Genotypic and Pathogenic Diversity of *Colletotrichum sublineola* Isolates from Sorghum (*Sorghum bicolor*) and Johnsongrass (*S. halepense*) in the Southeastern United States

K. V. Xavier, Department of Plant Pathology. University of Kentucky, Lexington 40546-0312; **E. S. G. Mizubuti,** Departamento de Fitopatologia, Universidade Federal de Viçosa, CEP 36570-900, Viçosa, MG Brazil; **M. V. Queiroz,** Departamento de Microbiologia, Laboratório de Genética Molecular de Fungos/BIOAGRO, Universidade Federal de Viçosa, Av. PH. Rolfs s/n, CEP 36570-900, Viçosa, MG Brazil; **S. Chopra,** Department of Plant Science, Pennsylvania State University, University Park 16802; and **L. Vaillancourt,**[†] Department of Plant Pathology, University of Kentucky

Abstract

Anthracnose caused by *Colletotrichum sublineola* is an important disease of cultivated sorghum (*Sorghum bicolor*) worldwide. Anthracnose is also common on the ubiquitous wild sorghum relative Johnsongrass (*S. halepense*). Analysis of repetitive molecular fingerprinting markers revealed that isolates of *C. sublineola* from both hosts in the southeastern United States were genotypically diverse, with relatively few haplotypes found in more than one location. With few exceptions, isolates recovered from *S. bicolor* belonged to a population that was genetically distinct from the population recovered from *S. halepense*. Twenty-three isolates from cultivated sorghum were all pathogenic to at least one of 13 heritage inbred lines of *S. bicolor*. In all, 4 of 10 isolates from *S. halepense* were also pathogenic to one or more of the lines, while the rest caused no disease in greenhouse assays. The four pathogenic isolates from *S. halepense*

were less aggressive, on average, than isolates from *S. bicolor*, although the ranges overlapped. Pathogenicity tests involving 15 representative pathogenic isolates from *S. bicolor* and *S. halepense* on eight heritage inbred lines of *S. bicolor* identified 12 races. The combined results of this study demonstrated that *C. sublineola* comprises two separate host-associated subpopulations in the field, even though some isolates from *S. halepense* were able to cause disease on *S. bicolor* under ideal greenhouse conditions. Nonetheless, the apparent existence of infrequent cross-infection events in the field, indicated by molecular fingerprinting, suggests that Johnsongrass has the potential to serve as a refuge and an incubator for genetic diversity in *C. sublineola*, which can complicate efforts to develop and deploy resistant sweet sorghum varieties in the region.

Anthracnose, caused by the fungus *Colletotrichum sublineola* Henn. ex Sacc. & Trotter 1913, is among the most important diseases of sorghum (*Sorghum bicolor* (L.) Moench) worldwide (Ali and Warren 1987; Chala et al. 2010b; Frederiksen and Odvody 2000; Jamil and Nicholson 1987; Warren 1986). Anthracnose can reduce yields of grain sorghum by 50% or more under warm and humid conditions in a susceptible variety (Ali and Warren 1987; Harris et al. 1964; Tesso et al. 2012). *C. sublineola* can infect all parts of the plant, including the stalk, peduncle, panicle, grain, and leaves (Ferreira and Warren 1982; Tesso et al. 2012). The pathogen can be spread over long distances via infested seed (Cardwell et al. 1989; Zhang et al. 2017). The primary control strategy for anthracnose in grain sorghum is the use of resistant cultivars (Chala et al. 2010a). Resistance is controlled mostly by single dominant genes, some with multiple allelic forms (Boora et al. 1998; Coleman and Stokes 1954; Le Beau and Coleman 1950; Tenkouano et al. 1993).

Although resistance is key for the management of anthracnose in grain sorghum, it frequently fails, and genotypes that provide high levels of resistance in one location often succumb to the disease in

another. This is thought to be related to the high degree of variability in the population of *C. sublineola* affecting grain sorghum (Casela and Frederiksen 1993; Casela et al. 1995, 1998, 2000, Chala et al. 2011; Prom et al. 2012). Genetic diversity within the pathogen population has been characterized by the use of repetitive molecular fingerprinting markers, including random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP), and amplified fragment length polymorphisms (Chala et al. 2011; Guthrie et al. 1992; Prom et al. 2012; Vaillancourt and Hanau 1992; Valèrio et al. 2005).

The ability of *C. sublineola* to infect a differential series of grain sorghum cultivars has been used to identify a large number of races in this species (Ali and Warren 1987; Boora et al. 1998; Chala et al. 2011; Rosewich et al. 1998; Valèrio et al. 2005). The interaction between sorghum and *C. sublineola* was suggested to follow a gene-for-gene pattern (Casela et al. 1995).

Sweet sorghum has been grown in the southeastern United States for more than 150 years on a relatively limited scale, primarily for the production of table syrup (Winberry 1980). During the Second World War, production was increased in the region to provide a substitute for imported cane sugar. Anthracnose disease became a serious problem in these plantations (Hunter and Anderson 1997; LeBeau et al. 1951). Although cultivation of sweet sorghum in the Southeast decreased dramatically after the war, it has continued to be an important specialty crop in Kentucky, which is one of the top producers of sorghum syrup in the United States (Bitzer 1997, 2009). Recently, interest in sweet sorghum as a potential source of biofuel has increased in the United States and worldwide (Bitzer 1997, 2009; Cutz et al. 2013; Kim and Day 2011; Koçar and Civaş 2013; Pederson 2010; Sarath et al. 2008; Sipos et al. 2009; Wheals et al. 1999; Yu et al. 2014). Anthracnose is listed as one of the most important diseases of sweet sorghum in Kentucky (Bitzer 2009), and it is also common on the hybrids that are increasingly grown in the Southeast for feedstock (Hagan et al. 2014). If sweet sorghum production continues to expand in the region, anthracnose has the potential to become a significant limiting factor.

[†]Corresponding author: L. Vaillancourt; E-mail: vaillan@uky.edu

Current address of K. V. Xavier: University of Florida, Gulf Coast Research and Education Center, 14625 CR 672, Wimauma, 33598.

C. sublineola is also a pathogen of the weed Johnsongrass (*S. halepense* (L.) Pers), which is related to *S. bicolor* (Paterson et al. 1995) and is ubiquitous across the Southeast. Johnsongrass is genetically diverse (Morrell et al. 2005). This host diversity could lead to the selection and maintenance of multiple *C. sublineola* races in the population; thus, Johnsongrass could serve as a refuge and an incubator for genetic variation in the pathogen.

There is currently no information about the population structure of *C. sublineola* in the southeastern United States. The goal of this work was to characterize genotypic and pathogenic diversity among *C. sublineola* isolates from *S. bicolor* and *S. halepense* in Kentucky and other southeastern states where sweet sorghum production is increasing, and to investigate the frequency of cross-infection among the two plant species. This information will help to improve management recommendations for anthracnose in sweet sorghum if it is more widely grown in the region in the future.

Materials and Methods

Fungal isolates. A collection of 384 fungal isolates was compiled for this study (Supplementary Table S1). Most of the isolates were recovered in 2011 to 2015 from sweet, forage, or grain sorghum (*S. bicolor*) as well as from Johnsongrass (*S. halepense*) in Kentucky, Georgia, Alabama, and Florida. A few grain sorghum isolates previously collected in the 1980s up through 2013 from Indiana, Texas, Brazil, Burkina Faso, Zambia, South Africa, and Sudan were also included in parts of the study. The identities of the isolates as *C. sublineola* were confirmed based on morphological characters, including production of acervuli with setae on the diseased tissue and typical falcate conidia in planta and in culture.

Isolates were recovered from the margins of anthracnose lesions on leaves or stalks. Tissue pieces were soaked in 70% ethanol for 30 s, then 10% sodium hypochlorite (Clorox) for 2 min, followed by three washes with sterile water. Disinfected tissue pieces were dried on sterile paper towels and placed on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) containing ampicillin (0.1 mg/ml). Cultures were incubated at 23°C under continuous fluorescent light. Isolates were purified by single sporing with a spore-cutter device (Muse et al. 2003) and preserved on silica gel at –80°C (Tuite 1969). Fungal isolates were routinely cultured on PDA at 23°C under continuous fluorescent light.

Genomic DNA extraction. Fungal conidia were harvested by flooding a 14-day-old PDA culture with water and rubbing gently with a sterilized plastic minipestle. The conidia were washed three times with sterile water. A volume of 10 ml of potato dextrose broth (Difco Laboratories) was added to a 60-mm Petri plate and inoculated with a conidial suspension to achieve a final concentration of 5×10^4 conidia/ml. Plates were incubated at 23°C under continuous fluorescent light for 7 days, after which mycelial mats were collected, frozen, and lyophilized. Genomic DNA was extracted by using the protocol of Thon et al. (2000). Briefly, the lyophilized mycelium was crushed to a fine powder, then mixed with 1.2 ml of DNA extraction buffer (100 mM Tris, pH 7.5; 0.7 M NaCl; 10 mM EDTA; and 10 g of cetyltrimethylammonium bromide per liter). The mixture was incubated at 65°C for 30 min and then an equal volume of chloroform

was added to each sample. The samples were centrifuged at $3,000 \times g$ for 15 min, the upper phase was transferred to a 1.5-ml Eppendorf tube, and the DNA was precipitated with 0.8 volume of isopropanol. After centrifugation, the pellets were washed with 70% ethanol and resuspended in Tris-EDTA buffer.

RFLP fingerprints with transposon probes. Proteins containing predicted transposase domains were identified by using the Protein Family Database (Pfam) to screen the predicted proteome of *C. sublineola* isolate CgSl1 (Buiate et al. 2017). A partial putative transposase sequence related to transposon elements belonging to the *Tc1-Mariner* superfamily was identified that was similar to JMSE01000036, a predicted transposase sequence from *C. sublineola* isolate CsTX430BB (Baroncelli et al. 2014) (Supplementary Fig. S1). The reverse complement of the CgSl1 sequence was used to design the transposase primers CsTn1F and CsTn1R (Table 1). Polymerase chain reaction (PCR) was performed using as a template genomic DNA from CgSl1. PCR conditions consisted of 3 min of denaturation at 94°C; followed by 35 cycles of denaturation for 30 s at 94°C, 60 s of annealing at 57°C, and 40 s of extension at 72°C; and a final extension of 10 min at 72°C. The resulting amplicon was sequenced and submitted to GenBank as CsTn1 (accession number KY006076). The amplicon was labeled with ^{32}P to produce a probe for Southern hybridization. Genomic DNA (1 μg) from each fungal isolate was digested with the restriction enzyme *Hin*dIII. The digested DNA samples were separated on a 0.7% agarose gel and transferred to a positively charged nylon membrane. The membrane was cross-linked with UV light, and the DNA was hybridized with the radioactively labeled probe.

RFLP fingerprints with telomere probes. Telomere probes were produced by using a published PCR protocol (IJdo et al. 1991). PCR was performed with primers (TTAGGG)$_5$ and (CCCTAA)$_5$ in the absence of template. The 100-μl reactions contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$, 0.001% gelatin, 200 μM each dNTP, 0.1 μM each primer, and 2 U of *Taq* DNA polymerase. The PCR amplification protocol consisted of 10 cycles of 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C; followed by 30 cycles each consisting of 1 min at 94°C, 30 s at 60°C, and 90 s at 72°C; and a final step of 5 min at 72°C. The resulting amplicons were gel purified and labeled with ^{32}P. Conditions for the Southern blot were as described above.

RAPD. Genomic DNA of *Colletotrichum* isolates was amplified by using the RAPD-PCR method (Williams et al. 1990), with 10 different 10-base random primers (Browning et al. 1999; Vaillancourt and Hanau 1992) (Table 1). Reactions were carried out in a final volume of 25 μl containing 10 ng of genomic DNA, 10× PCR buffer, 50 mM MgCl$_2$, 200 μM dNTP, 5 μM primer, and 1 U of *Taq* DNA polymerase. The PCR cycling protocol consisted of 3 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 35°C, and 2 min for extension at 72°C; followed by 34 cycles of 1 min of denaturation at 94°C, 20 s of annealing at 40°C, and 2 min of extension at 72°C; with a final extension of 72°C for 10 min. Amplicons were resolved on 1.0% agarose gels, stained with ethidium bromide, and visualized under UV light.

Identification and characterization of haplotypes. Isolates with the same band patterns were visually identified as members of the

Table 1. Random amplified polymorphic DNA (RAPD) and transposase primers used in this study

Primer	Type	Primer sequence	Reference
OPA 1	RAPD	5′CAGGCCCTTC3′	Vaillancourt and Hanau 1992
OPA 2	RAPD	5′TGCCGAGCTG3′	Vaillancourt and Hanau 1992
OPA 3	RAPD	5′AGTCAGCCAC3′	Vaillancourt and Hanau 1992
UBC 356	RAPD	5′GCGGCCCTCT3′	Browning et al. 1999
UBC 384	RAPD	5′TGCGCCGCTA3′	Browning et al. 1999
UBC 391	RAPD	5′GCGAACCTCG3′	Browning et al. 1999
UBC 357	RAPD	5′AGGCCAAATG3′	Browning et al. 1999
UBC 345	RAPD	5′GCGTGACCCG3′	Browning et al. 1999
OPA 11	RAPD	5′CAATCGCCGT3′	This publication
OPA 18	RAPD	5′AGGTGACCGT3′	This publication
CsTn1F	Transposase	5′AATCCAGGCTGATCTTGGGC3′	This publication
CsTn1R	Transposase	5′CCTGGCGAGCCTTCTGATAG3′	This publication

same RFLP or RAPD haplotypes (Supplementary Table S2). A matrix including 105 clone-corrected *C. sublineola* isolates, generated by allele presence or absence for all 10 primers (Supplementary Table S3), was subjected to principal component analysis (PCA) as an exploratory tool to describe groups of individuals with genetic similarities. Subsequently, the discriminant analysis of principal components (DAPC) was performed to identify more robust groups of genetically similar isolates. The Bayesian information criterion was evaluated to define the optimal number of clusters based on visual assessment of a scree plot, followed by execution of 10 runs of the "find.clusters" function. The adegenet package for R was used for these analyses (Jombart 2008). Evidence for random mating was tested in the whole dataset considering no structure according to host of origin, or groups defined by statistical methods (see above), and also in the populations from *S. halepense* and from sweet sorghum (*S. bicolor*) separately. The r_D index, a standardized index of association that estimates linkage disequilibrium, was used to test for random mating using 1,000 bootstraps (Agapow and Burt 2001).

Pathogenicity assays. Thirty-one isolates representing the most common RFLP haplotypes from a range of hosts and locations were tested for pathogenicity on the sweet sorghum variety Sugar Drip in the greenhouse (Supplementary Table S4). Due to limited space availability, the isolates were tested in two groups in separate experiments. The first experiment was repeated three times and the second was repeated twice. One isolate of *C. graminicola* from maize (M30.001) was used as a negative control in all experiments: this *C. graminicola* isolate is nonpathogenic on healthy sorghum plants (Torres et al. 2014). An aggressive isolate of *C. sublineola* from

sweet sorghum (SS63.001) was included as a positive control in all experiments.

In all, 19 *C. sublineola* isolates from the original group of 31 were tested on between 7 and 12 additional inbred varieties of *S. bicolor*, including Honey, Simon, Bailey, Chinese Amber, Dale, Dwarf Yellow Milo, Cowper, Orange, Planter, N100, Della, and Keller. Isolates were again tested in two groups in separate experiments, each repeated twice, and each also including the positive and negative controls. Sugar Drip seed were obtained from Dr. Todd Pfeiffer at the University of Kentucky (UK). Seed of the other varieties were obtained from Dr. Gary Pederson at the Plant Genetic Resources Conservation Unit in Griffin, GA. All seed stocks were increased in 2012 at the UK Agronomy Farm in Lexington.

The fungal isolates were cultured on PDA at 23°C under continuous fluorescent light for 2 weeks. Production of inoculum was done according to a modified method of Prom et al. (2009). Sterile water (10 ml) was added to each plate and conidia were loosened with a minipestle. The conidia were washed three times with sterile water. Sugar Drip sorghum grains were prepared for inoculation by washing and soaking them in water for 48 h, then autoclaving twice (121°C for 30 min). A conidial suspension was used to inoculate 40 g of autoclaved grains in a Petri plate to achieve a final concentration of 5×10^6 conidia/g. The plates were incubated for 14 days at 23°C in the dark.

Pathogenicity experiments were performed in the greenhouse as described previously (Xavier et al. 2017). Four sorghum seeds were sown per 20.32-cm pot, in a 3:2 mixture of Pro-Mix BX (Premiere Horticulture, Ltd., Riviere du Loup, PQ, Canada) and sterile topsoil.
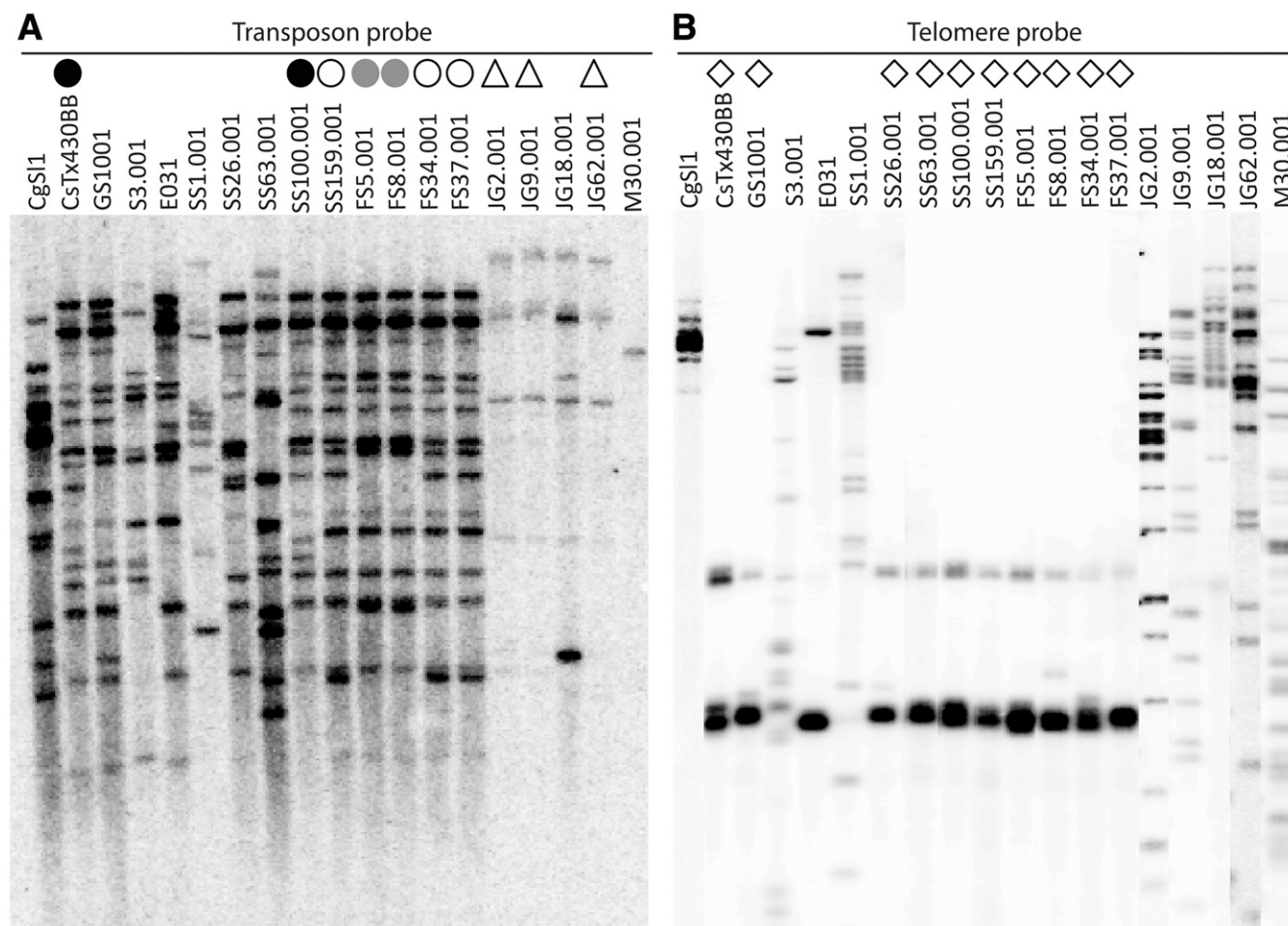

Fig. 1. Representative Southern blot analysis of *Colletotrichum* genomic DNA digested with *Hin*dIII. The strain name is indicated above each lane. The same blot was hybridized with the **A,** transposon and **B,** telomere probes. Matching symbols above the lanes in A indicate strains with the same transposon haplotype. Diamond symbols above the lanes in B indicate strains with similar patterns of hybridization with the telomere probe, classified as haplotype 33. Grain sorghum isolates of *Colletotrichum sublineola* were CgSl1, CsTx430BB, GS1.001, S3.001, and E031. Sweet sorghum isolates of *C. sublineola* are designated by the prefix "SS". Forage sorghum isolates are designated by the prefix "FS". Johnsongrass isolates are designated by the prefix "JG". M30.001 is an isolate of *C. graminicola* from maize.

Pots were arranged in a randomized complete block design with four blocks. Each treatment replicate included two pots containing a total of eight plants. Plants were grown to the V5 or V6 stage, then inoculated by placing 10 colonized sorghum grains into the whorl of each plant.

Disease development was evaluated weekly, beginning 14 days after inoculation, for a period of 4 weeks. The disease evaluation was performed based on incidence, expressed as the number of infected plants (compatible interactions). Plants were scored as infected when sporulating acervuli were observed on the foliage.

Incidence values from the combined data sets from the repetitions of each experiment were used to calculate the area under the disease progress curve (AUDPC) by the trapezoidal integration method (Campbell and Madden 1990). The AUDPC data were subjected to analysis of variance after square root transformation before further analysis, to stabilize the variance. Treatment means were compared with the negative control (M30.001) by using the Dunnett's test procedure of the SAS software package (SAS Users Guide, version 9.4; SAS Institute, Cary, NC, USA). P values < 0.05 were considered significant.

Results

Molecular marker analysis. *RFLP analyses, transposase probe.* The CsTn1 transposase probe hybridized to multiple polymorphic restriction fragments in all of the *Colletotrichum* isolates. However, hybridization to the maize isolate and to a majority of the Johnsongrass isolates was relatively weak, with fewer fragments, compared with most isolates from *S. bicolor*. Representative results are shown in Figure 1A. In total, 61 different haplotypes could be identified with this probe (Table 2).

RFLP analyses, telomere probe. The telomere probe hybridized to one or more fragments in all of the *Colletotrichum* isolates and identified 49 different haplotypes (Table 2). The Johnsongrass isolates had diverse patterns of hybridization, consisting of between 1 and 36 individual bands (Fig. 1B). Among the grain sorghum isolates, the probe hybridized to between 1 and 24 fragments. None of the patterns with multiple hybridizing fragments matched those from the Johnsongrass isolates. Most of the isolates from forage and sweet sorghum had just one major band of the same or similar size when hybridized with the telomere probe (Fig. 1B). For this reason, telomere

fingerprinting did not differentiate among the sweet and forage sorghum isolates. However, it could be used to distinguish between two groups: one with multiple bands, mostly from Johnsongrass and grain sorghum, and the other with a single major band, mostly from sweet and forage sorghum. The exceptions included seven grain sorghum isolates and one isolate from Johnsongrass that had the single major band, and seven isolates from sweet sorghum, representing three haplotypes, with multiple telomere bands.

Combined haplotypes. Among the 49 telomere haplotypes, only 2 included more than one transposon haplotype. One of these was type 33, which had only a single major hybridizing band and included the majority of sweet sorghum and forage sorghum isolates. The 273 isolates with telomere type 33 encompassed 35 transposon haplotypes. Among all 61 transposon haplotypes, 7 included more than one telomere haplotype but only three isolates had telomere haplotypes that were also found in combination with other transposon haplotypes. Thus, the transposon and telomere haplotypes were consistent for 381 of the 384 isolates (99%). To maximize the available information, the isolates were classified into 85 groups based on the combined transposon-telomere haplotype (Table 2).

Only three combined haplotypes were found in more than one state. The most common of these was 17:33 (Fig. 2), which included 83 isolates from sweet sorghum and 15 from forage sorghum and was found in Kentucky and Alabama (Fig. 2). Haplotype 17:33 also included two isolates from grain sorghum: one from Texas and one from South Africa (Fig. 2). Two other haplotypes (19:33 and 21:33) were found on both forage and sweet sorghum in Georgia, Florida, and Alabama. The remaining combined haplotypes were confined to a single host type and were found in only one location.

Among the Johnsongrass isolates, 34 different combined haplotypes could be discerned (Table 2). The Johnsongrass isolates displayed more diversity of combined haplotypes (haplotype richness) across most locations than the sweet sorghum isolates (Table 2). Nearly all of the haplotypes from Johnsongrass were found in only a single location (Fig. 2). However, there were four combined haplotypes that were more widely dispersed in Kentucky, including haplotypes 42:60 and 44:62, found in both Fayette and Franklin Counties (Fig. 2). None of these haplotypes was found outside Kentucky.

Analysis of genetic diversity among haplotypes. The telomere probe was not very informative for a large group of isolates from

Table 2. Combined haplotype richness by host and by location (locations with only one isolate were not included)[a]

Host	N[b]	Trans[c]	Telo[d]	Comb[e]	Haplotype richness	Location	Number of isolates	Number of combined haplotypes	Haplotype richness
Johnsongrass	87	14	33	34	0.39	Fayette, KY	18	6	0.33
	…	…	…	…	…	Franklin, KY	13	6	0.46
	…	…	…	…	…	Russell, KY	35	15	0.43
	…	…	…	…	…	Macon, AL	7	1	0.14
	…	…	…	…	…	Tift, GA	6	2	0.33
	…	…	…	…	…	Gadson, FL	7	6	0.86
Sweet sorghum	229	32	5	35	0.15	Brewton, AL	24	1	0.04
	…	…	…	…	…	Macon, AL	8	1	0.13
	…	…	…	…	…	Tift, GA	67	17	0.25
	…	…	…	…	…	Live Oak, FL	77	17	0.22
	…	…	…	…	…	Montgomery, KY	51	1	0.02
Forage sorghum	49	3	1	3	0.06	Brewton, AL	29	3	0.10
	…	…	…	…	…	Cullman, AL	8	2	0.25
	…	…	…	…	…	Macon, AL	12	2	0.17
Grain sorghum	18	15	12	16	0.88	United States	4	4	1
	…	…	…	…	…	Brazil	9	8	0.89
	…	…	…	…	…	Africa	5	5	1

[a] Haplotype richness = number of haplotypes divided by the number of strains.
[b] Total number of isolates.
[c] Total transposase haplotypes.
[d] Total telomere haplotypes.
[e] Total combined haplotypes.

S. bicolor that had only a single hybridizing fragment, while the transposon probe hybridized relatively poorly to the majority of isolates from *S. halepense*. Thus, we also characterized the isolates by amplifying them with RAPD primers. Isolates that shared the same patterns of bands for all 10 primers in multiple replicated experiments were considered to belong to the same RAPD haplotype. Representative results are shown in Supplementary Figure S2. RAPD analysis was performed for 358 of the isolates, identifying 281 haplotypes
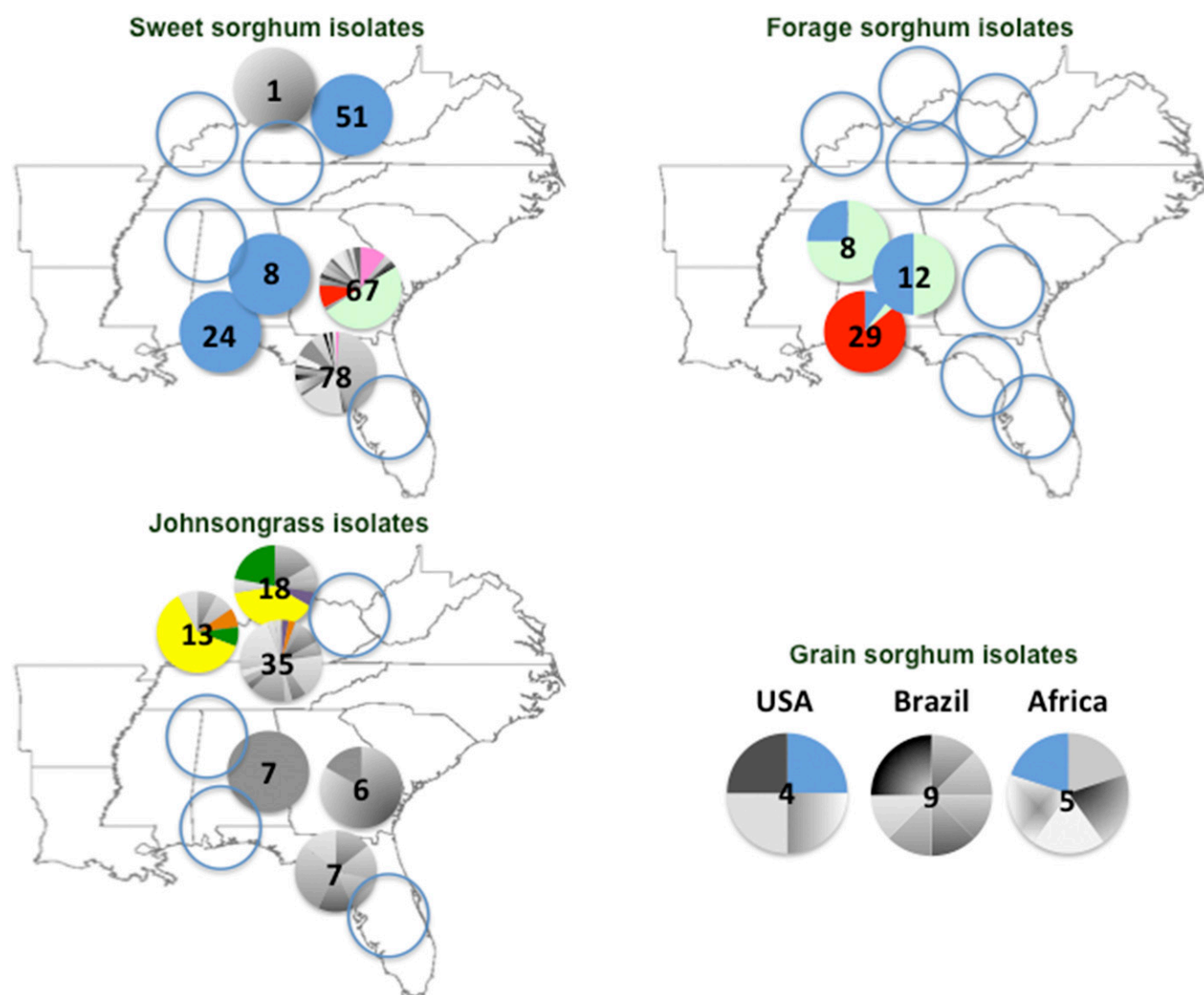

Fig. 2. Regional distribution of combined haplotypes. Pie slices that are the same colors represent the same combined haplotype found in different locations or on different host types. Slices in gray tones represent unique combined haplotypes that were found only in that location. The total number of isolates from each location is presented in the center of each pie graph. Empty circles represent locations from which no isolates of that category were obtained.

Table 3. Numbers of haplotypes identified among *Colletotrichum* isolates from Johnsongrass and from sweet, forage, and grain sorghum based on random amplified polymorphic DNA (RAPD) and on hybridization with the telomere or transposon probes

Plants	Number of strains	Number of locations	Probes	Individual haplotypes
Johnsongrass	85	7	RAPD	77
…	…	…	Telomere	33
…	…	…	Transposon	14
Sweet sorghum	214	6	RAPD	173
…	…	…	Telomere	5
…	…	…	Transposon	31
Forage sorghum	42	3	RAPD	22
…	…	…	Telomere	1
…	…	…	Transposon	3
Grain sorghum	16	15	RAPD	11
…	…	…	Telomere	11
…	…	…	Transposon	16
Total	358[a]	24	RAPD	281
…	…	…	Telomere	49
…	…	…	Transposon	59

[a] Total includes one maize strain of *Colletotrichum graminicola*.

(Table 3). Most of the haplotypes were represented by only one isolate. Among the 37 RAPD haplotypes that included two or more isolates (comprising a total of 114 isolates), 29 consisted only isolates with the same combined RFLP (transposase and telomere) haplotype. The other eight RAPD haplotypes included isolates with more than one combined RFLP haplotype, suggesting the possibility of homoplasy for some of the RAPD markers. Nonetheless, the RAPD haplotypes were consistent with the combined RFLP haplotypes for more than 95% of all isolates.

Individual polymorphic bands for 105 *C. sublineola* isolates with unique RAPD haplotypes, representing 76 combined RFLP haplotypes, were manually scored and entered into a matrix. Data were analyzed by PCA, initially without assuming any groupings by host or origin. The results provided evidence for a population structure that was strongly associated with host species (Fig. 3). Thus, the majority of isolates from *S. bicolor* (including isolates from grain, sweet, and forage sorghum) clustered together into one group, while the majority of isolates from *S. halepense* formed a second group. One Johnsongrass isolate (JG16.001) clustered with the *S. bicolor* isolates (Fig. 3). One sweet sorghum isolate (SS1.001) and one Johnsongrass isolate (JG14.001) were intermediate between the two groups (Fig. 3).

DAPC was performed to identify more robust groupings of genetically similar individuals. In all, 8 of 10 runs of the "find.cluster" function resulted in a most likely number of clusters (k) of 7. Confirming the association with host species, Johnsongrass isolates were grouped primarily into clusters 2, 3, and 7 whereas most isolates from *S. bicolor* were found in clusters 1, 4, 5, and 6 (Fig. 4; Supplementary Table S5). Exceptions included one isolate from sweet sorghum (SS1.001) in cluster 7 and two isolates from Johnsongrass (JG14.001 and JG16.001) in cluster 1. Most clusters included isolates with multiple origins, suggesting that gene flow had occurred among locations. All the forage sorghum isolates grouped together in cluster 5 and all but two of the grain sorghum isolates were in cluster 6.

There was no evidence for random mating in the total population (105 isolates; $r_D = 0.032$, *P* value < 0.001) or in the subpopulations on sweet sorghum (45 isolates; $r_D = 0.040$, *P* value < 0.001) or Johnsongrass (38 isolates; $r_D = 0.027$, *P* value < 0.001) (Supplementary Fig. S3). The other subpopulations were too small to analyze.

Pathogenicity assays. Thirty-one isolates from Johnsongrass and from sweet, grain, and forage sorghum, representing diverse geographic and host origins, were tested for pathogenicity on the sweet sorghum inbred Sugar Drip (Table 4). These isolates included members
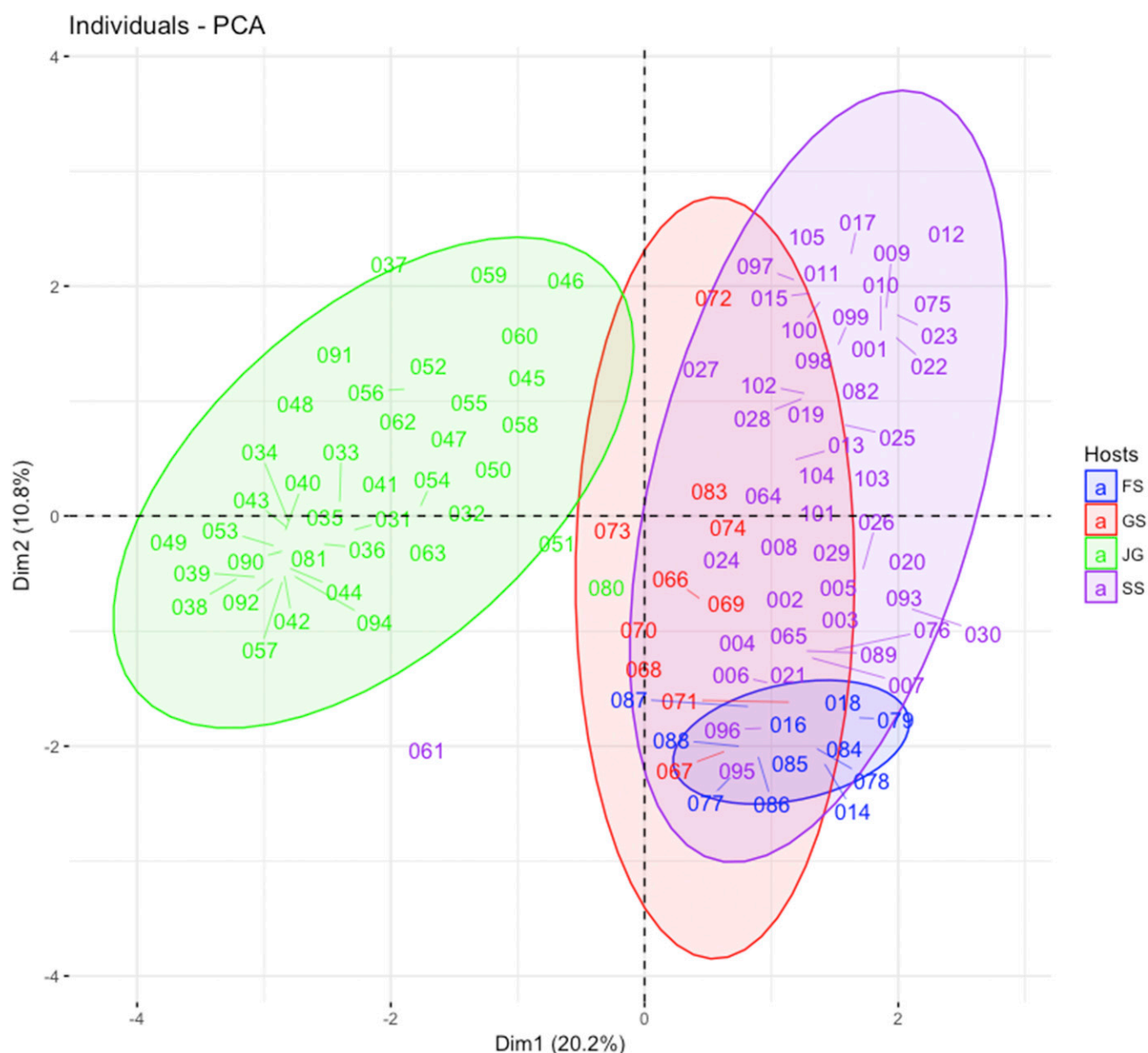

Fig. 3. Clusters of isolates of *Colletotrichum sublineola* based on random amplified polymorphic DNA fingerprint data subjected to principal components analysis (PCA). Isolates are colored-coded according to the host of origin: blue = forage sorghum, red = grain sorghum, green = Johnsongrass, and purple = sweet sorghum.

of the most common combined haplotypes. All of the isolates were compared with the negative control, the nonpathogenic maize isolate M30.001. Six isolates from *S. halepense* and four from *S. bicolor* did not differ statistically from the negative control when inoculated on Sugar Drip (Table 4; Supplementary Table S6). The other isolates caused varying levels of disease (expressed as AUDPC) in the greenhouse (Table 4). Most of these pathogenic isolates were from *S. bicolor*; however, there were three Johnsongrass isolates, all from Kentucky, that were also pathogenic to Sugar Drip (Table 5). All of the pathogenic isolates produced typical foliar anthracnose symptoms, consisting of elongated individual or coalescent reddish lesions with necrotic centers (not shown). On average, the Johnsongrass isolates were less aggressive (had lower AUDPC values) than the isolates from *S. bicolor* but the ranges overlapped among the two groups (Table 5).

A subset of 19 isolates from the experiments on Sugar Drip, including all but 2 of the isolates that were nonpathogenic on that variety, was used to inoculate several additional lines of *S. bicolor* (Table 5). Results revealed that all of the isolates from *S. bicolor* (including those that were nonpathogenic to Sugar Drip) could cause disease on at least one of these sorghum lines (Table 5).

Some isolates (e.g., SS63.001) were pathogenic to most of the sorghum lines tested, whereas others (e.g., SS100.001) were pathogenic to only a few (Table 5). Among the Johnsongrass isolates, some appeared to be completely nonpathogenic to *S. bicolor* because they didn't cause disease on any of the lines, whereas others were pathogenic on only some of the lines (Table 5). Honey was susceptible to all of the isolates from *S. bicolor* that were tested, as well as to four isolates from *S. halepense*. At the other extreme, Keller was resistant to all of the fungal isolates that were tested on it. Evaluation of pathogenicity patterns suggested the presence of at least 12 races among the 15 isolates that were pathogenic on *S. bicolor* (Fig. 5).

Discussion

Sorghum originated in Africa, and *C. sublineola* is specific to *Sorghum* spp.; thus, it is most likely that the pathogen was introduced to the United States together with its host, perhaps on seed because it has been reported to be seed-transmitted across long distances (Cardwell et al. 1989; Zhang et al. 2017). In 1857, Leonard Wray imported 16 varieties of sweet sorghum from Africa, including Honey and Sourless (Swanson and Laude 1934). Most of the types of sweet sorghum currently grown in the United States for syrup were developed
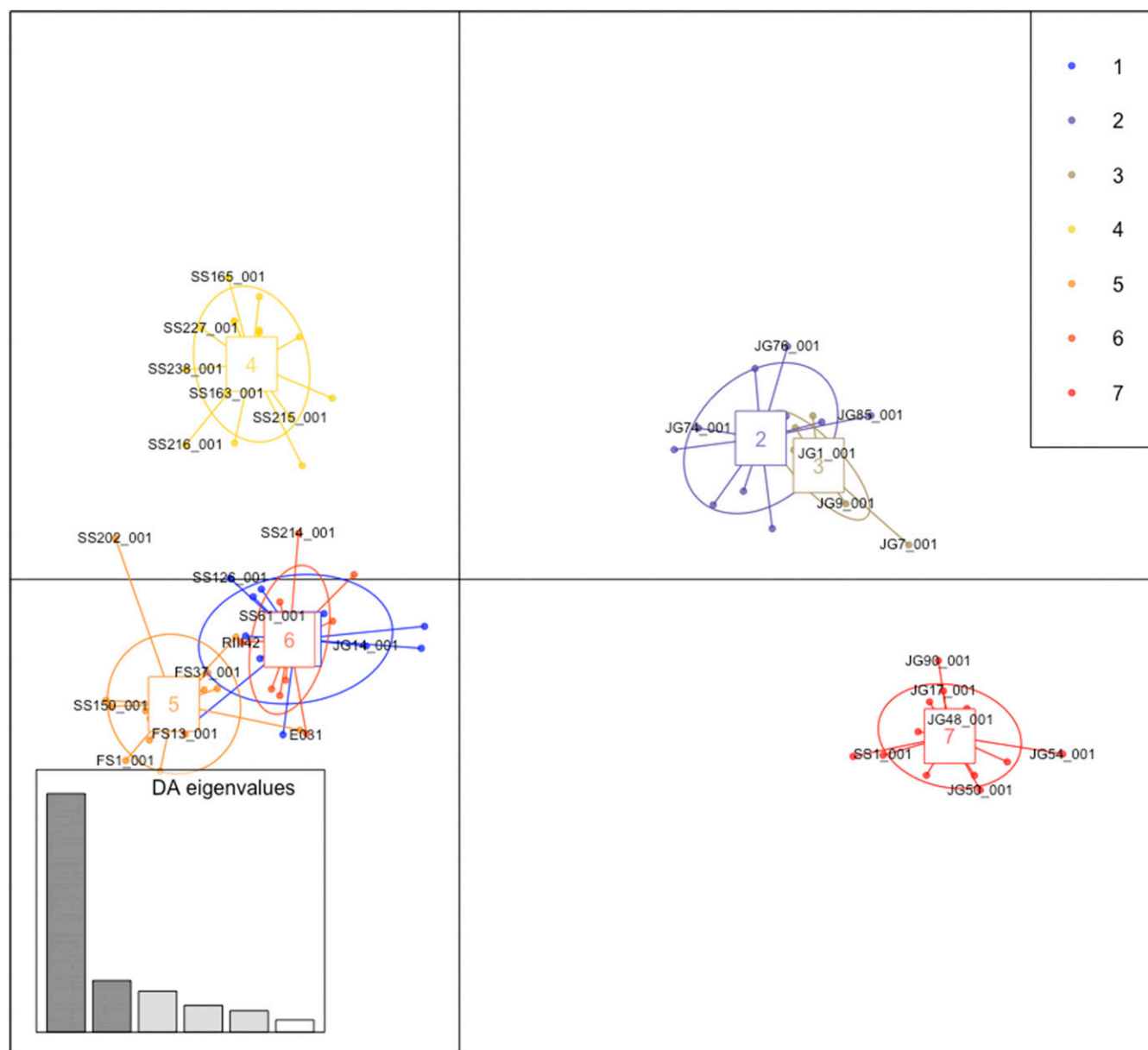

Fig. 4. Clusters of isolates of *Colletotrichum sublineola* based on random amplified polymorphic DNA fingerprint data subjected to discriminate analysis (DA) of principle components.

from these 16 varieties. Grain sorghum was introduced about 20 years after sweet sorghum, and forage sorghum was introduced in 1909 (Swanson and Laude 1934). The sorghum relative *S. halepense*, which is native to the Mediterranean, was brought to the United States for use as forage before 1830 (Ball 1902) and, by 1900, it had spread throughout the Southeast as a noxious weed. It has hybridized with cultivated *S. bicolor* varieties, so that the current population comprises numerous ecotypes containing various quantities of the *S. bicolor* genome (Morrell et al. 2005; Paterson et al. 1995). Johnsongrass is commonly infected by *C. sublineola* (Paterson et al. 1995). As a genetically diverse, open-pollinated perennial (Hill and Forer 1981; McWhorter 1981), it is possible that Johnsongrass could serve as an effective refuge for the anthracnose pathogen and as an incubator for the evolution of new races that could affect sweet sorghum production.

We recently reported that two isolates of *C. sublineola* from Johnsongrass in Kentucky were capable of completing their lifecycle when they were inoculated at a relatively high population density on Sugar Drip sweet sorghum in the field (Xavier et al. 2017). However, these isolates were significantly less aggressive than isolates from *S. bicolor*; thus, it was unclear whether or not cross-infection occurs under natural field conditions. To our knowledge, only one previous study has addressed this question. Rosewich et al. (1998) reported that isolates from Johnsongrass belonged to a population that was distinct from the isolates from grain sorghum in nearby fields in Texas, suggesting that cross-infection of grain sorghum

Table 4. Pathogenicity of 34 strains of *Colletotrichum sublineola* in comparison with the negative control *C. graminicola* on Sugar Drip in greenhouse inoculations

Strain[a]	AUDPC[b]	*P* value[c]
CgSl1 (2)	3.7*	<0.0001
CsTx430BB (1)	0.5	0.9980
GS1.001 (1)	3.2*	<0.0002
FS13.001 (1)	4.0*	<0.0001
FS34.001 (1)	1.3	0.2363
FS37.00 (1)	2.4*	0.0005
FS41.001 (1)	3.0*	<0.0001
FS5.001 (1)	3.1*	<0.0001
FS8.001 (1)	2.0*	0.0081
JG102.001 (2)	0.7	0.5951
JG18.001 (1)	0.7	0.9682
JG2.001 (1)	0.0	1.0000
JG34.001 (1)	2.1*	0.0062
JG42.001 (1)	2.6*	0.0002
JG62.001 (1)	1.2	0.3053
JG7.001 (1)	2.3*	0.0016
JG75.001 (2)	0.7	0.8607
JG9.001 (1)	0.6	0.9943
SS1.001 (1)	3.3*	<0.0001
SS100.001 (1)	1.5	0.1063
SS107.001 (1)	2.5*	0.0002
SS124.001 (1)	3.5*	<0.0001
SS159.001 (1)	3.7*	<0.0001
SS204.001 (2)	2.4*	0.0004
SS240.001 (2)	2.3*	0.0005
SS26.001 (1)	2.7*	<0.0001
SS29.001 (1)	3.2*	<0.0001
SS41.001 (1)	1.3	0.2690
SS45.001 (1)	4.5*	<0.0001
SS63.001 (1)	5.4*	<0.0001
SS63.001 (2)	2.7*	<0.0001
SS78.001 (1)	2.8*	<0.0001
M30.001 (1)	0.0	NA
M30.001 (2)	0.0	NA

[a] Number in parentheses refers to the experiment in which the strain was tested.
[b] Area under the disease progress curve. Asterisks indicate values that are significantly greater than the negative control (M30.001) at $P < 0.05$.
[c] NA = not applicable.

did not occur in that region. We tested the hypothesis that isolates from Johnsongrass belonged to the same population as those from *S. bicolor* by using several fingerprinting markers to analyze diversity among a collection of isolates. We also investigated their potential to cause anthracnose symptoms on a variety of sweet sorghum genotypes in the greenhouse.

Results of both RFLP and RAPD fingerprinting agreed that the population of *C. sublineola* in Kentucky and the southeastern United States is divided into two subpopulations that are associated with host species, and that cross-infection appears to be infrequent. Results with both types of markers also agreed that the populations on *S. bicolor* and on *S. halepense* are highly diverse, with multiple haplotypes in most locations, and relatively few occurring in more than one location. The DAPC cluster analysis based on RAPD markers suggested that there was gene flow among locations. This may indicate a role for regional seed transmission of the pathogen. Although phytosanitary inspections of seed for *C. sublineola* are not currently the norm, they should be explored as a mechanism for disease management in the future. The highest level of genetic diversity was among the grain sorghum isolates, although this could be because they were collected from locations around the world, across three decades. The least diverse were the forage sorghum isolates; however, these came from only three locations in Alabama, and they were collected in a single year. Sampling regimens were more similar for Johnsongrass and sweet sorghum isolates, and the fingerprinting analysis indicated that the more genetically diverse Johnsongrass plant population is associated with more genetic diversity in the pathogen. One contributor to diversity could be recombination: *C. sublineola* can undergo sexual development in the laboratory, although it has not been observed to do so in the field (Vaillancourt and Hanau 1992). However, analysis of the RAPD markers did not support the existence of random mating as a factor in either subpopulation or in the population as a whole.

Results from greenhouse pathogenicity assays indicated that some but not all isolates from Johnsongrass were capable of causing disease on *S. bicolor*. Johnsongrass isolates were less aggressive (expressed as AUDPC) than the majority of isolates from cultivated sorghum, although the ranges overlapped. One group of Johnsongrass isolates did not differ from the negative controls on Sugar Drip or on 12 other diverse sorghum genotypes. In a prior study, it was reported that two members of this apparently nonpathogenic group of Johnsongrass isolates (JG9.001 and JG18.001) produced limited numbers of sporulating acervuli in the field when inoculated at high population densities on susceptible Sugar Drip plants, even though they were nonpathogenic in greenhouse assays (Xavier et al. 2017). The current study indicates that there are two groups of isolates from Johnsongrass: one with very low levels of aggressiveness on *S. bicolor* and another that is intermediate in aggressiveness when compared with most isolates from cultivated sorghum. The demonstrated potential for cross-infection by some Johnsongrass isolates in the greenhouse assays contrasts with the molecular fingerprinting evidence, suggesting that cross-infection from *S. halepense* to *S. bicolor* is not common in the field. It seems likely that this relates to the relatively low levels of aggressiveness and reduced fitness of the Johnsongrass isolates on *S. bicolor*.

One sweet sorghum isolate (SS1.001) clustered together with the Johnsongrass isolates in the DAPC analyses. This isolate came from a single lesion on an *S. bicolor* plant of the heritage inbred variety Sourless growing on the UK experimental farm in Fayette County, KY. In the DAPC analysis, the isolate clustered with Johnsongrass isolates from Kentucky, including other isolates from Fayette County. The SS1.001 isolate was not clonal with any other isolate in the collection but it did have multiple telomere bands, similar to most of the Johnsongrass isolates. We propose that this isolate represents a cross-infection event from Johnsongrass. If so, the isolate is particularly well adapted to *S. bicolor*, with a level of aggressiveness similar to most other *S. bicolor* isolates and higher than any of the Johnsongrass isolates that were tested. This isolate also had the ability to infect six of the eight sorghum lines that were tested, whereas none of the Johnsongrass isolates was able to infect more than three

of the lines. Two genetically similar Johnsongrass isolates from the same field in Kentucky, JG14.001 and JG16.001, grouped with *S. bicolor* isolates. These two isolates may represent cross-infection events in the other direction, from *S. bicolor* to Johnsongrass. Beyond these three examples, representing less than 1% of the isolates we sampled, there was no evidence for cross-infection among the populations.

None of the RFLP or RAPD marker haplotypes was found in isolates from both *S. bicolor* and *S. halepense*, with the sole exception of the telomere RFLP haplotype 33, which was identified in one Johnsongrass isolate (JG35.001). Haplotype 33 was characterized by a single hybridizing band. It was the most common and widespread haplotype among *C. sublineola* isolates in the southeastern United States. It was also present in an isolate from Africa, which might indicate that it is an old haplotype that established itself prior to

divergence of the pathogen population on the different host species in our region. Its relative dominance on sweet and forage sorghum suggests the possibility of a fitness advantage on that host or of a founder population that has been spread along with cultivation of the crop.

An alternative hypothesis is that haplotype 33 has resulted from the horizontal transfer of DNA among unrelated isolates. The single telomere fragment in haplotype 33 could be due to integration at all of the chromosome ends of a telomere-targeted transposable element that contains a restriction site for the enzyme used for the Southern blot. In perennial ryegrass isolates of *Magnaporthe oryzae*, retrotransposons targeted specifically to the telomeres, called "MoTeR" elements, resulted in alterations in the RFLP patterns with the telomere probe (Starnes et al. 2012). Integration and propagation of a transposon could occur in isolates that are not closely related by

Table 5. Pathogenicity of 20 isolates of *Colletotrichum* on 12 genetically diverse inbred lines of *Sorghum bicolor* in greenhouse inoculations[a]

Strain[b]	Bailey	Chinese Amber	Cowper	Dale	Yellow Milo	Honey	N100	Orange	Planter	Simon	Della	Keller
CgSl1 (1)	0.0	0.0	1.3*	0.0	0.0	4.0*	0.7*	3.3*	4.0*	4.0*	0.0	0.0
CsTX430BB (2)	1.0*	0.7	0.0	0.2	0.4	1.6*	0.0	0.0	0.0	0.0	NT	NT
GS1.001 (1)	0.0	0.0	0.0	1.7*	4.0*	3.3*	0.3	0.3	0.0	1.3*	0.8*	0.0
FS34.001 (2)	0.0	0.0	0.0	0.0	1.7*	2.5*	0.0	0.0	0.8*	0.3	NT	NT
JG102.001 (1)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
JG18.001 (1)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
JG34.001 (2)	0.0	0.6	0.2	0.0	0.0	3.3*	0.0	0.0	1.2*	0.3	NT	NT
JG42.001 (2)	0.0	1.2*	0.0	0.0	0.2	1.7*	0.0	0.0	0.2	0.5	NT	NT
JG7.001 (2)	0.0	1.5*	0.0	0.0	0.0	1.0*	0.0	0.0	0.3	0.2	NT	NT
JG75.001 (1)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
JG9.001 (1)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
JG90.001 (2)	0.8	0.5	0.0	0.0	0.0	2.5*	0.0	0.5	1.3*	0.3	NT	NT
SS1.001 (1)	2.8*	3.3*	0.0	0.0	0.0	3.7*	0.0	0.3	3.5*	1.0*	0.0	0.0
SS100.001 (2)	2.5*	0.3	0.0	0.2	0.0	2.6*	0.0	0.2	0.3	0.0	NT	NT
SS204.001 (1)	3.5*	2.0*	0.0	0.8*	0.0	3.7*	0.0	0.0	0.0	1.0*	0.0	0.0
SS240.001 (1)	0.0	0.0	0.0	0.0	2.3*	4.0*	0.0	0.0	0.0	0.3	0.0	0.0
SS41.001 (2)	2.1*	0.0	0.0	0.3	2.4*	2.8*	0.0	0.0	0.8	0.2	NT	NT
SS63.001 (1)	3.3*	0.0	3.5*	0.0	3.5*	3.8*	0.3	2.2*	4.0*	4.0*	0.0	0.0
SS63.001 (2)	3.5*	0.0	2.8*	0.0	2.2*	3.8*	0.3	2.8*	3.2*	3.3*	NT	NT
SS90.001 (1)	3.7*	2.0*	0.0	1.7*	0.0	3.0*	0.0	0.0	0.0	0.5	0.0	0.0
M30.001 (1)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
M30.001 (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NT	NT

[a] Values represent the average disease incidence at 3 weeks after inoculation. Asterisks indicate values that are significantly greater than the negative control (M30.001) at *P* < 0.05. NT = not tested.
[b] Number in parentheses refers to the experiment in which the strain was tested.

Race	Strain	Combined Haplotype	Source	Honey	Sugar Drip	Bailey	Chinese Amber	Planter	Simon	Dwarf Yellow Milo	Dale
				Pathogenic On:						
1	SS63.001	4:33	Sweet Sorghum								
2	CgSl1	98:107	Grain Sorghum								
3	GS1.001	99:33	Grain Sorghum								
4	SS1.001	5:34	Sweet Sorghum								
5	SS204.001	17:33	Sweet Sorghum								
6	SS90.001	17:33	Sweet Sorghum								
7	FS34.001	19:33	Forage Sorghum								
8	SS240.001	19:33	Sweet Sorghum								
9	JG34.001	42:60	Johnsongrass								
9	JG90.001	49:83	Johnsongrass								
10	JG42.001	42:60	Johnsongrass								
10	JG7.001	39:56	Johnsongrass								
11	SS41.001	1:33	Sweet Sorghum								
12	CsTX430BB	17:33	Grain Sorghum								
12	SS100.001	17:33	Sweet Sorghum								
NP	JG9.001	38:57	Johnsongrass								
NP	JG75.001	47:78	Johnsongrass								
NP	JG102.001	50:84	Johnsongrass								
NP	JG18.001	43:61	Johnsongrass								
NP	M30.001	101:113	Maize								

Fig. 5. Race identities of 20 genetically and geographically diverse strains of *Colletotrichum*, as defined by greenhouse inoculations on eight differential cultivars of *Sorghum bicolor*. Dark gray boxes = virulent. White boxes = avirulent. NP = nonpathogenic strains (avirulent on all of the differential cultivars in greenhouse assays).

ancestry, a possibility that is supported by the large number of transposase haplotypes that were found in association with the telomere haplotype 33. We know that the telomere-targeted transposon is not CgTn1, because isolate JG35.001 does not hybridize strongly to the CgTn1 probe. If horizontal transfer of a telomere-targeted transposon explains the 33 haplotype, it is apparent that the movement from the *S. bicolor* population into the *S. halepense* population is not common, and this may imply that there are barriers to anastomosis between the members of the two groups.

The results of the current study imply that, although cross-infections are infrequent, Johnsongrass could nonetheless serve as a reservoir for *C. sublineola* during crop rotation schemes, and could also be a source of novel races. The example of SS1.001 indicates that such events can give rise to aggressive new races of the pathogen. This would complicate the implementation of an effective disease management program and efforts to develop new varieties of sweet sorghum with durable resistance.

Acknowledgments

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