense* were either sensitive or resistant to both thiophanate-methyl and azoxystrobin with the concentration of the fungicide at which fungal development is inhibited by 50% of ≥ 100 $\mu\text{g/ml}$. Resistant isolates revealed the E198A mutation in β -tubulin and the G143A mutation in cytochrome b. Nucleotide sequence analysis of the complete *CYTb* gene from genomic DNA of *C. siamense* isolates revealed an intronless

genotype (CsI) and a genotype revealing two introns (CsII) at amino acid positions 131 and 164. Resistance to thiophanate-methyl or azoxystrobin was not found in isolates of *C. fructicola* collected from peach fruit. The *CYTb* gene of isolates of this species was of the CjII genotype or revealed a unique CjIIa genotype. Phylogenetic analysis of *C. siamense* isolates from different locations and different crops showed that the resistant isolates were genetically closer to each other than to sensitive isolates, suggesting that field resistance to thiophanate-methyl and azoxystrobin fungicides is derived from a common ancestor.

Anthraxnose of peach has been reported throughout peach production areas of the United States and is primarily caused by *Colletotrichum acutatum* J. H. Simmonds, and *C. gloeosporioides* (Penz.) Penz. & Sacc. (Adaskaveg and Hartin 1997; Bernstein et al. 1995; Kim and Hong 2008). Both species are also found in the southeastern United States (Bernstein et al. 1995), where a third species (*C. truncatum*) was isolated from peach (Grabke et al. 2014). *C. gloeosporioides* is recognized as a species complex (Weir et al. 2012) and two species, *C. fructicola* and *C. siamense*, were identified to cause anthracnose of peach in South Carolina (Hu et al. 2015). Significant losses from peach anthracnose were prominent during the late 1940s, especially in the southeastern states (Petersen 1953).

Chemical control of preharvest disease is important for conventional peach production, and focuses on control of brown rot caused by *Monilinia fructicola* (G. Winter) Honey. Site-specific fungicides such as methyl benzimidazole carbamates (MBCs) and quinone outside inhibitors (QoIs) are effective against *M. fructicola*, and both chemical classes have been used since they have become available in the 1970s and 1990s, respectively. The MBCs include benzimidazoles and thiophanates and both act by inhibition of nuclear division via mitosis by binding to β -tubulin (*TUB2*) subunits (Davidse 1986; Köller 1999). Resistance to MBC fungicides has been detected in many fungal species (Chung et al. 2010; Ma and Michailides 2005). In most cases, resistance was associated with point mutations in the *TUB2* gene, which result in altered amino acid sequences at the binding site. Results from previous studies have shown that changes at codon 6, 50, 167, 198, 200, and 240 in the *TUB2* gene could cause MBC resistance in field isolates of pathogenic fungi (Ma and Michailides 2005). Among those amino acid positions, mutations at positions 198 and 200 are most commonly detected in plant fungal pathogens (Albertini et al. 1999; Banno et al. 2008; Baraldi et al. 2003; Chen et al. 2013; Koenraad et al. 1992; Luck and Gillings 1995; Ma and Michailides 2005). Mutations at different codons in

the β -tubulin may result in different resistance levels to MBC fungicides; E198A generally confers higher levels of resistance compared with F200Y (Chen et al. 2013; Chung et al. 2006, 2010). The direct involvement of the mutations in conferring resistance to benzimidazoles has been confirmed by site-directed mutagenesis followed by gene replacement (Li et al. 1996).

QoI fungicides inhibit mitochondrial respiration of fungi by binding to the cytochrome *bc1* enzyme complex (complex III) at the Qo site, blocking electron transfer and halting ATP synthesis (Bartlett et al. 2002). Resistance to QoIs was detected in field populations of several important plant pathogens soon after QoIs were first introduced in 1996 (Bartlett et al. 2002). In most cases, QoI resistance is conferred by point mutations in the mitochondrial cytochrome b (*CYTb*) gene (Ma and Michailides 2005). They include a mutation leading to an amino acid change from phenylalanine to leucine at codon 129 (F129L) (Kim et al. 2003) and from glycine to alanine at codon 143 (G143A) (Bardas et al. 2010; Bäuml et al. 2003; Bolton et al. 2013; Ma et al. 2003). F129L generally confers lower levels of resistance compared with G143A (Gisi et al. 2002; Kim et al. 2003; Sierotki et al. 2007). The direct involvement of this mutation in conferring resistance to QoIs has been confirmed by site directed mutagenesis in the *CYTb* of *Saccharomyces cerevisiae* (Fisher et al. 2004).

Although *C. acutatum* is naturally resistant to MBC fungicides (Peres et al. 2004), *C. gloeosporioides* isolates have been reported to be sensitive. Therefore, MBC fungicide thiophanate-methyl is recommended only for management of diseases caused by *C. gloeosporioides* (Brannen and Smith 2014b). However, *C. gloeosporioides* can become resistant to MBCs and resistant isolates were reported from several crops, including mango (Spalding 1982), avocado (Sanders et al. 2000), statice (Maymon et al. 2006), citrus (Peres et al. 2004), and strawberry (Chung et al. 2010). In these isolates, mutations E198A and F200Y in the *TUB2* gene were reported to be responsible for high and moderate levels of resistance, respectively (Chung et al. 2006, 2010; Kongtragoul et al. 2011; Nalumpang et al. 2010), and cross resistance among MBC fungicides was observed (Chung et al. 2010; Wong et al. 2008). Because MBCs do not control all species of *Colletotrichum*, the propensity for MBC resistance development in formerly sensitive species, and poor efficacy of many fungicide classes against *Colletotrichum* spp., QoI fungicides have become the primary choice for anthracnose control caused

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by *Colletotrichum* spp. in commercial peach orchards and commercial strawberry and blueberry fields in the United States (Brannen and Smith 2014a,b; Horton et al. 2014). Resistance in *C. gloeosporioides* to QoI fungicides is still rare and has only been found in isolates from strawberry in Japan. In these isolates, the G143A mutation in *CYTb* was associated with resistance, and cross resistance was found among QoI fungicides azoxystrobin and kresoxim-methyl (Inada et al. 2008). *C. gloeosporioides* isolates with resistance to QoI fungicides or with multiple resistance to both QoIs and MBCs have not been reported in the United States.

In most years, anthracnose is considered a minor disease of peach that occurs only sporadically on fruit. However, anthracnose has become an increasing problem for many commercial fruit producers in South Carolina with a history of spraying MBC and QoI fungicides. For example, during summer 2012 and 2013, anthracnose incidence rose to commercially unacceptable levels (more than 50% preharvest fruit loss) in some late-season peach cultivars. In these cases, orchards had received applications of MBC and QoI fungicides for anthracnose control. Additionally, five *Colletotrichum* isolates from a commercial 'Southern Highbush' blueberry farm in South Carolina and three isolates from strawberry fields in Florida were included in this study. The objective of this study was to (i) characterize the sensitivity of *Colletotrichum* isolates to thiophanate-methyl and azoxystrobin, (ii) investigate molecular mechanisms of resistance, and (iii) compare the efficacy of multiple chemical classes of fungicides for the control of thiophanate-methyl- and azoxystrobin-resistant isolates.

Materials and Methods

Fungal isolates and culture conditions. *C. siamense* and *C. fructicola* isolates of 'Sugar Lady' and 'Sweet Dream' peach fruit showing symptoms of anthracnose disease were collected from commercial peach farms near Chesnee, McBee, Ridge Spring, and Monetta, SC during summer 2012 and 2013 (Table 1). All collection sites were a minimum of 100 km from each other. Grower cooperators used MBCs, QoIs, sterol demethylation inhibitors (DMIs), and succinate dehydrogenase inhibitors (SDHIs) for summer disease control. Summer diseases included primarily peach anthracnose, brown rot, and Alternaria rot. MBCs and the QoIs are the only site-specific fungicides registered for control of *C. gloeosporioides* of those fungicides used for summer disease control. Single-spore isolations of *Colletotrichum* spp. were conducted as described previously (Hu et al. 2015).

Isolates from blueberry leaves were collected in 2012 from an unknown Southern Highbush blueberry near Fairfax, SC (Table 1). The farm also had used MBC and QoI fungicides for years to manage leaf spots and fruit diseases. The *Colletotrichum* isolates were recovered by surface sterilizing symptomatic leaves in 1% sodium hypochlorite for 2 min, rinsing leaves with sterile water twice, and placing a 5-by-5-mm disk of the necrotic area in potato dextrose agar (PDA) medium. Only one isolate was recovered from a single leaf. Resulting colonies were incubated at 22°C with a 12 h-cycle of fluorescent light, and single-spore isolates were prepared from spores produced on PDA, as described previously (Hu et al. 2015). Each single spore isolate was stored as dried mycelium on filter paper (Whatman Inc., Maidstone, UK) at -20°C, as previously described (Zhu et al. 2012).

C. gloeosporioides strains CG-162, FL98-139, and FL99-21 from strawberry were obtained from Dr. Barbara J. Smith, United States Department of Agriculture, Poplarville, MS (CG-162) and Dr. Natália A. Peres, University of Florida, Wimauma, FL (FL98-139 and FL99-21) and used as reference strains sensitive to thiophanate-methyl and azoxystrobin (Table 1). All *Colletotrichum* isolates were identified to the species level using a multiplex polymerase chain reaction (PCR) identification method (Hu et al. 2015).

Determination of azoxystrobin and thiophanate-methyl sensitivities using a microtiter assay. Conidial suspensions (2×10^4 conidia/ml) of all the isolates were prepared in double concentrated malt extract broth (pH 5.5). The active ingredients azoxystrobin and thiophanate-methyl were obtained as formulated compounds Abound Flowable and Topsin M 70WP, respectively. The following

final concentrations were used in the microtiter assay: 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3 ppm active ingredient for azoxystrobin and 0, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 ppm active ingredient for thiophanate-methyl. The addition of salicylhydroxamic acid (SHAM) was not deemed necessary for this spore germination assay because the assay with and without SHAM yielded similar results (data not shown). Fungicide dilution series were prepared in sterile deionized water. Each fungicide solution (50 µl) was transferred into 96-well microtiter plates and mixed with 50 µl of conidial suspension (final concentration: 1,000 conidia/well). Three replicates were used for each isolate and fungicide concentration. For each of the fungicide concentrations, three replicated wells were used as blanks (fungicide solution + medium). The plates were incubated for 4 days at 22°C in the dark, and mycelial growth was assessed measuring light absorption at A600 nm with a photometer. The experiment was repeated once. The values for each fungicide concentration were corrected using the corresponding blanks. The concentration of the fungicide at which fungal development is inhibited by 50% (EC₅₀) values were determined for each isolate.

DNA and RNA extraction from fungal isolates. *Colletotrichum* isolates were grown on PDA at 25°C for 4 days in the dark. Agar plugs containing actively growing mycelium were taken from the periphery of the advancing colonies and transferred to 250-ml flasks containing 40 ml of potato dextrose broth. Flasks were shaken at 110 rpm for 4 days at 25°C. Developing mycelium was removed from the broth and rinsed under sterile deionized water. Genomic DNA and total RNA were subsequently extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) and the MasterPure Yeast RNA Extraction Kit (Epicentre, Madison, WI), according to the manufacturer's recommendations. Quantification of all nucleic acids was carried out using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA).

Cloning of the full-length *CYTb* gene sequence. At the time of this study, the *CYTb* sequence from *C. gloeosporioides* or any of its subspecies was not available in GenBank. Therefore, we cloned the gene based on the full-length *CYTb* sequence from *C. graminicola* M1.001 genome sequence, which was available at the Broad Institute of MIT and Harvard (<http://www.broadinstitute.org>). Primers Cg_cytbF1, Cg_cytbF2, Cg_cytbR1, and Cg_cytbR2 were designed to amplify the corresponding fragment in *Colletotrichum* spp. (Table 2). Two "initial" fragments (fragments 1 and 2) were successfully amplified using primer pairs Cg_cytbF1/Cg_cytbR2 and Cg_cytbF2/Cg_cytbR1, respectively. After sequencing the initial fragments 1 and 2, primers Cg_cytbF3 and Cg_cytbR3 were designed to amplify a third fragment from *Colletotrichum* spp. Based on the aligned sequence from those three fragments, inverse PCR (IPCR) was conducted to amplify the 5' and 3' ends of the *CYTb* gene.

PCR volumes were 25 µl containing 1× PCR buffer provided by the manufacturer, 20 ng of template DNA, 0.8 µM each gene-specific primer, 200 µM each dNTP, and 1 U of Taq DNA polymerase (New England Biolabs, Ipswich, MA). The amplification parameters for fragment 1 and fragment 3 both consisted of an initial denaturation at 94°C for 4 min followed by 34 cycles of 94°C for 50 s, 53°C for 50 s, and 72°C for 2.5 min. A final elongation step of 5 min at 72°C was always incorporated. The parameters for amplification of fragment 2 were largely as described above, except that the extension time was reduced to 1 min.

*Bam*HI, *Eco*RI, *Eco*RV, and *Hind*III PCR libraries were synthesized for IPCR. Briefly, 500 ng of genomic DNA was digested with each of the four restriction enzymes (CUTSMART, Ipswich, MA) at 37°C for 12 h, then inactivated at 65°C for both *Eco*RI and *Eco*RV and 80°C for *Hind*III for 20 min. Products were ligated overnight using T4 DNA ligase (New England Biolabs) at 16°C, then inactivated at 65°C for 10 min. The resulting product (2 µl) (libraries) were used in IPCR as template. IPCR was carried out similarly to the above-described PCR, with minor changes. The annealing temperature was adjusted for each primer, and the 72°C extension step was performed for 3 min.

All PCR and IPCR products were separated on agarose gels in 1× Tris-acetate EDTA buffer at 100 V for 40 min. Unincorporated

primers and dNTPs remaining in both PCR and IPCR products were removed using ExoSAP-IT reagent (Affymetrix, Santa Clara, CA). The IPCR products were then cloned into the pCR4-TOPO vector according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Sequencing was conducted at Clemson University Genomics

Institute (Clemson, SC). After the *CYTb* gene was cloned, a set of sequencing primers was designed to sequence the entire genomic *CYTb* gene from *C. siamense* isolates OD13-5 and RR13-8, *C. fructicola* isolates SE12-2 and RR12-3. Three primer pairs were designed to amplify and sequence the entire *CYTb* gene by generating three

Table 1. Characterization of *Colletotrichum siamense* and *C. fructicola* isolates with respect to fungicide sensitivity and *CYTb* genotype

Species, isolate	Origin			Sensitivity (EC ₅₀ ; µg/ml) ^x		Phenotype ^y	Mutation ^z (cytochrome b, β-tubulin)	CYTB genotype
	Host	Location	Year	Azoxystrobin	T-methyl			
<i>C. siamense</i>								
EY12-1	Peach	Chesnee, SC	2012	0.020	3.352	S/S	–	CsII
EY12-2	Peach	Chesnee, SC	2012	0.020	4.363	S/S	–	CsII
EY12-3	Peach	Chesnee, SC	2012	0.017	5.496	S/S	–	CsII
EY12-4	Peach	Chesnee, SC	2012	0.037	4.152	S/S	–	CsII
EY12-5	Peach	Chesnee, SC	2012	0.019	4.338	S/S	–	CsII
EY12-6	Peach	Chesnee, SC	2012	0.017	5.341	S/S	–	CsII
EY12-7	Peach	Chesnee, SC	2012	0.025	4.883	S/S	–	CsII
EY12-8	Peach	Chesnee, SC	2012	0.022	4.682	S/S	–	CsII
EY12-9	Peach	Chesnee, SC	2012	0.021	5.378	S/S	–	CsII
EY12-10	Peach	Chesnee, SC	2012	0.017	5.333	S/S	–	CsII
IL12-1	Blueberry	Fairfax, SC	2012	>100	>100	R/R	G143A, E198A	CsII
IL12-2	Blueberry	Fairfax, SC	2012	>100	>100	R/R	G143A, E198A	CsII
IL12-3	Blueberry	Fairfax, SC	2012	>100	>100	R/R	G143A, E198A	CsII
IL12-4	Blueberry	Fairfax, SC	2012	>100	>100	R/R	G143A, E198A	CsII
IL12-5	Blueberry	Fairfax, SC	2012	>100	>100	R/R	G143A, E198A	CsII
OD12-1	Peach	McBee, SC	2012	>100	>100	R/R	G143A, E198A	CsII
OD12-2	Peach	McBee, SC	2012	>100	>100	R/R	G143A, E198A	CsII
OD12-3	Peach	McBee, SC	2012	>100	>100	R/R	G143A, E198A	CsII
OD12-4	Peach	McBee, SC	2012	>100	>100	R/R	G143A, E198A	CsII
OD12-5	Peach	McBee, SC	2012	>100	>100	R/R	G143A, E198A	CsII
OD12-6	Peach	McBee, SC	2012	>100	>100	R/R	G143A, E198A	CsII
OD12-7	Peach	McBee, SC	2012	>100	>100	R/R	G143A, E198A	CsII
OD12-8	Peach	McBee, SC	2012	>100	>100	R/R	G143A, E198A	CsII
OD12-9	Peach	McBee, SC	2012	>100	>100	R/R	G143A, E198A	CsII
OD12-10	Peach	McBee, SC	2012	>100	>100	R/R	G143A, E198A	CsII
OD13-1	Peach	McBee, SC	2013	>100	>100	R/R	G143A, E198A	CsII
OD13-2	Peach	McBee, SC	2013	>100	>100	R/R	G143A, E198A	CsII
OD13-3	Peach	McBee, SC	2013	>100	>100	R/R	G143A, E198A	CsII
OD13-4	Peach	McBee, SC	2013	>100	>100	R/R	G143A, E198A	CsII
OD13-5	Peach	McBee, SC	2013	>100	>100	R/R	G143A, E198A	CsII
OD13-6	Peach	McBee, SC	2013	>100	>100	R/R	G143A, E198A	CsII
OD13-7	Peach	McBee, SC	2013	>100	>100	R/R	G143A, E198A	CsII
OD13-8	Peach	McBee, SC	2013	>100	>100	R/R	G143A, E198A	CsII
OD13-9	Peach	McBee, SC	2013	>100	>100	R/R	G143A, E198A	CsII
OD13-10	Peach	McBee, SC	2013	>100	>100	R/R	G143A, E198A	CsII
RR12-2	Peach	Ridge Spring, SC	2012	0.015	4.945	S/S	–	CsII
RR12-4	Peach	Ridge Spring, SC	2012	0.030	5.514	S/S	–	CsII
RR12-6	Peach	Ridge Spring, SC	2012	0.016	3.988	S/S	–	CsII
RR13-1	Peach	Ridge Spring, SC	2013	0.016	5.877	S/S	–	CsII
RR13-2	Peach	Ridge Spring, SC	2013	>100	>100	R/R	G143A, E198A	CsI
RR13-3	Peach	Ridge Spring, SC	2013	>100	>100	R/R	G143A, E198A	CsI
RR13-4	Peach	Ridge Spring, SC	2013	>100	>100	R/R	G143A, E198A	CsI
RR13-5	Peach	Ridge Spring, SC	2013	>100	>100	R/R	G143A, E198A	CsI
RR13-6	Peach	Ridge Spring, SC	2013	>100	>100	R/R	G143A, E198A	CsI
RR13-7	Peach	Ridge Spring, SC	2013	>100	>100	R/R	G143A, E198A	CsI
RR13-8	Peach	Ridge Spring, SC	2013	>100	>100	R/R	G143A, E198A	CsI
RR13-9	Peach	Ridge Spring, SC	2013	0.017	4.874	S/S	–	CsII
RR13-10	Peach	Ridge Spring, SC	2013	0.020	5.951	S/S	–	CsII
FL98-139	Strawberry	FL	1998	0.017	3.537	S/S	–	CsII
FL99-21	Strawberry	FL	1999	0.017	3.838	S/S	–	CsII
<i>C. fructicola</i>								
RR12-1	Peach	Ridge Spring, SC	2012	0.021	4.661	S/S	–	CfIIa
RR12-3	Peach	Ridge Spring, SC	2012	0.022	4.312	S/S	–	CfII
RR12-5	Peach	Ridge Spring, SC	2012	0.032	4.743	S/S	–	CfIIa
SE12-1	Peach	Monetta, SC	2012	0.031	8.743	S/S	–	CfIIa
SE12-2	Peach	Monetta, SC	2012	0.027	8.662	S/S	–	CfIIa
CG-162	Strawberry	Unknown	N/A	0.019	2.378	S/S	–	CfII

^x EC₅₀ = the concentration of the fungicide at which fungal development is inhibited by 50%.

^y S/S and R/R indicate isolates sensitive or resistant, respectively, to both thiophanate-methyl and azoxystrobin.

^z Symbol: – indicates that no mutation was detected.

smaller fragments 1, 2, and 3, each less than 2 kb in size (Table 2). Primers used to amplify and sequence the *CYTb* gene are listed in Table 2. DNASTAR software (DNASTAR Inc., Nevada City, CA) was used to assemble and align the nucleotide and amino acid sequences. All nucleotide sequences were compared with previously reported sequences using BLAST (Altschul et al. 1990).

Investigation of intron-related variations in *CYTb* of *Colletotrichum* spp. isolates. *CYTb* genes with intron-related variations were identified in *C. siamense* and *C. fruticola* isolates using a two-step amplification system. Primer pair IPCR_F1/DetectR1 was located within introns flanking the amino acid 143-containing codon. It only amplified a fragment if two full-sized introns were present. The DNA not yielding a fragment with this primer was subjected to primer pairs D1F/D2R to amplify intronless coding regions and CF1/CR2 to amplify the amino acid 143-containing region from DNA with a deletion in intron 1. Reactions were incubated at 94°C for 3 min; followed by 34 cycles of 94°C for 40 s, 52°C for 40 s, and 72°C for 1 min; with a final elongation step of 72°C for 5 min.

Cloning of the full-length *CYTb* coding sequences. First-strand cDNA synthesis was carried out using 1 µg of total RNA from *C. siamense* isolate OD13-5 and *C. fruticola* isolate RR12-3 using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's recommendations. Amplification of *CYTb*-specific cDNA was performed using 2 µl of cDNA in a final volume of 25 µl. Primers Cytb-F1/Cytb-R1 were designed to amplify the *CYTb* sequence from translational start codon to translational stop codon on the basis of comparison of the full-length *CYTb* gene sequences from our *Colletotrichum* isolates (in this study) and the coding sequences from closely related species *C. graminicola* (strain M1.001). Reactions were incubated at 94°C for 3 min; followed by 34 cycles of 94°C for 40 s, 50°C for 40 s, and 72°C for 1.5 min; with a final elongation step of 72°C for 5 min.

Screening of *TUB2* and *CYTb* sequences for the presence of mutations conferring resistance to thiophanate-methyl and azoxystrobin. Primer pair TubF1/TubR1 was designed based on the *TUB2* sequence of *C. aeshynomene* from GenBank (U14138) to amplify the *TUB2* gene fragment containing the region of interest (corresponding to codons 198 to 200). The *CYTb* sequences containing amino acid position 143 of *C. siamense* isolates were amplified with primer pairs Cg_cytbF3/DetectR1, CF1/DetectR1, and D1F/Cg_cytbR3. Primer pair Cg_cytbF3/DetectR1 was used to amplify the fragment from genotypes containing two full-sized introns, primer pair CF1/DetectR1 was used to amplify the fragment from isolates with a short intron 1, and primer pair D1F/Cg_cytbR3 was used to obtain the fragment from intronless isolates. Thermal cycling parameters were identical to those described in the "Investigation of intron-related variations in *CYTb* of *Colletotrichum* spp. isolates" section.

Phylogenetic analysis. The phylogenetic relationships between sensitive and resistant isolates from different locations were investigated using a combined data matrix of three coding loci (Hu et al. 2015). The primers, PCR parameters, and conditions used to amplify the calmodulin (*CAL*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and *TUB2* genes were as previously described (Hu et al. 2015), except that TubI2 (5' to 3': GGA TCT TGG AGA TCA GGA GG) was used as the reverse primer instead of TubR1 to amplify and sequence the *TUB2* fragment from the *Colletotrichum* isolates RR13-2 to RR13-8. Multiple sequence alignments were conducted using DNASTAR (DNASTAR Inc.) and CLUSTALX v. 2.1 (Larkin et al. 2007; Thompson et al. 1997). *CAL*, *GAPDH*, and *TUB2* sequences of *C. aeshynomenes* strain ICMP 17673 (JX009721, JX009930, and JX010392), *C. fruticola* strain ICMP 18645 (JX009666, JX009992, and JX010408), and *C. gloeosporioides* strain ICMP 17821 (JX009731, JX010056, and JX010445) were obtained from GenBank as reference sequences. Maximum likelihood

Table 2. Primers utilized in this study

Primer name	Sequence (5' → 3')	Description
Cg-cytbF1	ATGAGAATTTTAAAAAGTCATCC	Amplified and sequenced initial fragment 1
Cg-cytbR2	ACGATATCTGTCCAATTCATGG	Amplified and sequenced initial fragment 1
Cg-cytbF2	AGGTGTTTCAGGTAATTACG	Amplified and sequenced initial fragment 2
Cg-cytbR1	TTAACGAAACCGCAAGTAC	Amplified initial fragment 2
Cg-cytbF3	ATCGCCTTGGTCTTACTACTATCG	1) Amplified and sequenced initial fragment 3
	...	2) Amplified the "hot spot" region of the <i>CYTb</i>
Cg-cytbR3	GCAGCTGGAGTTTGCATAGGGTT	1) Amplified and sequenced initial fragment 3
	...	2) Amplified and sequenced the "hot spot" region of the <i>CYTb</i>
Cytb-F1	ATGAGAATATTTAAAAAGCCATCC	1) Amplified and sequenced fragment 1
	...	2) Amplified and sequenced the entire cDNA
Cytb-walkR1	TGCAGTACATCTGAAGTACCAC	1) Amplified and sequenced fragment 1
	...	2) Sequenced initial fragment 1
CR2	TCTTGTTCAATTCATGGGATAGC	1) Amplified and sequenced the fragment 1 from <i>C. fruticola</i> isolates with deletion in intron 1
	...	2) Identified the deletion in intron 1 in the <i>C. fruticola</i> isolates
CF1	ATCTACCAAGACAAGACCGTC	1) Amplified and sequenced fragment 2
	...	2) Amplified the "hot spot" region of the <i>CYTb</i>
	...	3) Identified the deletion in intron 1 in the <i>C. fruticola</i> isolates
3EndIR	ACTTCTTGACAGATCAGTTCT	Amplified and sequenced fragment 2
CF2	TGTAGGACTAATAGATGGAG	Amplified and sequenced fragment 3
Cytb-R1	TTATTTACTGGCTTGAATCGATG	1) Amplified and sequenced fragment 3
	...	2) Amplified and sequenced the entire cDNA
IPCR-R1	TGCTTGGATTGTAATGCATAGC	1) Amplified 5' ends sequence in IPCR
	...	2) Amplified 3' ends sequence in IPCR
IPCR-F1	AGAGACTCGTGCAACACGTGT	1) Amplified and sequenced 5' ends sequence in IPCR
	...	2) Investigated the introns of <i>CYTb</i> in the <i>Colletotrichum</i> spp.
IPCR-F2	TGGCTAACCCATGCAAACTCC	Amplified and sequenced 3' ends sequence in IPCR
Detect-R1	CATGGTTATATATCCTTTTGGTG	1) Investigated the introns of <i>CYTb</i> in the <i>Colletotrichum</i> spp.
	...	2) Amplified and sequenced the "hot spot" region of the <i>CYTb</i>
D1F	AGCTATGCATTACAATCCAAGC	1) Verified the intron free <i>CYTb</i> genotype in the <i>C. siamense</i> isolates
	...	2) Amplified the "hot spot" region of the <i>CYTb</i>
D2R	CCAGAAACACCTAATGGGTTAC	Same as D1F
TubF1	ACTTCGTCTTCGGCCAGTCTG	Amplified and sequenced the "hot spot" region of β-tubulin gene
TubR1	TTCTGGACGTTGCGCATCTG	Amplified the "hot spot" region of β-tubulin gene

(ML) methods were used to carry out phylogenetic reconstruction using MEGA version 6.0 (Tamura et al. 2013). The ML tree was inferred using the following settings: the Tamura 3-parameter model, γ distributed (five categories), and heuristic method using Subtree-Pruning-Regrafting-extensive. The bootstrap consensus tree inferred from 1,000 replicates was used to represent the evolutionary history of the taxa analyzed (Felsenstein 1985).

Fungicide sensitivity assay on detached peach fruit. Commercial formulations of azoxystrobin (Abound Flowable; Syngenta Crop Protection, LLC, Greensboro, NC), cyprodinil (Vanguard WG; Syngenta Crop Protection, LLC), propiconazole (AmTide Propiconazole 41.8% EC; AmTide, LLC, Irvine, CA), thiophanate-methyl (Topsin M 70WP; United Phosphorus, Inc., King of Prussia, PA), cyprodinil plus fludioxonil (Switch 62.5WG; Syngenta Crop Protection, LLC), difenoconazole plus cyprodinil (Inspire Super; Syngenta Crop Protection, LLC), and difenoconazole plus propiconazole (Inspire XT; Syngenta Crop Protection, LLC) were used in vivo for fungicide sensitivity assays.

Ripe but still firm peach fruit were washed with soap and water to remove fungicide residue, then surface sterilized for 2 min in 1% sodium hypochlorite and rinsed with sterile, distilled water. After the fruit had been air dried, they were sprayed to runoff with Abound Flowable at a concentration of 2.42 ml/liter, AmTide Propiconazole 41.8%EC at 0.62 ml/liter, Inspire Super at 3.12 ml/liter, Inspire XT at 1.09 ml/liter, Switch 62.5WG at 2.09 g/liter, Topsin M 70WP at 3.60 g/liter, or

Vanguard WG at 0.75 g/liter using a hand sprayer (Consolidated Plastic Company, Inc., Twinsburg, OH). All the fungicides were dissolved in sterile, distilled water. Untreated fruit were sprayed with sterile, distilled water. Sensitive isolates included one *C. fructicola* isolate (CG-162 from Florida) and two *C. siamense* isolates (EY12-3 from South Carolina and FL98-139 from Florida). They represented isolates from both species and different hosts (Table 1). All resistant isolates were from location McBee (OD isolates), where the most severe outbreaks of anthracnose were observed. The methods for inoculation and incubation were as described previously (Hu et al. 2015). Control fruit received sterile water without conidia. After 6 days of incubation, lesion diameters were measured using a digital caliper. Three replicates of three fruit per replicate were used for each treatment and each isolate. The experiment was repeated once. For the data analysis, a statistical model was developed that related the in vivo responses in lesion diameter to the experimental replication, isolate, fungicide treatment, and their combination. The method of least squares was used to estimate the model terms connected with the factors. If a factor was found to be significant, mean separation (using Tukey's honestly significant difference) was used to further determine the nature of the effect of the factor on the responses. Bartlett's test for equal variances was performed to test whether the lesion diameter data of the two independent experiments could be combined. Analysis of variance and significant difference procedures were conducted using JMP Pro 10.0.0 software (SAS Institute Inc., Cary, NC) and all tests were performed with $\alpha = 0.05$.

Results

Identification of isolates to species level. Isolates sensitive or resistant to fungicides from peach collected in 2012 were identified to the species level previously (Hu et al. 2015). Isolates collected in 2013 from peach and isolates collected in 2012 from blueberry leaves were identified as *C. siamense* using a multiplex PCR method and sequencing of the *CAL*, *GAPDH*, and *TUB2* genes (Fig. 1). Similarly, the three reference strains from strawberry sensitive to thiophanate-methyl and azoxystrobin from Florida were identified as *C. fructicola* (CG-162) and *C. siamense* (FL98-139 and FL99-21). No *C. fructicola* isolates were recovered in 2013 in South Carolina.

Sensitivity to azoxystrobin and thiophanate-methyl in *C. siamense* and *C. fructicola* isolates. All *C. fructicola* isolates used in this study were sensitive to both azoxystrobin and thiophanate-methyl, with EC_{50} values of 0.019 to 0.032 and 2.378 to 8.743 $\mu\text{g/ml}$, respectively (Table 1). In all, 7 of the 10 *C. siamense* isolates from Ridge Spring (RR-) isolated in 2013 and all isolates from McBee (OD-) and Fairfax (IL-) were resistant to both azoxystrobin and thiophanate-methyl. EC_{50} values were greater than 100 $\mu\text{g/ml}$ for both fungicides (Table 1). All *C. siamense* isolates from Chesnee (EY-), and 3 of 10 isolates from Ridge Spring were sensitive to both azoxystrobin and thiophanate-methyl, with EC_{50} values of 0.015 to 0.037 and 3.352 to 5.951 $\mu\text{g/ml}$, respectively. Interestingly, isolates resistant to thiophanate-methyl were always resistant to azoxystrobin and vice versa. For the remainder of this article, isolates resistant to thiophanate-methyl and azoxystrobin are referred to as dual-resistant isolates.

Isolation and analysis of the full-length *CYT8* sequences from *C. siamense* and *C. fructicola*. To investigate molecular mechanisms of resistance to azoxystrobin and gene polymorphism based on intron-related variations, the target gene *CYT8* was cloned from *C. siamense* isolates sensitive and resistant to azoxystrobin. The same gene was amplified in its entirety from *C. fructicola* isolates to investigate potential gene polymorphism. Four genotypes were identified based on intron-related variations. Genotypes CsI and CsII were found in *C. siamense* isolates and CfII and CfIIa in *C. fructicola* isolates. CsI lacked introns, CsII and CfII each had two introns with minor nucleotide sequence variations, and CfIIa had two introns that also differed in sequence from CfII and CsII and that revealed a 641-bp deletion in intron 1 (Fig. 2; Tables 3 and 4).

The *CYT8* gene sequences from *C. siamense* isolates were 1,176 bp (CsI) or 4,354 bp (CsII) in length (Fig. 2; Table 3). Dual-resistant RR13-2 to RR13-8 isolates from 2013 were genotype CsI but OD and IL isolates were genotype CsII. The two introns were

Fig. 1. Maximum likelihood (ML) tree inferred from the data set containing the combined DNA sequences of *CAL*, *GAPDH*, and *TUB2* genes of *Colletotrichum siamense* isolates from different hosts and geographical areas. *C. siamense* isolates resistant to thiophanate-methyl and azoxystrobin are indicated. The host of *C. fructicola* isolate CG-162 and *C. siamense* isolates coded IL and FL are indicated in the figure; all other *C. siamense* isolates were from peach fruit collected in South Carolina. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying the neighbor-join (NJ) and BioNJ method. A discrete γ distribution was used to model evolutionary rate differences among sites (five categories [+G, parameter = 0.1126]). There were 1,653 positions in the final dataset.

located at amino acid positions (5' to 3') 131 and 164, with sizes of 1,912 and 1,266 bp, respectively (Fig. 2; Table 3). *C. siamense* isolates sensitive to both fungicides were genotype CsII (Fig. 2). Isolates resistant to azoxystrobin (RR13-, OD, and IL) possessed a cytosine instead of guanine at nucleotide position 428 for the CsI genotype and position 2,340 for the CsII genotype. Both mutations encoded a change of glycine to alanine at amino acid position 143 (G143A).

The *CYTb* gene sequences of *C. fructicola* genotypes CfII and CfIIa were 4,332 and 3,701 bp in length, respectively (Fig. 2; Table 3). Introns were located at the amino acid positions 131 and 164 as well. The nucleotide sequences of intron 1 and intron 2 from CsII were 99.5 and 97.7% identical, respectively, to those of CfII (Table 4). Introns 1 and 2 from CfII were 91.5% (excluding missing fragment) and 98.9% identical, respectively, to those of CfIIa (Table 4). The *CYTb* coding sequences from the two species were identical and both were 1,176 bp in length. The complete coding sequences of *CYTb* from *C. siamense* isolate OD13-5 and *C. fructicola* isolate RR12-3 were deposited in GenBank with accession numbers KM885303 and KM885304, respectively. The exon and intron sequences of *C. siamense* isolates OD13-5 and RR13-8 and *C. fructicola* isolates RR12-3 and SE12-2 are available in GenBank under accession numbers KM885299, KM885300, KM885301, and KM885302, respectively.

Analysis of intron-related variations in *CYTb* in *Colletotrichum* spp. isolates. The primer pair IPCR_F1/DetectR1 amplified a 531-bp region from all CsII and CfII genotypes, and primer pair D1F/D2R amplified a 497-bp fragment from CsI genotypes. The CF1/CR2 primer pair amplified a fragment 295 bp in length from CfIIa. The *CYTb* gene from *C. siamense* isolates OD, EY, IL, FL, and RR (13-1, -9, and -10) were all genotype CsII, while *C. siamense* isolates RR13-2 to RR13-8 were genotype CsI. The 641-bp deletion in intron 1 (genotype CfIIa) was detected in *C. fructicola* isolates RR12-1, RR12-5, SE12-1, and SE12-2 but not in genotype CfII isolates RR12-3 and CG-162 (Table 1).

Mutations E198A and G143A confer resistance to thiophanate-methyl and azoxystrobin in *C. siamense*. The primer pair TUB_F1/TUB_R1 was used to amplify a 792-bp fragment of the *TUB2* gene. This fragment contained codons 198 and 200 of *C. siamense* and *C. fructicola* isolates. The dual-resistant *C. siamense* isolates had a single adenine-to-cytosine substitution at position 328 of the PCR product. The predicted translation of this sequence indicates that this mutation results in a substitution of alanine for glutamic at amino acid position 198 (E198A; Table 1).

Three primer pairs—Cg_cytbF3/DetectR1, CF1/DetectR1, and D1F/Cg_cytbR3—amplified fragments 568, 575, and 657 bp in size from the *CYTb* gene from genotypes CsII and CfII, CfIIa, and CsI, respectively. Each fragment included codons 129 to 143. G143A was detected in dual-resistant isolates but not in sensitive isolates (Table 1). The *TUB2* fragment from *C. siamense* isolates EY12-6, OD12-5, and *C. fructicola* isolate RR12-5 were deposited in GenBank with accession numbers KM885296, KM885297, and KM885298, respectively.

Genetic relationships among *C. siamense* isolates sensitive and resistant to fungicides. Phylogenetic trees were generated from a data set of combined *G3PDH*, *CAL*, and *TUB2* gene sequences from *C. siamense* isolates and reference strains ICMP 17673, ICMP 18645, and ICMP 17821. In the ML phylogenetic tree (Fig. 1), *C. siamense* OD13 and RR13 isolates of peach collected in 2013 and isolates from blueberry coded IL12 collected in 2012, as well as isolates from strawberry coded FL, formed a large clade together with *C. siamense* isolates OD12, EY12, and RR12 collected from 2012. This clade was in close proximity to the *C. aescynomenes* reference isolate. The dual-resistant *C. siamense* isolates OD, RR13-2 to RR13-8, and IL12 from different locations and crops were more closely related to each other than to sensitive isolates (Fig. 1). The dual-resistant isolates RR13-2 to RR13-8 of genotype CsI from Ridge Spring were most closely related to the dual-resistant isolates OD12 and OD13 from McBee. The *CAL* gene (accession numbers KM885287, KM885288, and KM885289), *GAPDH* gene (accession numbers KM885290, KM885291, and KM885292), and *TUB2* gene (accession numbers KM885293, KM885294, and KM885295) sequences of isolates IL12-2, RR13-1, and RR13-6, respectively, were submitted to GenBank.

Efficacy of fungicides against *Colletotrichum* spp. isolates sensitive or resistant to thiophanate-methyl and azoxystrobin in detached fruit assays. Lesion diameter data of each isolate of the two independent experiments were merged, because variances between experiments were equal ($P > 0.05$). Sensitive isolates were most effectively controlled by thiophanate-methyl, propiconazole, difenoconazole plus propiconazole, and cyprodinil plus fludioxonil. They were controlled, although less effectively, by azoxystrobin, cyprodinil, and difenoconazole plus cyprodinil. Isolates resistant to thiophanate-methyl and azoxystrobin were not controlled by thiophanate-methyl or azoxystrobin but they were controlled by propiconazole, difenoconazole plus propiconazole, and cyprodinil plus fludioxonil. Similar to

Fig. 2. *CYTb* genotypes CsI, CsII, CfII, and CfIIa of *Colletotrichum siamense* and *C. fructicola* isolates. Boxes indicate exonic sequences and lines signify introns. Dashed lines indicate the missing fragments. Vertical, solid triangles point to codon 143.

sensitive isolates, resistant isolates were controlled less effectively by cyprodinil, and difenoconazole plus cyprodinil (Table 5).

Discussion

Anthraxnose fruit rot of peach was observed in several orchards in South Carolina in 2012 but a significant outbreak was observed only in McBee. In the following year, economically unacceptable disease levels were observed in one more location, Ridge Spring. In vitro tests revealed that many of the *Colletotrichum* isolates collected in 2012 and 2013 from orchards with high disease incidence were up to 1,000 times less sensitive to both thiophanate-methyl and azoxystrobin compared with sensitive isolates (Table 1). Isolates from blueberry collected in 2012 were also resistant to both thiophanate-methyl and azoxystrobin fungicides. They had been collected from a commercial highbush cultivar with unusually high (>20%) incidence of anthracnose leaf spot (data not shown). To our knowledge, this is the first report of *Colletotrichum* isolates with confirmed resistance to two different chemical classes of fungicides and the first report of thiophanate-methyl- and azoxystrobin-resistant '*C. gloeosporioides*' isolates from peach and blueberry.

Several mutations in the *TUB2* gene can confer resistance to MBC fungicides but the level of resistance depends on the mutation (Chen

et al. 2013; Nalumpang et al. 2010). The E198A mutation is one of the most potent of all mutations and is able to confer high levels of MBC resistance in *C. gloeosporioides* (Chung et al. 2006, 2010). Similarly, the G143A mutation in the *CYT8* gene can cause higher resistance than F129L in plant-pathogenic fungi (Gisi et al. 2002; Kim et al. 2003; Sierotzki et al. 2007). The dual-resistant *C. siamense* isolates in this study possessed the E198A and G143A mutations. These isolates had EC₅₀ values equal to or greater than 100 µg/ml (Table 1) and were able to cause disease on thiophanate-methyl- and azoxystrobin-treated fruit (Table 5), indicating that these mutations confer qualitative resistance to both thiophanate-methyl and azoxystrobin. Consistent with this result, azoxystrobin-resistant Japanese *C. gloeosporioides* isolate could not be controlled on strawberry seedlings treated with azoxystrobin at 100 µg/ml (Inada et al. 2008).

Two *CYT8* gene genotypes were found in each of *C. siamense* and *C. fructicola* based on intron distribution. Different *CYT8* gene genotypes were also found in *Botrytis cinerea* isolates, one with three introns and the other with four introns (Banno et al. 2009). Mitochondrial introns are classified into two main groups, group I and group II, based on their distinct RNA structures that facilitates their self-splicing activity (Lang et al. 2007; Saldanha et al. 1993). The two introns found in *Colletotrichum* isolates both belong to group I and contain internal open reading frames which facilitate intron propagation to new sites in genes as mobile genetic elements (data not shown) (Dujon 1989; Lambowitz and Perlman 1990; Lazowska et al. 1980; Pellenz et al. 2002). For instance, at amino acid position 164 of the *CYT8* gene, the introns of *M. laxa* and *M. fructigena* were not homologous with introns of *M. yunnanensis* and *M. mumeicola*, although they are species of the same genus (Hu et al. 2011; Yin et al. 2012). The highly divergent sequences between introns at the same position that were observed in closely related species indicate the high mobility of these introns (Yin et al. 2012). The two introns in our *Colletotrichum* isolates were located at amino acid positions 131 and 164 of *CYT8*, respectively (Table 3). These are the same positions at which frequent intron loss events occur (Yin et al. 2012) but they were not located in positions known to protect G143 from being mutated into G143A (Grasso et al. 2006; Luo et al. 2010; Sierotzki et al. 2007). There was no correlation between the *C. siamense* type II genotype and fungicide sensitivity because there were sensitive and dual-resistant isolates in this group. However, all *C. siamense* isolates with intronless *CYT8* genes were resistant to both thiophanate-methyl and azoxystrobin. More isolates from different locations would have to be sampled and studied to determine whether this genotype is always linked to fungicide resistance.

Resistance to thiophanate-methyl and azoxystrobin was conferred by point mutations in the *TUB2* and the *CYT8* gene, respectively, and our isolates either had none or both of these mutations. MBC fungicides have been used for more than 40 years and a large population of *C. siamense* isolates resistant to MBC fungicides has built up over time, which was described for other pathogens (Fernández-Ortuño et al. 2014). It is likely that this population of azoxystrobin-resistant isolates has emerged since their introduction in 1998, and

Table 3. Locations of exonic regions within *CYT8* sequences in *Colletotrichum siamense* and *C. fructicola*^z

Exon	<i>C. siamense</i>		<i>C. fructicola</i>	
	CsI	CsII	CfII	CfIIa
Exon 1	1–1176	1–393	1–393	1–393
...	1–391	1–131	1–131	1–131
Exon 2	...	2,306–2,402	2,305–2,401	1,665–1,761
...	...	132–164	132–164	132–164
Exon 3	...	3,669–4,354	3,647–4,332	3,016–3,701
...	...	165–391	165–391	165–391

^z Numbers in nonbold type indicate the nucleotide locations of exonic regions and numbers in bold type indicate the corresponding amino acid positions of exonic regions.

Table 4. Nucleotide identities between intron 1 and intron 2 of *CYT8* gene from CsII, CfII, and CfIIa

Introns	Nucleotide identity (%) ^z					
	CsII		CfII		CfIIa	
	Intron 1	Intron 2	Intron 1	Intron 2	Intron 1	Intron 2
CsII						
Intron 1	...	–	99.5	–	91.5	–
Intron 2	–	...	–	97.7	–	98.5
CfII						
Intron 1	99.5	–	...	–	91.5	–
Intron 2	–	97.7	–	...	–	98.9

^z Symbol: – indicates no identity.

Table 5. Influence of fungicides of various chemical classes on growth inhibition of *Colletotrichum* spp. isolates sensitive or dual-resistant to thiophanate-methyl and azoxystrobin

Isolates	Phenotype ^z	Growth inhibition (%) ^y						
		Az	Pr	Di plus Cy	Di plus Pr	Cy plus Fl	Tm	Cy
EY12-3	S/S	61.1 a	91.3 a	72.7 a	91.8 a	90.8 a	66.1 a	32.0 a
CG-162	S/S	41.8 b	90.8 a	67.3 ab	93.2 a	81.5 a	77.3 a	30.6 a
FL98-139	S/S	39.6 b	89.5 a	66.8 ab	90.1 a	93.3 a	83.3 a	32.2 a
OD12-3	R/R	–5.0 c	91.3 a	65.6 b	92.0 a	88.4 a	–2.1 b	23.1 a
OD12-5	R/R	–13.3 c	91.2 a	65.7 b	92.1 a	83.4 a	–1.9 b	25.9 a
OD12-9	R/R	–8.8 c	93.0 a	66.6 b	91.6 a	88.3 a	–4.5 b	19.7 a

^y Numbers in each column followed by the same letter are not significantly different at $\alpha = 0.05$ as determined by analysis of variance. Mean separation was conducted using Tukey's honestly significant difference test. Az = azoxystrobin, Pr = propiconazole, Di = difenoconazole, Cy = cyprodinil, Fl = fludioxonil, and Tm = thiophanate-methyl.

^z S/S and R/R indicate isolates sensitive or resistant, respectively, to both thiophanate-methyl and azoxystrobin.

dual-resistant isolates were selected due to frequent rotation of MBC and QoI fungicides. A similar phenomenon was described for *B. cinerea* of strawberry by our research group (Li et al. 2014). In that study, a statistical model showed that resistance to QoI fungicides had likely derived from a pool of isolates that was already resistant to MBC fungicides. With a larger sample size, we would expect to find isolates that are just resistant to thiophanate-methyl. However, this missing link in the evolutionary path toward dual resistance was also rather rare in the *B. cinerea* system (Fernández-Ortuño et al. 2014), despite a large sample size used in the latter study.

In 2012, economically relevant losses to anthracnose disease were reported near McBee (peach) and Fairfax (blueberry), two locations where we found dual-resistant *C. siamense* isolates that year. Resistant isolates were identified in one additional location (Ridge Spring) on peach in 2013. The emergence of resistance in the additional location was caused by genetically similar but still distinguishable genotypes, indicating that resistant isolates emerged independently. As mentioned above, the resistant isolates from peach and blueberry grouped together and were genetically closer to each other than to sensitive isolates from the same crop and location (peach only), suggesting that the resistant isolates characterized in this study, although from different hosts, were derived from a common ancestor (Fig. 1). No isolates sensitive to the two fungicides were recovered from the blueberry farm. Kim et al. (2003) also found that, among the 20 QoI-resistant *Pyricularia grisea* isolates, 15 had identical Pot2 (a repetitive element) fingerprints matching the profile of fingerprint group 2, indicating that the *CYT2* mutations were linked to a certain genotype. The proposed common ancestor of our dual-resistant isolates may have been already resistant to two chemical classes of fungicides and may have emerged by random chance prior to the introduction of the two chemical classes or may have emerged in a stepwise fashion, as described above, as a result of selection pressure. The fact that no *C. fructicola* isolates were obtained from cultivars with high levels of anthracnose disease indicates that populations were controlled successfully with fungicides and that resistance in this species may not have yet developed.

The emergence and localized selection of resistance to two of the most efficacious chemical classes used for control of diseases caused by the *C. gloeosporioides* species complex (*C. siamense* in particular) has significant practical implications. In the southeastern United States, MBC fungicide resistance is already prevalent in *M. fructicola* (Schnabel et al. 2012) and, according to our findings, is likely also prevalent in *C. siamense* populations. QoI fungicides are typically applied in a mixture with SDHI fungicides for preharvest brown rot control. This combination will continue to select for QoI fungicide resistance in *C. siamense* because most SDHIs are not effective and, thus, not registered for anthracnose fruit rot control. DMI fungicides, in particular the triazoles, are widely used in rotation with QoI/SDHI premixtures. Some registered triazoles such as difenoconazole and propiconazole do have documented activity against *Colletotrichum* spp. of chili pepper and turfgrass (Gopinath et al. 2006; Wong and Midland 2007) and this activity was also confirmed in *C. siamense* in our detached-fruit assays. This emphasizes the importance of DMI fungicide use for summer disease and preharvest rot disease of peach. Whether other triazoles registered for peach are also effective against *C. siamense* was not investigated in this study. In regard to strawberry and blueberry, some DMI fungicides and fludioxonil may be viable options to control anthracnose diseases caused by MBC and QoI fungicide-resistant isolates.

In conclusion, the investigation of *C. gloeosporioides* species allowed the discovery of species-specific selection of resistance to two chemical classes, including MBCs and QoIs. The resistant populations observed on two crops, peach and blueberry, and different geographical areas phylogenetically clustered together and, thus, appeared to have been derived from a single common ancestor. From a disease management standpoint, this study stresses the importance of knowing what *Colletotrichum* sp. is present for resistance and

disease management in commercial peach or blueberry production, and also documents that certain DMI fungicides may be valuable rotation partners for resistance management.

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Literature Cited

- Adaskaveg, J. E., and Hartin, R. J. 1997. Characterization of *Colletotrichum acutatum* isolates causing anthracnose of almond and peach in California. *Phytopathology* 87:979-987.
- Albertini, C., Gredt, M., and Leroux, P. 1999. Mutations of the β -Tubulin gene associated with different phenotypes of benzimidazole resistance in the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis*. *Pestic. Biochem. Physiol.* 64:17-31.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Banno, S., Fukumori, F., Ichiishi, A., Okada, K., Uekusa, H., Kimura, M., and Fujimura, M. 2008. Genotyping of benzimidazole-resistant and dicarboximide-resistant mutations in *Botrytis cinerea* using real-time polymerase chain reaction assays. *Phytopathology* 98:397-404.
- Banno, S., Yamashita, K., Fukumori, F., Okada, K., Uekusa, H., Takagaki, M., Kimura, M., and Fujimura, M. 2009. Characterization of QoI resistance in *Botrytis cinerea* and identification of two types of mitochondrial cytochrome b gene. *Plant Pathol.* 58:120-129.
- Baraldi, E., Mari, M., Chierici, E., Pondrelli, M., Bertolini, P., and Pratella, G. C. 2003. Studies on thiabendazole resistance of *Penicillium expansum* of pears: Pathogenic fitness and genetic characterization. *Plant Pathol.* 52:362-370.
- Bardas, G. A., Veloukas, T., Koutita, O., and Karaoglanidis, G. S. 2010. Multiple resistance of *Botrytis cinerea* from kiwifruit to SDHIs, QoIs and fungicides of other chemical groups. *Pest Manag. Sci.* 66:967-973.
- Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. 2002. The strobilurin fungicides. *Pest Manag. Sci.* 58:649-662.
- Bäumler, S., Felsenstein, F. G., and Schwarz, G. 2003. CAPS and DHPLC analysis of a single nucleotide polymorphism in the cytochrome b gene conferring resistance to strobilurins in field isolates of *Blumeria graminis* f. sp. *hordei*. *J. Phytopathol.* 151:149-152.
- Bernstein, B., Zehr, E. I., Dean, R. A., and Shabi, E. 1995. Characteristics of *Colletotrichum* from peach, apple, pecan, and other hosts. *Plant Dis.* 79:478-482.
- Bolton, M. D., Rivera, V., and Secor, G. 2013. Identification of the G143A mutation associated with QoI resistance in *Cercospora beticola* field isolates from Michigan, United States. *Pest Manag. Sci.* 69:35-39.
- Brannen, P., and Smith, P. 2014a. 2015 Southeast Regional Blueberry Integrated Management Guide. Online publication. <http://www.smallfruits.org/SmallFruitsRegGuide/>
- Brannen, P., and Smith, P. 2014b. 2015 Southeast Regional Strawberry Integrated Pest Management Guide. Online publication. <http://www.smallfruits.org/SmallFruitsRegGuide/>
- Chen, F., Liu, X., and Schnabel, G. 2013. Field strains of *Monilinia fructicola* resistant to both MBC and DMI Fungicides isolated from stone fruit orchards in the Eastern United States. *Plant Dis.* 97:1063-1068.
- Chung, W.-H., Chung, W.-C., Peng, M.-T., Yang, H.-R., and Huang, J.-W. 2010. Specific detection of benzimidazole resistance in *Colletotrichum gloeosporioides* from fruit crops by PCR-RFLP. *New Biotechnol.* 27:17-24.
- Chung, W.-H., Ishii, H., Nishimura, K., Fukaya, M., Yano, K., and Kajitani, Y. 2006. Fungicide sensitivity and phylogenetic relationship of anthracnose fungi isolated from various fruit crops in Japan. *Plant Dis.* 90:506-512.
- Davide, L. C. 1986. Benzimidazole fungicides: Mechanism of action and biological impact. *Annu. Rev. Phytopathol.* 24:43-65.
- Dujon, B. 1989. Group I introns as mobile genetic elements: Facts and mechanistic speculations—A review. *Gene* 82:91-114.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
- Fernández-Ortuño, D., Grabke, A., Bryson, P. K., Amiri, A., Peres, N. A., and Schnabel, G. 2014. Fungicide resistance profiles in *Botrytis cinerea* from strawberry fields of seven Southern U.S. states. *Plant Dis.* 98:825-833.
- Fisher, N., Brown, A. C., Sexton, G., Cook, A., Windass, J., and Meunier, B. 2004. Modeling the Qo site of crop pathogens in *Saccharomyces cerevisiae* cytochrome b. *Eur. J. Biochem.* 271:2264-2271.
- Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Manag. Sci.* 58:859-867.
- Gopinath, K., Radhakrishnan, N. V., and Jayaraj, J. 2006. Effect of propiconazole and difenoconazole on the control of anthracnose of chilli fruits caused by *Colletotrichum capsici*. *Crop Prot.* 25:1024-1031.
- Grabke, A., Williamson, M., Henderson, G. W., and Schnabel, G. 2014. First report of anthracnose on peach fruit caused by *Colletotrichum truncatum* in South Carolina. *Plant Dis.* 98:1154.
- Grasso, V., Palermo, S., Sierotzki, H., Garibaldi, A., and Gisi, U. 2006. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Manag. Sci.* 62:465-472.

- Horton, D., Brannen, P., Bellinger, B., Lockwood, D., and Ritchie, D., eds. 2014. Southeastern Peach, Nectarine and Plum Pest Management and Culture Guide. Univ. Ga. Bull. 1171:33.
- Hu, M. J., Grabke, A., and Schnabel, G. 2015. Investigation of the *Colletotrichum gloeosporioides* species complex causing peach anthracnose in South Carolina. Plant Dis. 99:797-805.
- Hu, M.-J., Cox, K. D., Schnabel, G., and Luo, C.-X. 2011. *Monilinia* species causing brown rot of peach in China. PLoS One 6:e24990.
- Inada, M., Ishii, H., Chung, W. H., Yamada, T., Yamaguchi, J., and Furuta, A. 2008. Occurrence of strobilurin-resistant strains of *Colletotrichum gloeosporioides* (*Glomerella cingulata*), the causal fungus of strawberry anthracnose. Jpn. J. Phytopathol. 74:114-117.
- Kim, W. G., and Hong, S. K. 2008. Occurrence of anthracnose on peach tree caused by *Colletotrichum* species. Plant Pathol. J. 24:80-83.
- Kim, Y. S., Dixon, E. W., Vincessi, P., and Farman, M. L. 2003. Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome b gene. Phytopathology 93:891-900.
- Koenraadt, H., Somerville, S. C., and Jones, A. L. 1992. Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. Phytopathology 82:1348-1354.
- Köller, W. 1999. Chemical approaches to managing plant pathogens. Pages 337-376 in: Handbook of Pest Management. J. R. Ruberson, ed. Marcel Dekker, Inc., New York.
- Kongtragoul, P., Nalumpang, S., Miyamoto, Y., Izumi, Y., and Akimitsu, K. 2011. Mutation at codon 198 of *tub2* gene for carbendazim resistance in *Colletotrichum gloeosporioides* causing mango anthracnose in Thailand. J. Plant Prot. Res. 51:377-384.
- Lambowitz, A. M., and Perlman, P. S. 1990. Involvement of aminoacyl-tRNA synthetases and other proteins in group I and group II intron splicing. Trends Biochem. Sci. 15:440-444.
- Lang, B. F., Laforest, M.-J., and Burger, G. 2007. Mitochondrial introns: A critical view. Trends Genet. 23:119-125.
- Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947-2948.
- Lazowska, J., Jacq, C., and Slonimski, P. P. 1980. Sequence of introns and flanking exons in wild-type and *box3* mutants of cytochrome b reveals an interlaced splicing protein coded by an intron. Cell 22:333-348.
- Li, J., Katiyar, S. K., and Edlind, T. D. 1996. Site-directed mutagenesis of *Saccharomyces cerevisiae* β -tubulin: Interaction between residue 167 and benzimidazole compounds. FEBS Lett. 385:7-10.
- Li, X., Fernández-Ortuño, D., Chen, S., Grabke, A., Luo, C.-X., Bridges, W. C., and Schnabel, G. 2014. Location-specific fungicide resistance profiles and evidence for stepwise accumulation of resistance in *Botrytis cinerea*. Plant Dis. 98:1066-1074.
- Luck, J. E., and Gillings, M. R. 1995. Rapid identification of benomyl resistant strains of *Botrytis cinerea* using the polymerase chain reaction. Mycol. Res. 99:1483-1488.
- Luo, C.-X., Hu, M.-J., Jin, X., Yin, L.-F., Bryson, P. K., and Schnabel, G. 2010. An intron in the cytochrome b gene of *Monilinia fructicola* mitigates the risk of resistance development to QoI fungicides. Pest Manag. Sci. 66:1308-1315.
- Ma, Z., Felts, D., and Michailides, T. J. 2003. Resistance to azoxystrobin in *Alternaria* isolates from pistachio in California. Pestic. Biochem. Physiol. 77:66-74.
- Ma, Z., and Michailides, T. J. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Prot. 24:853-863.
- Maymon, M., Zveibil, A., Pivonia, S., Minz, D., and Freeman, S. 2006. Identification and characterization of benomyl-resistant and -sensitive populations of *Colletotrichum gloeosporioides* from *Statice* (*Limonium* spp.). Phytopathology 96:542-548.
- Nalumpang, S., Miyamoto, Y., Miyake, C., Izumi, Y., Akitmitsu, K., and Kongtragoul, P. 2010. Point mutations in the beta-tubulin gene conferred carbendazim-resistant phenotypes of *Colletotrichum gloeosporioides* causing 'Nam Dok Mai' mango anthracnose. J. Agric. Technol. 6:365-378.
- Pellenz, S., Harington, A., Dujon, B., Wolf, K., and Schäfer, B. 2002. Characterization of the I-Spom I endonuclease from fission yeast: Insights into the evolution of a group I intron-encoded homing endonuclease. J. Mol. Evol. 55:302-313.
- Peres, N. A. R., Souza, N. L., Peever, T. L., and Timmer, L. W. 2004. Benomyl sensitivity of isolates of *Colletotrichum acutatum* and *C. gloeosporioides* from Citrus. Plant Dis. 88:125-130.
- Petersen, D. H. 1953. Plant Diseases, the Yearbook of Agriculture. U.S. Department of Agriculture, Washington, DC.
- Saldanha, R., Mohr, G., Belfort, M., and Lambowitz, A. M. 1993. Group I and group II introns. FASEB J. 7:15-24.
- Sanders, G., Korsten, L., and Wehner, F. C. 2000. Survey of fungicide sensitivity in *Colletotrichum gloeosporioides* from different avocado and mango production areas in South Africa. Eur. J. Plant Pathol. 106:745-752.
- Schnabel, G., Amiri, A., and Brannen, P. M. 2012. Fungicide resistance in crop protection: Risk and management. Pages 116-132 in: Field Kit- and Internet-Supported Fungicide Resistance Monitoring. T. S. Thind, ed. CAB International, Oxfordshire, UK.
- Sierotzki, H., Frey, R., and Wullschlegel, J. 2007. Cytochrome b gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. Pest Manag. Sci. 63:225-233.
- Spalding, D. H. 1982. Resistance of mango pathogens to fungicides used to control post harvest diseases. Plant Dis. 66:1185-1186.
- Tamura, K., Stecher, G., Peterson, D., Filipinski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725-2729.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.
- Weir, B. S., Johnston, P. R., and Damm, U. 2012. The *Colletotrichum gloeosporioides* species complex. Stud. Mycol. 73:115-180.
- Wong, F. P., de la Cerdá, K. A., Hernandez-Martinez, R., and Midland, S. L. 2008. Detection and characterization of benzimidazole resistance in California populations of *Colletotrichum cereale*. Plant Dis. 92:239-246.
- Wong, F. P., and Midland, S. L. 2007. Sensitivity distributions of California populations of *Colletotrichum cereale* to the DMI Fungicides propiconazole, myclobutanil, tebuconazole, and triadimefon. Plant Dis. 91:1547-1555.
- Yin, L.-F., Hu, M.-J., Wang, F., Kuang, H., Zhang, Y., Schnabel, G., Li, G.-Q., and Luo, C.-X. 2012. Frequent gain and loss of introns in fungal cytochrome b genes. PLoS One 7:e49096.
- Zhu, F., Bryson, P. K., and Schnabel, G. 2012. Influence of storage approaches on instability of propiconazole resistance in *Monilinia fructicola*. Pest Manag. Sci. 68:1003-1009.