Characterization of *Colletotrichum* Species Causing Anthracnose of Pomegranate in the Southeastern United States

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Abstract

Anthracnose fruit rot and leaf blight caused by *Colletotrichum* species are important diseases of pomegranate in the southeastern United States. In this study, 26 isolates from pomegranate were identified based on pathological and molecular characterization. Isolates were identified to species based on multilocus sequence analysis with the internal transcribed spacer region, glyceraldehyde-3-phosphate dehydrogenase, β -tubulin, and chitin synthase genomic genes. Pomegranate isolates grouped within the *C. acutatum* and *C. gloeosporioides* species complexes, with more than 73% belonging to the latter group. Three species were identified within the *C. acutatum* species complex (*C. nymphaeae* [$n = 5$], *C. fioriniae* [$n = 1$], and *C. simmondsii* [$n = 1$]), and three other species were identified within the *C. gloeosporioides* species complex (*C. theobromicola* [$n = 11$], *C. siamense* [$n = 6$], and *C. gloeosporioides*

[$n = 2$]). Inoculations of pomegranate fruit showed that isolates from the *C. acutatum* species complex were more aggressive than isolates from the *C. gloeosporioides* species complex. Interestingly, opposite results were observed when leaves of rooted pomegranate cuttings were inoculated. In addition, *Colletotrichum* isolates from pomegranate, strawberry, blueberry, mango, and citrus were cross-pathogenic when inoculated to fruit. This is the first study identifying six different species of *Colletotrichum* causing pomegranate leaf blight and fruit anthracnose in the southeastern United States and the potential cross-pathogenic capability of pomegranate isolates to other commercially important crops.

Keywords: *Punica granatum*, *Colletotrichum*, anthracnose, phylogeny, Southeastern, pathogenicity, fruit rot, leaf blight

Pomegranate (*Punica granatum* L.) is widely grown in many subtropical to tropical countries where it is well-adapted. India, Iran, China, the United States (U.S.), and Turkey are the top five pomegranate-producing countries, with 76% of the total production worldwide (Jain and Desai 2018). The largest producers in Asia are India and Iran. In Europe, Spain is the largest producer of pomegranate (Stover and Mercure 2007). California is the largest producer of pomegranate in the U.S. (NASS 2012). In the southeastern U.S., pomegranate production is relatively small; however, it has emerged as an alternative crop for growers dealing with devastating diseases of citrus and avocado.

Several fungal pathogens have been reported to cause disease on pomegranate worldwide (Munhuweyi et al. 2016). *Colletotrichum* spp. can infect pomegranate leaves and fruit, causing anthracnose leaf blight and fruit rot, respectively (Day and Wilkins 2011; Munhuweyi et al. 2016). Fruits infected by this pathogen become unmarketable, leading to economic losses (Munhuweyi et al. 2016).

Anthracnose fruit rot caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *C. theobromicola* Delacr., and *C. tropicale* Rojas, Rehner & Samuels has been reported in the U.S. (Alfieri 1984; Anonymous 1960), Greece (Thomidis 2014), Brazil (Mendes 1998; Silva-Cabral et al. 2019), India (Munhuweyi et al. 2016;

Sataraddi et al. 2011; Sharma et al. 2015), Iran (Rahimlou et al. 2014), and Australia (Shivas et al. 2016). This disease is favored by conditions of high humidity, ranging from 62 to 95%, and temperatures ranging from 20 to 30°C, which are common in tropical and subtropical regions (Arauz 2000; Cannon et al. 2012; Whitelaw-Weckert et al. 2007). In most of the pomegranate-growing areas around the world, a Mediterranean climate with warm, dry summers predominates. For this reason, in regions where pomegranate is produced on a commercial scale, such as California and the Middle East, anthracnose is not a limiting factor (Day and Wilkins 2011). The warm, wet, humid conditions in regions such as in the southeastern U.S., India, and southeast Asia present favorable conditions for *Colletotrichum* spp. infection and dissemination, and as a result commercial pomegranate production in the southeastern U.S. has been severely limited by anthracnose (KC and Vallad 2015). Anthracnose symptoms start as small circular leaf spots with yellow halos. Infected leaves then become chlorotic and drop from the tree, leading to premature defoliation. Fruit symptoms are characterized by brown lesions that later progress through the rind and arils, resulting in fruit decay. Gray to orange spore masses often become visible on lesions of either green or ripe fruit (Munhuweyi et al. 2016; Thomidis 2014).

Colletotrichum spp. are also known as the causal agent of anthracnose on multiple fruit crops such as blueberry (*Vaccinium corymbosum* L.), strawberry (*Fragaria × ananassa* Duch.), citrus (*Citrus* spp. L.), peach (*Prunus persica* [L.] Batsch), apple (*Malus pumila* Mill.), grape (*Vitis vinifera* L.), and mango (*Mangifera indica* L.) (Arauz 2000; Chung et al. 2003; MacKenzie et al. 2007; Peres et al. 2008; Polashock et al. 2005; Sergeeva et al. 2008; Whitelaw-Weckert et al. 2007). Although some of these fruit crops are cultivated in close proximity to pomegranate in Florida and in the southeastern U.S., it is unknown if cross-infection occurs between isolates from pomegranate and other fruit crops. Cross-infection among *Colletotrichum* species, however, has been reported on several other fruit crops (Alahakoon et al. 1994; Freeman et al. 1998). *Colletotrichum acutatum* J.H. Simmonds isolates from strawberry and blueberry were able to infect nonwounded grapefruits, and grape isolates were able to infect blueberries and strawberries under laboratory conditions (Whitelaw-Weckert et al. 2007).

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Morphological characteristics are not sufficient to discriminate between species in the genus *Colletotrichum*; thus, in recent years elucidation of species has been performed using molecular approaches (Cai et al. 2009; Cannon et al. 2012; Damm et al. 2012; Du et al. 2005; Weir et al. 2012). Several species associated with anthracnose

on fruit crops were identified using multilocus phylogenetic data (Baroncelli et al. 2015; Lima et al. 2013; Weir et al. 2012). The species *C. acutatum* is the causal agent of two diseases of citrus: post-bloom fruit drop and key lime anthracnose. However, based on multilocus phylogenetic data, these two diseases are caused by two

Table 1. *Colletotrichum* isolates used in this study

Species	Isolate ^y	Host ^z	Year	Location	GenBank accession number				Reference
					ITS	GAPDH	CHS-1	TUB2	
<i>C. nymphaeae</i>	02-152	Strawberry	2002	USA: Florida	MK130726	MK240040	MK177230	MK239989	This study
<i>C. lupini</i>	03-36	Citrus	2003	USA: Florida	MK130727	MK240041	MK177231	MK239990	This study
<i>C. theobromicola</i>	C4	Pomegranate	2014	USA: Florida	MK130728	MK240042	MK177232	MK239991	This study
<i>C. lupini</i>	05-04	Citrus	2005	USA: Florida	MK130729	MK240043	MK177233	MK239992	This study
<i>C. siamense</i>	05-48	Blueberry	2005	USA: Florida	MK130730	MK240044	MK177234	MK239993	This study
<i>C. fiorinae</i>	05-88	Blueberry	2005	USA: Florida	MK130731	MK240045	MK177235	MK239994	This study
<i>C. siamense</i>	05-92	Blueberry	2005	USA: Florida	MK130732	MK240046	MK177236	MK239995	This study
<i>C. siamense</i>	05-93	Blueberry	2005	USA: Florida	MK130733	MK240047	MK177237	MK239996	This study
<i>C. fiorinae</i>	05-218	Blueberry	2005	USA: Florida	MK130734	MK240048	MK177238	MK239997	This study
<i>C. fiorinae</i>	05-219	Blueberry	2005	USA: Florida	MK130735	MK240049	MK177239	MK239998	This study
<i>C. lupini</i>	05-263	Citrus	2005	USA: Florida	MK130736	MK240050	MK177240	MK239999	This study
<i>C. siamense</i>	06-01	Strawberry	2006	USA: Florida	MK130737	MK240051	MK177241	MK240000	This study
<i>C. siamense</i>	07-05	Strawberry	2007	USA: Florida	MK130738	MK240052	MK177242	MK240001	This study
<i>C. nymphaeae</i>	07-19	Strawberry	2007	USA: Florida	MK130739	MK240053	MK177243	MK240002	This study
<i>C. gloeosporioides</i>	07-79	Citrus	2007	USA: Florida	MK130740	MK240054	MK177244	MK240003	This study
<i>C. gloeosporioides</i>	07-81	Citrus	2007	USA: Florida	MK130741	MK240055	MK177245	MK240004	This study
<i>C. gloeosporioides</i>	07-82	Citrus	2007	USA: Florida	MK130742	MK240056	MK177246	MK240005	This study
<i>C. theobromicola</i>	C7	Pomegranate	2014	USA: Florida	MK130743	MK240057	MK177247	MK240006	This study
<i>C. nymphaeae</i>	08-21	Strawberry	2008	USA: Florida	MK130744	MK240058	MK177248	MK240007	This study
<i>C. nymphaeae</i>	08-48	Mango	2008	USA: Florida	MK130745	MK240059	MK177249	MK240008	This study
<i>C. nymphaeae</i>	08-76	Mango	2008	USA: Florida	MK130746	MK240060	MK177250	MK240009	This study
<i>C. nymphaeae</i>	08-81	Mango	2008	USA: Florida	MK130747	MK240061	MK177251	MK240010	This study
<i>C. theobromicola</i>	C10	Pomegranate	2014	USA: Florida	MK130748	MK240062	MK177252	MK240011	This study
<i>C. siamense</i>	C15	Pomegranate	2014	USA: Florida	MK130749	MK240063	MK177253	MK240012	This study
<i>C. theobromicola</i>	C16	Pomegranate	2014	USA: Florida	MK130750	MK240064	MK177254	MK240013	This study
<i>C. gloeosporioides</i>	C17	Pomegranate	2014	USA: Florida	MK130751	MK240065	MK177255	MK240014	This study
<i>C. siamense</i>	C18	Pomegranate	2014	USA: Florida	MK130752	MK240066	MK177256	MK240015	This study
<i>C. siamense</i>	C19	Pomegranate	2014	USA: Florida	MK130753	MK240067	MK177257	MK240016	This study
<i>C. siamense</i>	C28	Pomegranate	2014	USA: Florida	MK130754	MK240068	MK177258	MK240017	This study
<i>C. gloeosporioides</i>	C30	Pomegranate	2014	USA: Florida	MK130755	MK240069	MK177259	MK240018	This study
<i>C. siamense</i>	C33	Pomegranate	2014	USA: Florida	MK130756	MK240070	MK177260	MK240019	This study
<i>C. siamense</i>	C34	Pomegranate	2014	USA: Florida	MK130757	MK240071	MK177261	MK240020	This study
<i>C. theobromicola</i>	C38	Pomegranate	2014	USA: Florida	MK130758	MK240072	MK177262	MK240021	This study
<i>C. theobromicola</i>	C41	Pomegranate	2014	USA: Florida	MK130759	MK240073	MK177263	MK240022	This study
<i>C. theobromicola</i>	C43	Pomegranate	2014	USA: Florida	MK130760	MK240074	MK177264	MK240023	This study
<i>C. theobromicola</i>	C44	Pomegranate	2014	USA: Florida	MK130761	MK240075	MK177265	MK240024	This study
<i>C. simmondsii</i>	C45	Pomegranate	2014	USA: Florida	MK130762	MK240076	MK177266	MK240025	This study
<i>C. fiorinae</i>	C46	Pomegranate	2015	USA: Georgia	MK130763	MK240077	MK177267	MK240026	This study
<i>C. theobromicola</i>	C62	Pomegranate	2015	USA: Florida	MK130764	MK240078	MK177268	MK240027	This study
<i>C. theobromicola</i>	C63	Pomegranate	2015	USA: South Carolina	MK130765	MK240079	MK177269	MK240028	This study
<i>C. nymphaeae</i>	C64	Pomegranate	2015	USA: South Carolina	MK130766	MK240080	MK177270	MK240029	This study
<i>C. nymphaeae</i>	C65	Pomegranate	2015	USA: South Carolina	MK130767	MK240081	MK177271	MK240030	This study
<i>C. nymphaeae</i>	C66	Pomegranate	2015	USA: South Carolina	MK130768	MK240082	MK177272	MK240031	This study
<i>C. nymphaeae</i>	C67	Pomegranate	2015	USA: South Carolina	MK130769	MK240083	MK177273	MK240032	This study
<i>C. nymphaeae</i>	C68	Pomegranate	2015	USA: South Carolina	MK130770	MK240084	MK177274	MK240033	This study
<i>C. theobromicola</i>	C71	Pomegranate	2015	USA: Florida	MK130771	MK240085	MK177275	MK240034	This study
<i>C. lupini</i>	96-50	Citrus	1996	USA: Florida	MK130772	MK240086	MK177276	MK240035	This study
<i>C. siamense</i>	97-15A	Strawberry	1997	USA: Florida	MK130773	MK240087	MK177277	MK240036	This study
<i>C. siamense</i>	97-63A	Strawberry	1997	USA: Florida	MK130774	MK240088	MK177278	MK240037	This study
<i>C. siamense</i>	98-285	Strawberry	1998	USA: Florida	MK130775	MK240089	MK177279	MK240038	This study
<i>C. nymphaeae</i>	99-37	Strawberry	1999	USA: Florida	MK130776	MK240090	MK177280	MK240039	This study
<i>C. gloeosporioides</i>	CBS:112999*	Citrus	NA	Italy	JQ005152	JQ005239	JQ005326	JQ005587	Damm et al. (2012)
<i>C. acutatum</i>	CBS:127602	Strawberry	1988	Australia	JQ948359	JQ948690	JQ949020	JQ950010	Damm et al. (2012)
<i>C. lupini</i>	CBS:109225**	Lupine	NA	Ukraine	JQ948155	JQ948485	JQ948816	JQ949806	Damm et al. (2012)
<i>C. nymphaeae</i>	CBS:515.78*	Water lily	1978	Netherlands	JQ948197	JQ948527	JQ948858	JQ949848	Damm et al. (2012)
<i>C. simmondsii</i>	IMI:313840	Mango	NA	Australia	JQ948284	JQ948614	JQ948945	JQ949935	Damm et al. (2012)
<i>C. fiorinae</i>	CBS:127537	Blueberry	1990	USA	JQ948318	JQ948648	JQ948979	JQ949969	Damm et al. (2012)
<i>C. siamense</i>	C1315.2#	Coffee	2008	Thailand	JX010171	JX009924	JX009865	JX010404	Weir et al. (2012)
<i>C. theobromicola</i>	C1316.13**	Cocoa	2008	Panama	JX010294	JX010006	JX009869	JX010447	Cannon et al. (2012)
<i>C. fruticola</i>	C1315.3#	Coffee	2007	Thailand	JX010165	JX010033	JX009866	JX010405	Weir et al. (2012)
<i>M. infusans</i>	CBS:869.96 [§]	Sweet potato	1996	South Africa	JQ005780	JX546612	JQ005801	JQ005864	O'Connell et al. (2012)

^y * = culture from epitype; ** = culture from neotype; # = culture from exotype; and [§] = culture from type material.

^z *Theobroma cacao* (cocoa), *Coffea arabica* (coffee), *Vaccinium* sp. (blueberry), *Mangifera indica* (mango), *Nymphaea alba* (white water lily), *Lupinus albus* (lupine), *Fragaria × ananassa* (strawberry), *Citrus sinensis* (citrus), *Ipomoea batatas* (sweet potato), and *Punica granatum* (pomegranate).

distinct phylogenetic lineages (Peres et al. 2008). Another species frequently associated with postharvest anthracnose of citrus is *C. gloeosporioides* (Munhuweyi et al. 2016; Sataraddi et al. 2011). Difference in sensitivity of *C. acutatum* and *C. gloeosporioides* isolates from citrus to fungicides has been reported. *C. gloeosporioides* isolates were found to be highly sensitive and resistant to benomyl, whereas the *C. acutatum* isolates were moderately resistant (Peres et al. 2004). Thus, the importance of species differentiation is critical for the implementation of correct management strategies (Cai et al. 2009).

Currently, anthracnose of pomegranate is attributed to *C. gloeosporioides*, *C. acutatum*, *C. fructigenum*, and *C. theobromicola* (Bellé et al. 2018; Gorter 1977; Mendes 1998; Munhuweyi et al. 2016; Sataraddi et al. 2011; Sharma et al. 2015; Shivas et al. 2016; Thomidis 2014); however, there are no studies on the molecular characterization of *Colletotrichum* species associated with pomegranate fruit and leaves in the southeastern U.S. Furthermore, there are no studies on cross-infection among pomegranate and other fruit crop isolates. Therefore, the objectives of this study were to (i) characterize *Colletotrichum* isolates infecting pomegranate in the southeastern U.S., (ii) compare the aggressiveness of isolates on pomegranate fruit and leaves, and (iii) determine the cross-infectivity on other fruit crops in order to develop effective disease management strategies.

Materials and Methods

Fungal isolates. Pomegranate isolates used in this study were recovered from symptomatic leaves, stems, pedicels, buds, flowers, and fruit of pomegranate collected from several orchards throughout the southeastern U.S. during 2014 and 2015 (Table 1). Excised symptomatic tissues were surface disinfested for 2 min in a 1% solution of sodium hypochlorite (NaOCl), rinsed three times in sterile distilled water, air dried under aseptic conditions, and placed on V8 juice agar (15.0 g of agar, 200 ml of V8 juice, and 2.0 g of CaCO₃ per liter) amended with 0.01 mg of rifampicin and 0.25 g of ampicillin per liter. Cultures were incubated at 25°C under continuous fluorescent light for 3 to 5 days, and single-spore cultures were obtained from colonies emerging from the infected tissues and examined morphologically. *Colletotrichum* isolates recovered from blueberry, strawberry, citrus, and mango were obtained from Natalia Peres at the University of Florida Gulf Coast Research and Education Center. The isolates were stored in 30% glycerol at –80°C at Gary E. Vallad's laboratory located at the University of Florida Gulf Coast Research and Education Center. Fungal cultures were grown on V8 agar at 23°C under continuous fluorescent light for 2 weeks.

Genomic DNA extraction. DNA extraction was performed using the Dellaporta protocol with modifications (Dellaporta et al. 1983). Extraction buffer (1 M Tris, pH 8.0; 0.25 M EDTA, pH 8.0; 5 M NaCl; and 10 mM β-mercaptoethanol) and 10% (v/v) sodium dodecyl sulfate were added to mycelial mats for homogenization. Three SPEX SamplePrep 4-mm beadbeater beads (Metuchen, NJ) were added to the tubes, and the samples were homogenized at 1,500 rpm for 2 min using a SPEX SamplePrep 1600 MiniG, frozen for 10 min at –80°C, and then incubated at 65°C for 15 min. For purification, samples were treated with 8 M potassium acetate and

precipitated with isopropanol. The DNA pellet was washed twice in 70% ethanol, dried, dissolved in 10 mM TE buffer (10 mM Tris-HCl, 1 mM EDTA-Na₂) and stored at –20°C.

Phylogenetic analysis. A multilocus sequence analysis was performed with isolates from pomegranate (*n* = 26), strawberry (*n* = 9), blueberry (*n* = 6), citrus (*n* = 7), and mango (*n* = 3). Four different loci were used to elucidate relationships among these *Colletotrichum* isolates: (i) the internal transcribed spacer (ITS) sequence of the rDNA repeats; (ii) chitin synthase (CHS-1); (iii) β-tubulin (TUB2); and (iv) glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Polymerase chain reaction (PCR) amplifications were performed with the respective primer pairs ITS1 and ITS4 (White et al. 1990), CHS-79F and CHS-234R (Carbone and Kohn 1999), T1 (O'Donnell and Cigelnik 1997) and Bt-2b (Glass and Donaldson 1995), and GDF1 and GDR1 (Guerber et al. 2003) (Table 2).

PCR was performed in a final volume of 25 μl containing 25 ng of genomic DNA, 12.5 μl of GoTaq Green Master Mix (2×), and 1 μl of each primer (10 μM). The PCR cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing for 30 s at a determined temperature for each primer (Table 2), and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were purified by using ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific). The purified PCR amplicons were submitted to Elim Biopharmaceuticals (Hayward, CA) for sequencing. Forward and reverse primers were used to sequence the amplicons with the BigDye Terminator cycle-sequencing chemistry (Applied Biosystems, Foster City, CA) on an ABI 3730 DNA Capillary Sequencer following the manufacturer's protocol in a 10-μl reaction volume.

In addition to sequences obtained in this study, sequences of each of the four loci from exotype, neotype, and epitype isolates (Cannon et al. 2012; Damm et al. 2012; O'Connell et al. 2012; Weir et al. 2012) were retrieved from NCBI as references (Table 1). *Monilochaetes infusans* (CBS 869.96) from *Ipomoea batatas* was used as the outgroup. Sequences were aligned and examined in Geneious Pro 6.0.6 (Kearse et al. 2012), followed by trimming and manual editing and optimization of the alignments as required. All sequences were deposited in GenBank under accession numbers MK130726 to MK130776 for ITS, MK177230 to MK177280 for CHS-1, MK239989 to MK240039 for TUB2, and MK240040 to MK240090 for GAPDH.

Phylogenetic analyses were conducted using maximum likelihood (ML) in MEGA6 (Tamura et al. 2011) and Bayesian inference (BI) in MrBayes version 3.0b4 software package (Ronquist and Huelsenbeck 2003). Analyses were performed for each locus (CHS-1, GAPDH, TUB2, and ITS) individually and for the concatenated alignment of the four loci. After completing the multiple alignments via MUSCLE (Edgar 2004), the best substitution models were estimated using default parameters. The ML method was based on the Kimura two-parameter model assuming a discrete gamma distribution (K2 + G) for the combined data set, TUB2, and ITS; the Kimura two-parameter model assuming an invariant sites model (K2 + I) for CHS-1; and the Kimura two-parameter model (K2) for GAPDH. Gaps were treated as missing data. The support for each node was determined by 1,000 bootstrap replicates. For BI, searches

Table 2. Primers used in this study^z

Gene	Primer name	Sequence (5' to 3')	Tm (°C)	Reference
ITS	ITS1	TCCGTAGGTGAACCTGCGG	57.3	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	52.1	
CHS-1	CHS-79F	TGGGGCAAGGATGCTTGAAGAAG	61.6	Carbone and Kohn (1999)
	CHS-234R	TGGAAGAACCATCTGTGAGAGTTG	56.6	
TUB2	T1	AACATGCGTGAGATTGTAAGT	52.3	O'Donnell and Cigelnik (1997)
	Bt-2b	ACCCTCAGTGTAGTGACCCCTGGC	62.5	
GAPDH	GDF1	GCCGTCAACGACCCCTTCATTGA	61.7	Guerber et al. (2003)
	GDR1	GGGTGGAGTCGTACTTGAGCATGT	60.6	

^z Tm = melting temperature; ITS = internal transcribed spacer sequence of the rDNA repeats; CHS-1 = chitin synthase; TUB2 = β-tubulin; and GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

using Metropolis-coupled Markov chain Monte Carlo sampling were performed. For these analyses, one cold and three heated Markov chains were used. Bayesian analyses were run for 5,000,000 generations with trees sampled every 100 generations. BI and ML produced similar topologies; thus, in this work we are only presenting ML tree topologies. Bootstrap and posterior probability are used for branch values based on BI and ML, respectively.

Inoculum preparation. Fungal strains were routinely cultured on V8 juice agar at 23°C under continuous fluorescent light for 2 weeks. For inoculations performed on ripe pomegranate fruit, a 7-mm mycelial plug was excised from the 2-week-old cultures and placed on wounded fruits. For inoculations performed on pomegranate plants, and on strawberry, blueberry, citrus, and mango fruits, a spore suspension was prepared by adding sterile distilled water to each fungal

Fig. 1. Maximum likelihood tree of *Colletotrichum* spp. based on combined ITS, GAPDH, CHS-1, and TUB2 loci. The tree generated by Bayesian inference had a similar topology. Bootstrap values ($\geq 70\%$) are shown after the slash (/), and posterior probabilities ($\geq 90\%$) are shown before. Bootstrap values ($< 70\%$) and posterior probabilities ($< 90\%$) are not shown or are represented by a minus sign (-). Isolate names from this study and reference are indicated in bold. Black vertical bars on the right delineate the phylogenetic clades. *Monilochaetes infuscans* was used as outgroup.

culture, and spores were loosened with a mini-pestle. The spores were washed three times with sterile water. Spore suspensions at a concentration of 10^6 conidia/ml were prepared from the cultures and used immediately for inoculations.

Fruit inoculation assays. *Colletotrichum* isolates from pomegranate ($n = 10$), strawberry ($n = 2$), blueberry ($n = 2$), and citrus ($n = 2$) were used for inoculation of mature fruit of pomegranate cultivar Wonderful. The same set of isolates, including one more isolate from mango, was also used to check cross-infectivity on fruit of strawberry cultivar Camarosa, blueberry cultivar Jewel, navel orange, and organic mango. Strawberry and blueberry fruit were harvested in February and April, respectively, at the Gulf Coast Research and Education Center, University of Florida. Mango and citrus fruit, originating from Mexico (Crespo organic) and California, respectively, were purchased at a retail supermarket. All the fruits were physiologically mature. Fruits were surface disinfested with 1% aqueous solution of chlorine and air dried under a laminar flow hood. Pomegranate fruits were inoculated using the standard method described by Thomidis and Exadaktylou (2011) with modifications. Each isolate treatment consisted of five replications per treatment, with one fruit per replicate. Pomegranate fruits were wounded, in two locations spaced 5 cm apart, with a cork borer 7 mm in diameter and 3 mm in depth. Inoculation was performed with a mycelial plug of 7 mm, except for controls, which were treated with a noncolonized piece of V8 juice agar.

Citrus and mango fruits were wounded with a sterile needle (3 mm depth) and inoculated with a 20- μ l drop of spore suspension

(10^6 conidia/ml); controls consisted of wounded fruits treated with a 20- μ l drop of water. There were four replications per treatment, consisting of one fruit per replicate. Nonwounded strawberry and blueberry fruits were inoculated with a 20- μ l drop of spore suspension (10^6 conidia/ml); controls consisted of nonwounded fruits treated with a 20- μ l drop of water. The drop of spore suspension or water was placed on the side of the strawberry or on the blueberry calyx. There were eight and three replications per treatment, consisting of one and four fruits per replicate, for strawberry and blueberry experiments, respectively.

All inoculated fruits were enclosed in clear plastic boxes lined with wet paper towels and incubated at 12-h photoperiod at 24°C. The pathogenicity and aggressiveness of an isolate was determined by measuring the lesion diameter of pomegranate, strawberry, citrus, and mango fruits 3 weeks after inoculation. In the blueberry experiment, the disease evaluation was performed based on the number of symptomatic fruits, 11 days after inoculation. Each fruit inoculation experiment was performed twice.

Foliar inoculation assays. Three-month-old rooted pomegranate cuttings were used for the inoculations. One pomegranate cutting of cultivar Wonderful was planted per pot (15 cm) in Pro-Mix BX (Premiere Horticulture, Riviere du Loup, PQ, Canada). Pots were arranged in a randomized complete block design with three blocks. Each treatment replicate included one pot containing one plant. The same isolates tested on pomegranate fruits were also tested on the leaves. Cuttings were inoculated with a spore suspension

Fig. 2. Examples of anthracnose symptoms on fruits inoculated with representative isolates: **A**, *Colletotrichum acutatum* species complex (C67); **B**, *C. gloeosporioides* species complex (C10); and **C**, control (V8 mycelial plug).

(10^6 conidia/ml) and incubated for 72 h in box containers with 100% humidity under a 12-h photoperiod at 24°C. Three cuttings were sprayed with water and used as a negative control. Disease severity was evaluated once at 7 days postinoculation by measuring the percentage of symptomatic leaf surface. This experiment was performed twice.

Statistical analysis. The effect of isolate on disease incidence (blueberry assay) and severity (pomegranate fruit and leaf assays, strawberry, citrus, and mango assays) was analyzed using a generalized linear mixed model (PROC GLIMMIX, SAS, version 9.4, SAS Institute, Cary, NC) with blocking as a random effect. When the effect of isolates on disease severity or incidence was significant, differences among isolates were compared using Fisher's protected least square difference (LSD) method for mean separation at the 95.0% level of confidence.

Results

Phylogenetic analysis of *Colletotrichum* species. Relationship among 51 *Colletotrichum* isolates from pomegranate and other fruit crops was determined based on a combined alignment of ITS, GAPDH, CHS-1, and TUB2 loci. Sequences from reference isolates of *C. acutatum* and *C. gloeosporioides* species complexes were included (Table 1) (Cannon et al. 2012; Damm et al. 2012; O'Connell et al. 2012; Weir et al. 2012). Individual phylogenetic trees were also generated from each locus: ITS, GAPDH, CHS-1, and TUB2 (Supplementary Fig. S1). The phylogenetic tree from the alignment

of concatenated sequences separated isolates from pomegranate into two well-supported clades (100% posterior probability for BI and 99% bootstrap support for ML) corresponding to *C. gloeosporioides* and *C. acutatum* species complexes (Fig. 1). Interestingly, the isolates from this study were identified as six different *Colletotrichum* species between the two species complexes: *C. siamense*, *C. gloeosporioides*, *C. theobromicola*, *C. fioriniae*, *C. simmondsii*, and *C. nymphaeae*. Isolates from pomegranate and other fruit crops were distributed into three distinct clades within the *C. gloeosporioides* species complex: *C. siamense*, *C. gloeosporioides*, and *C. theobromicola* (Fig. 1). The *C. siamense* clade contained isolates from pomegranate ($n = 6$), blueberry ($n = 3$), and strawberry ($n = 5$). Based on the concatenated sequence there were 12 single-nucleotide polymorphisms among the pomegranate isolates within the *C. siamense* clade. The *C. gloeosporioides* clade contained pomegranate ($n = 2$) and citrus ($n = 3$) isolates. However, there was no nucleotide variation among the two pomegranate isolates within *C. gloeosporioides*. Notably, the *C. theobromicola* clade consisted of 11 isolates, all from pomegranate that were 100% identical at each locus. Four other distinct clades were formed within the *C. acutatum* species complex (Fig. 1). The first one was formed by a unique isolate from pomegranate (C46), the *C. fioriniae* clade, together with three isolates from blueberry (Fig. 1). Based on the individual CHS-1, ITS, and GAPDH gene trees, pomegranate isolate C46 was 100% similar to the reference isolate *C. fioriniae*, as well as to the blueberry isolates. *C. simmondsii* was another distinct group, with only one pomegranate isolate. The last one containing pomegranate isolates

Fig. 3. Aggressiveness of 16 *Colletotrichum* spp. isolates on fruit of pomegranate 'Wonderful.' Isolates used for the pomegranate fruit inoculations were recovered from the following fruits: pomegranate (Pom) (C67, C66, C64, C45, C41, C30, C18, C17, C15, and C10), blueberry (blueb.) (05-88 and 05-48), citrus (03-36 and 07-82), and strawberry (strawb.) (07-19 and 98-285). Gray bars represent isolates belonging to the *C. acutatum* species complex, white bars represent isolates belonging to the *C. gloeosporioides* species complex, and the black bar represent mock inoculation. Bars followed by the same letter are not statistically significant ($P = 0.05$) according to Fisher's protected LSD test.

($n = 5$) was *C. nymphaeae* clade, which also contained mango ($n = 3$) and strawberry ($n = 4$) isolates. All pomegranate isolates within the *C. nymphaeae* clade were 100% identical at each locus. Another separate clade identified within the *C. acutatum* species complex was *C. lupini*, with all isolates originating from citrus ($n = 4$) (Fig. 1).

Cross-infectivity, pathogenicity, and virulence. Representative isolates of *C. gloeosporioides*, *C. theobromicola*, *C. siamense*, *C. simmondsii*, and *C. nymphaeae* recovered from pomegranate and other fruit crops were selected to inoculate fruit and leaves of pomegranate cultivar Wonderful.

Inoculated fruit of pomegranate began to show symptoms of anthracnose as dark brown to black necrotic lesions surrounding the inoculation point 1 week after inoculation. Lesion size at 3 weeks after inoculation ranged from 0.3 to 3.5 cm (Fig. 2). Fruit inoculation assays were performed twice, and there was no significant difference between the two experiments ($P = 0.1936$). All isolates tested from pomegranate and other fruit crops were able to infect pomegranate fruit at different levels of aggressiveness in comparison with the control treatments. Thus, significant differences were detected in disease severity among the isolates belonging to different *Colletotrichum* species ($P < 0.0001$). Overall, isolates from the *C. acutatum* species complex were more aggressive than isolates from the *C. gloeosporioides* species complex in the pomegranate fruit assay (Fig. 3). Exceptions were isolates C41 (*C. simmondsii*) and C45 (*C. theobromicola*), which were not different from one another. Isolates from the *C. acutatum* species complex from

blueberry and citrus were more aggressive than isolates from the *C. gloeosporioides* species complex. However, the *C. gloeosporioides* species complex isolate from strawberry was more aggressive than the *C. acutatum* species complex isolate.

Based on the foliar inoculation assay on rooted pomegranate cuttings, inoculated leaves began to show symptoms 2 days after inoculation as small lesions with a brown center and yellow halo. As the symptoms developed, the lesions increased in number but little in size, and the centers became necrotic. The percentage of leaf damage was analyzed 1 week after inoculation. Results from the leaf inoculation were the opposite of fruit experiments, with isolates from the *C. gloeosporioides* species complex being more aggressive than the isolates from the *C. acutatum* species complex (Fig. 4). Exceptions were isolates C30 and C17, *C. gloeosporioides* species complex, which were less aggressive than isolate C64, *C. acutatum* species complex. All isolates from other fruit crops tested were also able to infect pomegranate when inoculated on the leaves, but at a lower disease severity level than the isolates from pomegranate ($P < 0.0001$). Based on leaf inoculation, isolates of *C. acutatum* and *C. gloeosporioides* species complexes, from either citrus or blueberry, were not significantly different from each other. The strawberry isolate from the *C. gloeosporioides* species complex was more aggressive to leaves than the one from the *C. acutatum* species complex.

All 17 representative isolates were also inoculated on strawberry, blueberry, citrus, and mango fruits (Table 3). From inoculations

Fig. 4. Aggressiveness of 17 *Colletotrichum* spp. isolates on leaves of pomegranate 'Wonderful.' Isolates used for the pomegranate whole plant inoculations were recovered from the following fruits: pomegranate (Pom) (C67, C66, C64, C45, C41, C30, C18, C17, C15, and C10), blueberry (blueb.) (05-88 and 05-48), citrus (03-36 and 07-82), strawberry (strawb.) (07-19 and 98-285), and mango (08-76). Gray bars represent isolates belonging to the *C. acutatum* species complex, white bars represent isolates belonging to the *C. gloeosporioides* species complex, and the black bar represent mock inoculation. Bars followed by the same letter are not statistically significant ($P = 0.05$) according to Fisher's protected LSD test.

performed on strawberry fruits, results showed differences among the isolates tested ($P < 0.0001$). The most aggressive isolate on strawberry fruits was isolate 07-19 recovered from strawberry and belonging to the *C. acutatum* species complex. Pomegranate isolates from the *C. acutatum* species complex were more aggressive than isolates from the *C. gloeosporioides* species complex, with the exception of isolate C41 (Table 3). From the citrus inoculation, the most aggressive isolate was the citrus one belonging to the *C. gloeosporioides* species complex. There was a group of isolates pathogenic to citrus, including five pomegranate isolates, one blueberry (05-48), and two strawberry isolates (Table 3). All 17 isolates from pomegranate, strawberry, blueberry, citrus, and mango were pathogenic to mango fruits in comparison with the water control ($P < 0.0001$). From inoculations performed on blueberry fruits, all pomegranate isolates and one blueberry isolate from the *C. acutatum* species complex were more aggressive than isolates from pomegranate *C. gloeosporioides* species complex (Table 3).

Discussion

Pomegranate is an experimental, alternative crop for the southeastern U.S., cultivated on a very limited scale, with an estimate of approximately 100 acres across four states (KC et al. 2017; C. Weinstein, *personal communication*), compared with the large-scale pomegranate production of almost 33,000 acres (NASS 2012) in California. Limited information exists on the economically important diseases of pomegranate, their biology, and management in production areas lacking a Mediterranean climate. In surveys conducted from 2014 to 2017 in Florida, Georgia, and South Carolina, anthracnose was identified as the most important yield-limiting factor in commercial production in the southeastern U.S., capable of causing more than 90% fruit loss if left unmanaged (KC and Vallad 2015).

The sampling was limited to 26 isolates because the production area is currently very limited in the region with approximately 100 acres.

Table 3. Aggressiveness of 17 *Colletotrichum* spp. isolates on strawberry, citrus, mango, and blueberry fruits

Isolates ^w	Host ^x	Lesion diameter (cm)			Infected fruits (#)
		Strawberry ^y	Citrus ^y	Mango ^y	Blueberry ^y
C67	Pomegranate ^A	0.7 ef ^z	0.4 bc	2.3 cde	3.8 a
C66	Pomegranate ^A	1.1 c	0.3 c	2.0 e	3.5 ab
C64	Pomegranate ^A	1.4 b	0.3 c	1.9 ef	2.7 bc
C45	Pomegranate ^A	0.6 f	0.3 c	2.1 cde	3.5 ab
C41	Pomegranate ^G	0.9 de	0.3 c	1.8 ef	1.0 efg
C30	Pomegranate ^G	0.2 hi	0.6 bc	2.6 bcd	1.7 de
C18	Pomegranate ^G	0.3 gh	0.3 c	2.1 cde	2.8 bc
C17	Pomegranate ^G	0.3 gh	1.2 b	2.1 cde	1.3 def
C15	Pomegranate ^G	0.3 gh	0.7 bc	3.3 a	2.0 cd
C10	Pomegranate ^G	0.3 gh	0.5 bc	2.1 de	0.5 g
05-88	Blueberry ^A	1.1 cd	0.3 c	2.0 e	3.8 a
05-48	Blueberry ^G	0.5 fg	0.4 bc	2.0 e	2.7 bc
03-36	Citrus ^A	0.6 f	0.3 c	1.4 f	1.7 de
07-82	Citrus ^G	0.1 hi	3.6 a	2.1 cde	0.3 g
07-19	Strawberry ^A	1.6 a	0.4 bc	2.6 bc	3.5 ab
98-285	Strawberry ^G	1.2 bc	0.4 bc	3.1 ab	2.7 bc
08-76	Mango ^A	0.2 hi	0.2 c	2.0 e	2.7 bc
Water		0.0 i	0.1 c	0.2 g	0.5 g
P value		<0.0001	<0.0001	<0.0001	<0.0001

^w Isolates used for the fruit inoculations were recovered from pomegranate (C67, C66, C64, C45, C41, C30, C18, C17, C15, and C10), blueberry (05-88 and 05-48), citrus (03-36 and 07-82), strawberry (07-19 and 98-285), and mango (08-76).

^x Superscript A indicates isolates from the *C. acutatum* species complex, and superscript G indicates isolates from the *C. gloeosporioides* species complex.

^y Fruits used for the inoculations.

^z Values followed by the same letter within columns are not statistically significant ($P = 0.05$) according to Fisher's LSD test.

Based on our results, the distribution of *C. acutatum* and *C. gloeosporioides* species complexes on pomegranate across the southeastern U.S. appears to be associated with geographic regions. However, this is likely owing to sampling limitations, because the production area of pomegranate across the southeastern U.S. is relatively low.

This study is the first in which *Colletotrichum* species associated with pomegranate across the southeastern U.S. were characterized using pathological and molecular tools. Based on multilocus sequence analysis, the pomegranate isolates were separated into two major species complexes, *C. acutatum* and *C. gloeosporioides*, with 73% belonging to the latter group. Moreover, this study revealed that *Colletotrichum* isolates causing anthracnose of pomegranate belong to six distinct *Colletotrichum* species: *C. gloeosporioides*, *C. theobromicola*, *C. fiorinae*, *C. simmondsii*, and *C. nymphaeae*, and *C. siamense*. The latter was defined as *C. siamense*, and not *C. fructicola*, based on the TUB2 individual gene tree, which has been reported to be the best to distinguish between these two species (Weir et al. 2012).

To date, *C. gloeosporioides* (Mendes 1998; Munhuweyi et al. 2016; Thomidis 2014), *C. acutatum* (Bellé et al. 2018), *C. fructigenum* (Gorter 1977), *C. theobromicola* (Sharma et al. 2015; Shivas et al. 2016), and *C. tropicale* (Silva-Cabral et al. 2019) are the species reported to cause anthracnose of pomegranate worldwide. In the U.S., the current *Colletotrichum* species infecting pomegranate are unknown; however, *C. gloeosporioides* and *C. theobromicola* were previously reported based on morphological evidence (Alfieri 1984; Anonymous 1960). Thus, this report expands to include the *C. acutatum* species complex as a cause of preharvest anthracnose on pomegranate.

The detached fruit inoculation experiment was performed using the standard method to inoculate pomegranate fruits in other published studies using several fungal isolates, including *Colletotrichum* (Chen et al. 2014; Mincuzzi et al. 2017; Palou et al. 2010; Thomidis and Exadaktylou 2011). Our results from the foliar inoculation assay confirmed the results obtained from the detached pomegranate fruit assay, with all the isolates tested being able to infect pomegranate at different levels of aggressiveness in comparison with the control treatments. Furthermore, our results from inoculation assays on pomegranate fruit and leaves also confirmed the separation of the pomegranate isolates into two groups. Representative isolates from the *C. acutatum* species complex were more aggressive than isolates from the *C. gloeosporioides* species complex when inoculated on fruit and less aggressive when inoculated on the intact leaves. It has been indicated that *C. acutatum* is more important in causing fruit rot of strawberry than *C. gloeosporioides* (Peres et al. 2005). In citrus, *C. acutatum* is able to cause postbloom fruit drop and key lime anthracnose. Although these two diseases are caused by distinct phylogenetic lineages, postbloom fruit drop clade isolates were not able to cause disease on key lime foliage (Peres et al. 2008). Furthermore, *Colletotrichum* spp. can form latent infections on many fruit crops early in the season that can later develop, resulting in fruit rots at harvest or even postharvest (Jeffries et al. 1990; Peres et al. 2005; Wharton and Diéguez Uribeondo 2004).

In Florida, it is common that other fruit crops, such as citrus, blueberry, strawberry, and mango, are grown in close proximity to pomegranate orchards. Because *Colletotrichum* spp. are also able to cause anthracnose on citrus, blueberry, strawberry, and mango (Arauz 2000; MacKenzie et al. 2007; Polashock et al. 2005), we evaluated the possibility of cross-infection between isolates from pomegranate and other fruit crops. Pomegranate isolates were already reported to cause anthracnose on mango leaves and fruits (Lakshmi et al. 2011). All pomegranate isolates tested were able to infect wounded mango fruit, as well as nonwounded blueberry and strawberry fruits, with the exception of isolate C10 on blueberry fruit. Five pomegranate isolates were able to infect wounded citrus fruit. All the nonpomegranate isolates tested in this study were able to infect wounded pomegranate fruit and intact leaves. Phylogenetic analysis performed in this study showed a close relationship among isolates from pomegranate and other hosts. Combined with the laboratory results that

demonstrate cross-pathogenicity, these findings suggest a wide host range of *Colletotrichum* species causing anthracnose on pomegranate and may assist in disease management decisions while considering initial sources of inoculum.

In conclusion, the presence of six different species of *Colletotrichum* on pomegranate can complicate disease management and breeding efforts. Differences in fungicide sensitivity among species within the *C. acutatum* and *C. gloeosporioides* complexes have been demonstrated (Munir et al. 2016; Peres et al. 2004; Valero et al. 2010); thus, the presence of species within both of these two complexes could affect fungicide spray regimes recommended to growers. At-bloom fungicide applications are critical for effective disease management in many *Colletotrichum*–host systems (Arauz 2000; Peres et al. 2002). Therefore, it will be essential to identify preventive and curative fungicide applications as well as to determine the application timing necessary for managing fruit losses. Additional studies will also need to address potential differences in fungicide sensitivity among isolates from the various *Colletotrichum* species identified in this study. At this point, there are no conventional fungicides labeled for anthracnose of pomegranate in the U.S., although efforts are in progress to pursue labeling of effective fungicides for pomegranate.

In the meantime, however, it will be critical that growers adopt cultural management strategies, such as field sanitation, to minimize seasonal carryover of pathogens, and that research efforts continue to further understand the biology and epidemiology of anthracnose on pomegranate.

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