Spatial Genetic Structure and Population Dynamics of Gummy Stem Blight Fungi Within and Among Watermelon Fields in the Southeastern United States

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ABSTRACT

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The epidemiology of gummy stem blight (GSB) of cucurbits, particularly the sources of inoculum for epidemics, and the regional population genetic structure of the causal fungi *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*), *S. citrulli*, and *S. caricae* are not well understood. Our goal was to better understand the population structure and fine-scale spatial genetic structure of *Stagonosporopsis* spp. in the southeastern United States. Overall, 528 isolates collected from nine fields in 2012, 2013, and 2014 were genotyped with 16 microsatellite markers. In 2013, *S. caricae*

was first detected in the southeastern United States; however, *S. citrulli* remained the dominant species, representing 96.4% of the isolates. Principal coordinates analysis, discriminant analysis of principle components, and analysis of molecular variance indicated that most populations of *S. citrulli* were genotypically diverse, yet dominated by widely distributed clones that contributed to regional population structure. Spatial genetic structure resulting from aggregation of clonal genotypes at distances of less than 10 meters was detected within two of three fields in which isolate location was recorded. Studies on the epidemiological and fitness differences between *S. citrulli* and *S. caricae* and of prevalent and widespread clones will provide insight into the population structure and species dynamics observed in GSB epidemics.

Many plant-pathogenic fungi have mixed modes of reproduction that result in both asexual and sexual reproduction. Sexual reproduction enables the formation of novel genotypes within populations through recombination, while cycles of asexual reproduction lead to the rapid accumulation of fit clonal genotypes with coadapted alleles (Smith et al. 1993). Homothallic, or self-compatible, fungi also reproduce clonally through sexual reproduction by selfing. Both the reproductive mode and rate of migration, or gene flow, of pathogens influence their evolutionary potential (McDonald and Linde 2002). Pathogens that have a mixed reproductive mode, frequently outcross, and have high rates of migration, especially of clonal genotypes, are predicted to have a high evolutionary potential. These pathogens can readily overcome host resistance and rapidly evolve fungicide resistance (McDonald and Linde 2002). The fungi that cause gummy stem blight (GSB) may fit these characteristics and are, therefore, potentially high-risk pathogens with a rapid rate of evolution.

GSB is a devastating disease of cucurbits worldwide (Keinath 2011; Koike 1997; Lee et al. 1984; van Steekelenburg 1983), leading to heavy losses during severe epidemics. However, the geographic distribution and dispersal patterns of the causal fungi and the major sources of inoculum for disease epidemics are not well understood. GSB is caused by three morphologically indistinguishable, yet genetically distinct, *Stagonosporopsis* species, *S. citrulli*, *S. cucurbitacearum* (syn. *Didymella bryoniae*), and *S. caricae* (Stewart et al. 2015). These fungi belong to the phylum Ascomycota and class Dothidiomycetes, which includes many notorious plant pathogens (Ohm et al. 2012). The GSB fungi are necrotrophic and

have a wide host range among cucurbits (Chiu and Walker 1949a; Keinath 2011). Additionally, *S. caricae* is a pathogen of papaya (*Carica papaya*) causing leaf spot and fruit rot (Stewart et al. 2015). GSB fungi are known pathogens only of cucurbits and papaya, but pathogenicity to other hosts has not been studied extensively, so they cannot be ruled out as sources of inoculum. The GSB fungi are homothallic, or self-compatible (Chiu and Walker 1949a; Keinath 2012), producing ascospores within pseudothecia on leaves and stems of cucurbits. Throughout the growing season, abundant conidia are produced asexually within pycnidia on the leaves and stems of cucurbit hosts. GSB can be introduced into greenhouses and fields on infested or infected seed (Lee et al. 1984) and, then, into fields on infected transplant seedlings (Keinath 2011). The fungi can overwinter as conidia, ascospores, or mycelium in plant debris in the field (Chiu and Walker 1949b; Keinath 2002, 2008; van Steekelenburg 1983). Conidia are splash-dispersed short distances within fields (Café-Filho et al. 2010; Wiant 1945) and ascospores can also be locally dispersed (Café-Filho et al. 2010; Schenck 1968; van Steekelenburg 1983). Long-distance dispersal of ascospores has not been studied. Nonetheless, ascospores and conidia from field debris are potential sources of epidemics in fields and greenhouses. A better understanding of the population structure of GSB fungi among cucurbit fields and the spatial genetic structure of populations within fields could provide insight into dispersal of the pathogens and one or more major sources of inoculum for GSB epidemics. We expect that, if inoculum derives from an introduced source, such as seed, transplant seedlings, or ascospores that have dispersed long distances, panmixia will result from frequent migrants preventing geographic population differentiation. Conversely, if epidemics are strictly initiated by local sources of inoculum, such as locally derived ascospores, conidia, or mycelium from plant debris in the field, geographic populations will be genetically structured with no apparent temporal population subdivision. If local inoculum is the sole source of field epidemics, we expect to see isolation-by-distance (IBD), by which increasing genetic differentiation would occur with increasing geographic

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distances (Slatkin 1987; Wright 1943), due to limited spore dispersal over longer distances.

Symptoms of GSB can occur on any vegetative structure of cucurbits above the soil (Wiant 1945). Leaf symptoms are chlorotic margins that gradually develop into dark brown, circular necrotic spots. On transplant seedlings produced in greenhouses, water-soaked lesions can develop on petioles and stems (Koike 1997). In the field, cankers may develop in cortical tissues, leading to wilting, girdling, and eventually cracking of the stems, causing the plant to produce an amber-colored gummy exudate from which the name of the disease arose. Black rot on fruits is typically irregular shaped and yellowish in color at first and more frequently occurs on pumpkins and squash (*Cucurbita* spp.) (Wiant 1945). GSB can have severe economic impacts in warm and humid regions (Keinath 2011) such as the southeastern United States, where the disease is particularly devastating (Sitterly and Keinath 1996). GSB decreases the yield of cucurbit crops and reduces the marketable quality of fruit (Chiu and Walker 1949b; Keinath 2000). It is a major threat to cucurbit production in the southeastern United States, because the states of Georgia, Florida, South Carolina, and North Carolina produce over half of the commercial cucurbit crops, contributing approximately \$600 million annually to the United States economy (NASS 2014). Within the southeastern United States, watermelon (*Citrullus lanatus*) is most susceptible to GSB (Sitterly and Keinath 1996).

In prior studies on GSB, isolates collected worldwide showed a higher level of genetic diversity than was expected for homothallic fungi based on different molecular markers, including amplification fragment length polymorphism (Kothera et al. 2003), random amplified polymorphic DNA (Somai et al. 2002), and intergenic transcribed spacer sequence (Babu et al. 2015; Somai et al. 2002; Stewart et al. 2015). Populations of *S. citrulli* from two watermelon fields in Georgia showed high genotypic diversity and evidence of recombination, despite abundant clones, and population differentiation between the fields was detected, indicating limited dispersal (Brewer et al. 2015). However, additional studies on fine-scale spatial structure within fields as well as on a regional scale across the southeastern United States and over multiple years are needed to better understand the diversity, distribution, and dispersal of these fungi to provide insight into the epidemiology of GSB. Therefore, the objectives of this study were to: (i) determine the distribution of *Stagonosporopsis* spp. in the southeastern United States, (ii) understand the regional and temporal population structure of GSB fungi, and (iii) investigate the fine-scale spatial genetic structure within watermelon fields in the southeastern United States.

MATERIALS AND METHODS

Sampling and DNA extraction. All isolates used in this study were collected from watermelon because it is highly susceptible to GSB and host specialization among cucurbit species has not been detected (Brewer et al. 2015; Keinath et al. 1995; Kothera et al. 2003; Stewart et al. 2015). One hundred watermelon leaves with lesions characteristic of GSB were collected from watermelon fields in four counties in the southeastern United States (Fig. 1): Tift County (GAT) and Cook County (GAC) in southern Georgia, Suwannee County in northern Florida (FL), and Colleton County in coastal South Carolina (SC). A single field from each county was sampled in July 2012 and GAT, GAC, and FL were sampled in 2013. Samples were collected haphazardly at distances of 5 m or greater, unless otherwise indicated. Twenty-two single-conidia isolates recovered from a field in Colleton County, South Carolina in 2013 were provided by A. Keinath at the Clemson University Coastal Research & Education Center. An additional sampling was conducted in Cook County, Georgia in July 2014. The sites in Cook County, Georgia were from adjacent fields in 2012 and 2013 (GAC12 and GAC13) and 2.0 km from the site sampled in 2014 (GAC14). The sites sampled in 2012 and 2013 from the other counties could not be collected as near to each other because

watermelons were not planted in the immediate area or GSB epidemics did not occur nearby. The Tift County, Georgia sites (GAT12 and GAT13) were 7.4 km apart, the Suwannee County, Florida sites (FL12 and FL13) were 29.3 km apart, and the Colleton County, South Carolina sites (SC12 and SC13) were 10.3 km apart.

To investigate the fine-scale spatial genetic structure of GSB fungi, the within-field location of each symptomatic leaf collected in 2013 and 2014 from the sites in Georgia (GAT13, GAC13 and GAC14) was recorded. For GAT13 and GAC13, 10 transects along watermelon rows were placed at approximately 5.5-m intervals (Supplementary Fig. S1). A single symptomatic leaf was collected every 5.5 m for a total of 10 leaves along each of the 10 transects. The sampling for GAC14 was carried out within a smaller area with 10 transects along watermelon rows placed at intervals of approximately 2 m. A symptomatic leaf was collected every 2 m along each transect for a total of 10 leaves along each of the 10 transects.

A 1-cm² lesion from each symptomatic leaf was surface-sterilized in 0.6% NaClO for one min, followed by two rinses with sterile distilled H₂O. Three 0.25-cm² segments from the lesion margin were excised and placed onto quarter-strength potato dextrose agar (qPDA) in a 100-mm petri dish. A 4-mm² agar plug containing hyphal tips from the edge of each colony was transferred to a second dish of qPDA after 3 days of growth at 25°C. Isolates were initially confirmed as a *Stagonosporopsis* species based on colony morphology and the rapid growth associated with GSB fungi (Aveskamp et al. 2010). Isolates were maintained for long-term storage on filter paper at -20°C.

Genomic DNA was extracted from fresh mycelium incubated at 25°C in the dark for 4 days on qPDA covered with sterile cellophane, using a modified method (Lee et al. 1988). Approximately 100 mg of mycelium was incubated in 1 ml of lysis buffer (50 mM EDTA, pH 8, 100 mM Tris, pH 8, 3.5% sodium dodecyl sulfate, 500 µg of proteinase K per milliliter, and 1% sodium bisulfite) for 15 min at 65°C. After vortexing for 1 min and centrifugation for 5 min at 14,000 × g, 200 µl of ammonium acetate (7.5 M) was added to the supernatant. The mixture was vortexed 10 s, was placed on ice for 15 min, and then, was centrifuged at 14,000 × g for 3 min. The supernatant was precipitated with an equal volume of isopropanol and was centrifuged at 14,000 × g for 5 min. The pellet was rinsed twice with 70% ethanol, was air-dried, and was resuspended in 100 µl of sterile H₂O. The DNA quality was measured using a Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE). The DNA was stored at -20°C.

Fig. 1. Map of watermelon fields sampled for gummy stem blight fungi. The nine populations include Tift County, Georgia (GAT12, GAT13), Cook County, Georgia (GAC12, GAC13, GAC14), Suwannee County, Florida (FL12, FL13), and Colleton County, South Carolina (SC12, SC13). The digits in the population name indicate the sampling year.

Multilocus genotyping and identification of *Stagonosporopsis* species. All isolates were genotyped with 20 previously developed microsatellites markers in three multiplex reactions (*Db01* to *Db20*) (Brewer et al. 2015). Each of the reactions was carried out in a 10- μ l volume containing 1 μ l of genomic DNA (3 to 20 ng), 5 μ l of 2 $\times$ Type-it Multiplex PCR master mix (Qiagen, Germantown, MD), and 1 μ l of 10 $\times$ primer mixture. The thermal cycling conditions included initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 90 s, and extension at 72°C for 30 s, with a final extension step at 60°C for 30 min. One microliter of a 1:15 dilution of the polymerase chain reaction (PCR) products was added to 9.9 μ l of Hi-Di formamide (Life Technology, Grand Island, NY) and 0.1 μ l of GeneScan-500 LIZ internal size standard (Applied Biosystems, Foster City, CA). The PCR products were denatured at 95°C for 5 min, and the samples were submitted to the Georgia Genomics Facility (Athens, GA) for fragment analysis on an Applied Biosystems 3730xl 96-capillary DNA analyzer. The alleles were scored in GeneMapper v4.0 (Applied Biosystems). Loci were distinguished by fluorescent dye and allele size range.

The microsatellite markers were developed for *S. citrulli* and do not work consistently for *S. caricae* or *S. cucurbitacearum* (Brewer et al. 2015); therefore, a PCR-based marker that can distinguish the three *Stagonosporopsis* species causing GSB (Brewer et al. 2015) was used to help identify the species of four *Stagonosporopsis* isolates from GAT13 that did not produce amplicons for most of the microsatellite loci. The marker was used on all 76 GSB isolates recovered from GAC14, because 15 *Stagonosporopsis* isolates from this field did not produce amplicons for most of the microsatellite loci.

Identification of clones and measures of genetic diversity and linkage disequilibrium (LD). Clones are genetically identical individuals that can arise in *S. citrulli* by both asexual reproduction and sexual reproduction when selfing occurs. Depending on the sample size, the number of markers used, and the diversity of alleles for each marker, the same multilocus genotype (MLG) may arise by recombination or outcrossing. To infer which repeated MLG may not be clones and could have occurred as the result of a sexual outcrossing event, we used MLGsim v2.0 (Stenberg et al. 2003) to estimate the probability (p_{sex}) that each MLG occurring more than once could have arisen from an outcrossing event (Parks and

Werth 1993). Values that are significantly low indicate that the repeated MLG likely arose by clonal reproduction. The P value for testing the significance of p_{sex} was estimated by 1,000 random simulations. In order to investigate the impact of clonal reproduction on populations, two datasets were compiled for our analyses, the complete dataset containing MLG of all isolates and the clone-corrected dataset with only one representative from each MLG, unless there was a high and nonsignificant p_{sex} value indicating that the repeated MLG could have arisen through outcrossing.

Genotypic diversity ($\hat{G}$), which is the probability that two randomly selected individuals have unique MLG, was calculated for each complete population using MultiLocus v1.3b (Agapow and Burt 2001). Allelic richness and private allelic richness were estimated for the datasets using HP-Rare v1.0 (Kalinowski 2004, 2005). Rarefaction was used to adjust for biases in diversity estimates due to differences in sample sizes among populations (Hurlbert 1971).

LD, also known as gametic disequilibrium, can be used to provide insight on deviations from random mating within populations (Brown et al. 1980; Milgroom 1996; Weir 1979). Pairwise LD between polymorphic loci within the complete and clone-corrected total population was tested for significance, using the log likelihood ratio statistic. A Markov chain method with a dememorization number of 1,000, with 100 batches each iterated 1,000 times, was conducted in GENEPOP v4.2 (Raymond and Rousset 1995; Rousset 2008). The Bonferroni correction $\beta = \alpha/n$, where α is the level of significance for a single comparison and n is the number of comparisons, was applied to account for multiple comparisons. Multilocus LD for the complete and clone-corrected datasets for each population and the total population was measured by the index of association (I_A) (Brown et al. 1980; Smith et al. 1993) in MultiLocus v1.3b (Agapow and Burt 2001). I_A increases based on the number of loci analyzed, so a modified version, $\bar{r}_d$, that is independent of locus number was estimated (Agapow and Burt 2001). P values for both I_A and $\bar{r}_d$ were determined by 1,000 random permutations of the data.

Analyses of population structure. To determine genetic structure among *S. citrulli* populations, analysis of molecular variance (AMOVA) using the Φ -statistics in GenAlEx v6.501 (Peakall and Smouse 2006, 2012) was determined for the complete and clone-corrected datasets. Pairwise Φ_{PT} values, which are analogous to Wright's F_{ST} on molecular data (Excoffier et al. 1992; Peakall et al. 1995), were calculated between the nine populations. Principal coordinate analysis (PCoA) of pairwise genetic distances of MLG of isolates from populations sampled in 2012 and 2013 was conducted with GenAlEx v6.501. Differentiation among populations was estimated by analysis of variance (ANOVA) of the first two principal coordinates of isolates in each population, using JMP Pro v11 (SAS Institute, Cary, NC). To investigate underlying population structure without a priori assignment of individuals to populations, the number of clusters among *S. citrulli* sampled from the southeastern United States and assignment of isolates to each cluster was determined by discriminant analysis of principal components (DAPC), a multivariate analysis that has no underlying assumptions based on evolutionary models (Jombart et al. 2010). DAPC was conducted on the complete dataset in the R package adegenet v1.4-1 (Jombart 2008; Jombart and Ahmed 2011). Clusters were determined by K -means clustering of principal components. The optimal number of clusters, K , was inferred as the number of clusters where the Bayesian information criterion increases or decreases by a negligible amount.

Analyses of spatial genetic structure. To investigate an IBD model of dispersal (Rousset 1997; Wright 1943) among *S. citrulli* populations, regression analysis of standardized genetic distance, $\Phi_{PT}/(1 - \Phi_{PT})$, between populations plotted against the logarithm of geographic distance in meters was conducted in JMP Pro v11 on the complete and clone-corrected datasets of pairwise genetic distances (Φ_{PT}).

TABLE 1. Estimates of genetic diversity for *Stagonosporopsis citrulli* populations from the southeastern United States sampled in 2012, 2013, and 2014

Population ^a	No. isolates (N)	No. MLG (g)	Repeated fraction ^b	$\hat{G}^c$	Allelic richness ^d	Private allelic richness ^d
GAT12	83	44	0.47	0.904	2.23	0.18
GAT13	95	49	0.48	0.976	2.15	0.04
GAC12	72	40	0.51	0.926	2.11	0.02
GAC13	53	28	0.47	0.931	2.17	0.02
GAC14	61	32	0.48	0.950	2.18	0.06
FL12	17	17	0	1.000	2.63	0.46
FL13	72	13	0.82	0.702	1.74	0.03
SC12	34	26	0.15	0.955	2.04	0.04
SC13	22	13	0.41	0.883	2.13	0.02
Total	509	199	0.61	0.973	2.25	NA

^a Nine field populations were sampled: Tift County, Georgia (GAT12, GAT13), Cook County, Georgia (GAC12, GAC13, GAC14), Suwannee County, Florida (FL12, FL13), and Colleton County, South Carolina (SC12, SC13). The digits in the population name indicate the sampling year.

^b Repeated fraction = $1 - (g/N)$.

^c Genotypic diversity ($\hat{G}$), which is the probability that two randomly selected individuals have unique multilocus genotypes (MLG), was calculated using MultiLocus v1.3b (Agapow and Burt 2001).

^d Rarefaction was applied to allelic richness and private allelic richness estimates to account for differences in population sample sizes (Kalinowski 2004, 2005).

To identify within-field genetic structure of *S. citrulli*, spatial autocorrelation (Heywood 1991; Sokal and Oden 1978) of genotypes was conducted in GenAlEx v6.501 for the Georgia populations (GAT13, GAC13, GAC14) in which the within-field location of each isolate was recorded. The coefficient r (Smouse and Peakall 1999) measures the genetic similarity of pairs of isolates within specific distance classes. Values of r less or greater than the 95% confidence intervals of the null hypothesis of no genetic structure determined by 1,000 random permutations of the data indicate spatial genetic structure within that distance class.

RESULTS

Species composition, genetic diversity, clonal composition, and LD. From the 800 leaves with lesions symptomatic of GSB, 506 *Stagonosporopsis* isolates were recovered. Isolation frequency was very low in some populations (Table 1), especially FL12 (17%) and SC12 (34%), due to secondary infections, most often by *Alternaria* species. An additional 22 isolates were obtained for SC13. Of the 528 field isolates, 509 (96.4%) were identified as *S. citrulli* based on amplification by the microsatellite markers. The remaining 19 isolates that did not consistently produce amplicons for most of the microsatellite loci were identified as *S. caricae* by the PCR-based marker that distinguishes the three morphologically similar *Stagonosporopsis* species. Four of the *S. caricae* isolates were from Tift County, Georgia, sampled in 2013 (4% of the GAT13 population), and 15 were from the Cook County, Georgia field sampled in 2014 (20% of the population GAC14).

For population genetic analyses of *S. citrulli* in the southeastern United States, four of the microsatellite loci were discarded because they were monomorphic (*Db02*, *Db03*, and *Db04*) or did not amplify for the majority of isolates (*Db01*). Thus, analyses were based on MLG of *S. citrulli* isolates for 16 microsatellite loci. The number of alleles for the 16 loci varied from two to 11. Among the 509 total *S. citrulli* isolates, there were 199 MLG. Although 61% of the genotypes were repeated, genotypic diversity ($\hat{G} = 0.973$) was high. For each population, genotypic diversity was relatively high ($\hat{G} > 0.9$), except for SC13 ($\hat{G} = 0.883$) and FL13 ($\hat{G} = 0.702$). There were clonal MLG with multiple members in these two populations (Table 2), contributing to the low genotypic diversity. FL12 was the most genotypically diverse population ($\hat{G} = 1.0$), with each isolate represented by a unique MLG. Rarefied allelic richness ranged from 1.74 for FL13 to 2.63 for FL12, indicating that most

loci for populations, when standardized for sample size, showed just over two alleles. GAT12 and FL12 both had more private alleles, which are alleles detected in one population, than the other populations (Table 1), with richness of 0.18 and 0.46, respectively, compared with a range of 0.02 to 0.06 for the other populations.

TABLE 3. Estimates of multilocus linkage disequilibrium for *Stagonosporopsis citrulli* populations in the southeastern United States

Population ^a	<i>N</i>	<i>I</i> _A ^b	$\bar{r}_d$ ^c
GAT12	83	1.571***	0.108***
Clone corrected	44	0.881***	0.060***
GAT13	95	0.362***	0.026***
Clone corrected	53	0.162 ^{ns}	0.011*
GAC12	72	1.166***	0.087***
Clone corrected	42	0.271*	0.020*
GAC13	53	1.473***	0.110***
Clone corrected	30	0.393**	0.287**
GAC14	61	0.772***	0.054***
Clone corrected	33	0.195*	0.013*
FL12	17	0.934***	0.063***
Clone corrected	17	0.934***	0.063***
FL13	72	3.202***	0.267***
Clone corrected	13	0.468**	0.036**
SC12	34	0.572***	0.040***
Clone corrected	27	0.297*	0.022*
SC13	22	1.750***	0.148***
Clone corrected	13	0.192*	0.016*
Total population	509	-0.190***	-0.015***
Clone corrected	216 ^d	0.373***	0.026***

^a Nine populations were sampled including: Tift County, GA (GAT12, GAT13); Cook County, GA (GAC12, GAC13, GAC14); Suwannee County, FL (FL12, FL13); and Colleton County, SC (SC12, SC13). The digits in the population name indicate the sampling year. Clone-corrected datasets include a single representative of each multilocus genotype (MLG) unless the repeated MLG showed a high and nonsignificant p_{sex} value (Parks and Werth 1993), calculated in MLGsim2.0 (Stenberg et al. 2003), suggesting that the repeated MLG could have arisen through recombination.

^b Index of association (Brown et al. 1980; Smith et al. 1993) measured in MultiLocus v1.3b (Agapow and Burt 2001). *P* values (ns = not significant, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$) are based on 1,000 permutations.

^c Modified index of association (I_A), which is independent of the number of loci analyzed, was measured in MultiLocus v1.3b (Agapow and Burt 2001). Significance levels (ns = not significant, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$) are based on *P* values determined by 1,000 permutations of the data.

^d Clone-corrected for the total population.

TABLE 2. Clonal composition of *Stagonosporopsis citrulli* populations from the southeastern United States sampled in 2012, 2013 and 2014

Population ^a	<i>N</i>	<i>g</i> ^b	<i>g</i> ₂ ^c	Number of isolates in each repeated MLG ^d
GAT12	83	44	12	19, 5, 4, 4, 3, 3, 3, 2 ^{x1} , 2 ^{x2} , 2 ^{y1} , 2 ^{y2} , 2, (1 ^{x3} , 1 ^{x4} , 1 ^{x5} , 1 ^{x6})
GAT13	95	49	19	8 ^{x5} , 6 ^{x7} , 5 ^{x1} , 5, 5, 4 ^{x4} , 4 ^{x6} , 3, 3, 3, 3, 2 ^{x2} , 2 ^{x8} , 2, 2, 2 ^{ns} , 2 ^{ns} , 2 ^{ns} , 2 ^{ns} , (1 ^{x9} , 1 ^{x10} , 1 ^{x11} , 1 ^{x12} , 1 ^{x15} , 1 ^{y3} , 1 ^{y4} , 1 ^{y5} , 1 ^{y6} , 1 ^{y13})
GAC12	72	40	9	17 ^{x1} , 5 ^{x5} , 4 ^{x4} , 4 ^{x13} , 3 ^{x7} , 2 ^{y9} , 2, 2 ^{ns} , 2 ^{ns} , (1 ^{x8} , 1 ^{x9} , 1 ^{x10} , 1 ^{x14} , 1 ^{y3} , 1 ^{y7} , 1 ^{y8} , 1 ^{y10})
GAC13	53	28	11	13 ^{x5} , 4 ^{x12} , 3 ^{x14} , 2 ^{x1} , 2 ^{x4} , 2 ^{x10} , 2 ^{y10} , 2 ^{y11} , 2, 2 ^{ns} , 2 ^{ns} , (1 ^{x3} , 1 ^{x6} , 1 ^{x11} , 1 ^{x13} , 1 ^{y2} , 1 ^{y5} , 1 ^{y7} , 1 ^{y9})
GAC14	61	32	12	10 ^{x6} , 7, 3 ^{x2} , 3 ^{x5} , 3 ^{y6} , 3, 2 ^{x1} , 2 ^{x7} , 2 ^{x11} , 2 ^{x13} , 2, 2 ^{ns} , (1 ^{x14} , 1 ^{x15} , 1 ^{y1} , 1 ^{y4})
FL12	17	17	0	(1 ^{x7} , 1 ^{y12})
FL13	72	13	4	34, 19 ^{x4} , 8, 2, (1 ^{x13} , 1 ^{x15} , 1 ^{y12})
SC12	34	26	4	5 ^{x4} , 3, 2, 2 ^{ns} , (1 ^{x3} , 1 ^{y8})
SC13	22	13	2	7 ^{x5} , 4 ^{x4} , (1 ^{x7} , 1 ^{x8} , 1 ^{x9} , 1 ^{x11} , 1 ^{x12} , 1 ^{x13} , 1 ^{y11} , 1 ^{y13})
Total	509	199	66	39 ^{x4} , 37 ^{x5} , 34, 28 ^{x1} , 19, 16 ^{x6} , 13 ^{x7} , 9 ^{x13} , 8, 7 ^{x2} , 7, 6 ^{x12} , 5 ^{x11} , 5 ^{x14} , 5, 5, 5, 4 ^{x8} , 4 ^{x10} , 4 ^{y6} , 4, 4, 4 ^{ns} , 3 ^{x3} , 3 ^{x9} , 3 ^{x15} , 3 ^{y1} , 3 ^{y2} , 3 ^{y9} , 3 ^{y10} , 3 ^{y11} , 3, 3, 3, 3, 3, 3, 3, 3, 3 ^{ns} , 2 ^{y3} , 2 ^{y4} , 2 ^{y5} , 2 ^{y7} , 2 ^{y8} , 2 ^{y12} , 2 ^{y13} , 2, 2, 2, 2, 2, 2, 2, 2 ^{ns} , 2 ^{ns} , 2 ^{ns} , 2 ^{ns} , 2 ^{ns} , 2 ^{ns} , 2 ^{ns} , 2 ^{ns} , 2 ^{ns}

^a Nine populations were sampled: Tift County, Georgia (GAT12, GAT13), Cook County, Georgia (GAC12, GAC13, GAC14), Suwannee County, Florida (FL12, FL13), and Colleton County, South Carolina (SC12, SC13). The digits in the population name indicate the sampling year.

^b Number of multilocus genotypes (MLG) in each population.

^c Number of MLG represented by more than one member.

^d Superscripts of 'x' or 'y' indicate MLG that were present in more than one population with 'x' indicating repeated MLG present in three or more different populations and 'y' indicating repeated MLG present in two different populations; the superscript number indicates the repeated MLG to which the isolates belong. The MLG with high and nonsignificant p_{sex} values (Parks and Werth 1993) calculated in MLGsim2.0 (Stenberg et al. 2003), which could have arisen through recombination and may not have been clonally produced, are indicated with the superscript 'ns'. Single MLG within a population that are shared with another population are listed in parentheses.

All of the populations had repeated fraction values higher than 0.40, except for FL12 and SC12. For each of the nine populations except FL12, there were repeated MLG. However, there were two isolates in FL12 that shared MLG with isolates from other populations (Table 2). There were 66 repeated MLG (g_2) across the entire southeastern United States population. Most of the repeated MLG were likely due to clonal reproduction, yet p_{sex} estimates indicated that 12 repeated MLG had a high probability to have arisen by recombination (Table 2, labeled as ns), so these were not considered clones. Within each of the sampled field populations, there were many clonal MLG containing multiple members (g_2) and some with a very large number of members. For example, FL13 contained clonal MLG with 34 and 19 members and GAT12, GAC12, GAC13, and GAC14 also contained MLG with 10 or more members. Many MLG were widespread and shared among multiple populations over multiple years. Thirteen MLG were shared among two populations (Table 2, labeled as $y_1, y_2, \dots, y_{13}$) and 15 MLG were shared among three or more populations (Table 2, labeled as $x_1, x_2, \dots, x_{15}$). The most abundant and widespread were MLG x_1, x_4, x_5, x_7 , and x_{13} , which contained 28, 39, 37, 13, and nine members across five, seven, six, five, and five populations, respectively (Table 2).

All *S. citrulli* populations in the southeastern United States were in multilocus LD based on the I_A and the modified I_A ($\bar{r}_d$) (Table 3). Even after clone correction, all *S. citrulli* populations were in multilocus LD based on $\bar{r}_d$. Among the 120 pairs of loci in the population, 66 pairs (55%) in the complete dataset and 22 pairs (18%) in the clone-corrected dataset showed significant LD after Bonferroni correction ($P < 0.0001$).

Population structure. Significant pairwise genetic differentiation (Φ_{PT}) was detected between all *S. citrulli* populations, except GAC13 and SC13, when clones were included in the analyses (Table 4). However, after clone correction, the amount of genetic differentiation between populations decreased, with many no longer significantly different. None of the populations from 2013 were

significantly different after clone correction. The overall Φ_{PT} between 2012 and 2013 across all populations was significant for the complete dataset ($\Phi_{\text{PT}} = 0.073, P < 0.001$) and the clone-corrected dataset ($\Phi_{\text{PT}} = 0.015, P < 0.001$), although there was a decrease in the variance partitioned between the two years. In some cases, populations from fields in the same county were genetically structured between subsequent years (GAT12 v. GAT13, and SC12 v. SC13), but in other cases, the geographically proximal populations were not structured (GAC12 v. GAC13, GAC13 v. GAC14, and FL12 v. FL13).

PCoA showed an overlap of genotypes in both 2012 and 2013 (Fig. 2). In 2012 (Fig. 2A), however, there was clustering of isolates within populations along the principal coordinates. This was

Fig. 2. Principal coordinates analysis of *Stagonosporopsis citrulli* isolates collected from watermelon fields in the southeastern United States in **A**, 2012 and **B**, 2013. Sampling locations include Tift County, Georgia (GAT12, GAT13), Cook County, Georgia (GAC12, GAC13), Suwannee County, Florida (FL12, FL13), and Colleton County, South Carolina (SC12, SC13).

Fig. 3. Discriminant analysis of principal components for *Stagonosporopsis citrulli* from the southeastern United States. **A**, Scatterplot of the 10 assigned clusters based on Bayesian information criterion and **B**, histogram of assignment probability of 509 *S. citrulli* isolates from nine field populations from the southeastern United States into the 10 genetic clusters. The nine populations include Tift County, Georgia (GAT12, GAT13), Cook County, Georgia (GAC12, GAC13, GAC14), Suwannee County, Florida (FL12, FL13), and Colleton County, South Carolina (SC12, SC13). The digits in the population name indicate the sampling year.

confirmed by ANOVA by population for PC1 and PC2, in which differences were significant ($P < 0.0001$) at the two major coordinates. In 2013 (Fig. 2B), differences among the four populations were also detected by ANOVA for PC1 ($P = 0.0006$) and PC2 ($P < 0.0001$). After clone correction, the differences among populations from 2012 remained significant for both PC1 ($P = 0.0002$) and PC2 ($P = 0.0112$). There were no longer significant differences among the four 2013 *S. citrulli* populations at PC1 ($P = 0.7435$) or PC2 ($P = 0.6161$).

DAPC supported K = 10 optimal clusters. Clusters 1, 3, 5, 7, and 8 overlapped, while the remaining five clusters were isolated (Fig. 3A). The number of *S. citrulli* isolates assigned to each cluster, in order of cluster number, was 33, 54, 92, 78, 60, 17, 58, 11, 39, and 67, respectively. Most of the clusters were usually represented by isolates that are members of a dominant or widespread clone, members of other clones with genotypes containing similar alleles, and unique genotypes sharing some alleles with the dominant clone. Most of the clusters were distributed across multiple populations; however, there were some noticeable exceptions, with some clusters that were more abundant in some fields. For example, cluster 10 contained one of the dominant clones (34 members), detected only in the FL13 population (Table 2). Cluster 2 contained the other dominant clone, with 19 members from FL13 and 39 total members among seven of the populations. These two clusters that contained the dominant clones from FL13 are represented by orange (cluster 2) and magenta (Fig. 3B, cluster 10). Cluster 7, which is represented in blue, was also dominated by a large clone (19 members) found within a single population (GAT12). The other clusters contained many MLG with few members.

Spatial genetic structure. Although there was a trend of increasing genetic distance with geographic distance (Fig. 4), the genetic distance varied tremendously within similar geographic distances, and there was no significant association based on linear regression, when comparing the genetic distance of the complete ($R^2 = 0.01$, $P = 0.67$) and clone-corrected ($R^2 = 0.06$, $P = 0.16$) datasets.

Spatial autocorrelation of *S. citrulli* isolates collected from three Georgia watermelon fields—GAT13, GAC13 and GAC14—showed clustering of similar genotypes for some distance classes (Fig. 5). For GAT13 (Fig. 5A), isolates within 6-m clusters were

more genetically similar than expected at random. For GAC13 (Fig. 5B), isolates were not aggregated by genetic similarity. For GAC14 (Fig. 5C), isolates within 2-m clusters were more genetically similar than expected at random.

DISCUSSION

To our knowledge, this is the first study to investigate the population genetic structure of GSB at a regional level over multiple years as well as to assess the spatial genetic structure of these fungi within cucurbit fields. Previous studies showed that three morphologically identical but genetically distinct *Stagonosporopsis* species cause GSB of cucurbits; however, only *S. citrulli* had been previously identified in the southeastern United States (Stewart et al. 2015). Our results show that *S. citrulli* continues to be the dominant species in the southeastern United States, representing 96.4% of the isolates identified in this study. Interestingly, we first identified *S. caricae* in the southeastern United States in 2013. It represented 1.7% of the GSB isolates we collected that year. Moreover, the frequency of *S. caricae* identified among GSB isolates increased to 19.7% in 2014. However, the *S. caricae* isolates in 2013 and 2014 each came from a single field and only this field was sampled in 2014, so it is not clear what the overall frequency of this species is across the southeastern United States and if this increase in *S. caricae* is significant at a regional level. Surveys of GSB epidemics on a broader scale would need to be conducted over subsequent years in order to confirm if populations of *S. caricae* are increasing. The origin of *S. caricae* in the southeastern United States is not clear, but it may have been introduced through seed, which is a mechanism of long-distance dispersal for GSB fungi (Keinath 2011; Stewart et al. 2015). Studies on the genetic diversity of *S. caricae* in the southeastern United States and in major seed-producing regions would help determine if it was a single introduction event, multiple introductions, or if *S. caricae* has been present in the southeastern United States over the past few decades but at levels that were not previously detected. An increase in the abundance of *S. caricae* may be the result of a selective advantage, such as fungicide resistance, especially because fungicides are the main management strategy for GSB in the southeastern United States (Avenot et al. 2012; Keinath 2012; Thomas et al. 2012). An

TABLE 4. Geographic and temporal population structure measured by Φ_{PT} between *Stagonosporopsis citrulli* populations in the southeastern United States sampled in 2012, 2013, and 2014

	Population ^a								
Population	GAT12	GAC12	FL12	SC12	GAT13	GAC13	FL13	SC13	GAC14
GAT12	...								
GAC12	0.086*** (0.019*)	...							
FL12	0.086*** (0.023 ^{ns})	0.065** (0.022 ^{ns})	...						
SC12	0.168*** (0.101***)	0.110*** (0.059***)	0.088** (0.085***)	...					
GAT13	0.125*** (0.040***)	0.083*** (0.010 ^{ns})	0.105*** (0.069***)	0.080*** (0.001 ^{ns})	...				
GAC13	0.114*** (0.033*)	0.065*** (0.000 ^{ns})	0.087*** (0.056**)	0.100*** (0.054**)	0.014* (0.000 ^{ns})	...			
FL13	0.253*** (0.037*)	0.205*** (0.000 ^{ns})	0.214*** (0.029 ^{ns})	0.154*** (0.057*)	0.110*** (0.000 ^{ns})	0.134*** (0.000 ^{ns})	...		
SC13	0.118*** (0.026 ^{ns})	0.069*** (0.000 ^{ns})	0.077** (0.041*)	0.121** (0.060*)	0.027* (0.000 ^{ns})	0.000 ^{ns} (0.000 ^{ns})	0.131*** (0.000 ^{ns})	...	
GAC14	0.145*** (0.058***)	0.085*** (0.018*)	0.079*** (0.037*)	0.055*** (0.032**)	0.071*** (0.020*)	0.092*** (0.006 ^{ns})	0.182*** (0.003 ^{ns})	0.130*** (0.010 ^{ns})	...

^a Nine populations were sampled: Tift County, Georgia (GAT12, GAT13), Cook County, Georgia (GAC12, GAC13, GAC14), Suwannee County, Florida (FL12, FL13), and Colleton County, South Carolina (SC12, SC13). The digits in the population name indicate the sampling year. Clone-corrected dataset comparisons (in parentheses) include a single representative of each multilocus genotype (MLG) unless the repeated MLG showed a high and nonsignificant p_{sex} value (Parks and Werth 1993), calculated in MLGsim2.0 (Stenberg et al. 2003), suggesting that the repeated MLG could have arisen through recombination.

^b Pairwise population differentiation was measured by Excoffier's Φ_{PT} (Excoffier et al. 1992), an analog of F_{ST} measured via analysis of molecular variance, using GenAlEx v.6.501 (Peakall and Smouse 2006). Significance levels (ns = not significant, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$) are based on P values determined by 1,000 permutations of the data.

understanding of fitness differences between *S. citrulli* and *S. caricae* isolates when exposed to different fungicides or other potential selective pressures occurring in cucurbit production will help explain the population dynamics of these species. For two closely related species to coexist, it is expected that niche partitioning must occur, otherwise competition for the same resources will drive one of the species to extinction, according to the competitive exclusion principle (Feau et al. 2012; Fitt et al. 2006; Hamelin et al. 2011).

Among *S. citrulli* populations throughout the southeastern United States, we found clones that occurred at high frequency and were widespread at both regional and temporal scales. In seven of the nine *S. citrulli* populations, clones represented over 40% of the isolates (Table 1). Five populations had at least one high frequency clone with more than 10 members (Table 2). In total, 13 clones were distributed across two populations, 15 clones were distributed across at least three populations, and five clones were distributed across five of the nine populations. It is not clear why some clones of *S. citrulli* are prevalent and widespread or how they are dispersed, but it could be via seed, transplant seedlings, hurricane dispersal of conidia, or wind-dispersed ascospores produced via selfing. Prevalent and widespread clones have also been reported for *Sclerotinia sclerotiorum* (Clarkson et al. 2013; Kohli et al. 1995), which is also self-compatible but frequently outcrosses (Attanayake et al. 2014). High frequency clones of plant pathogenic fungi have been demonstrated to be the result of fitness advantages (Goyeau et al. 2007; Liang et al. 2014; Maciel et al. 2014) or founding events, for which the clones are prevalent because they established early in the epidemic by chance (Bardin et al. 2014; Berbegal et al. 2013). It would be of interest to investigate the aggressiveness to cucurbits and fungicide resistance profiles of dominant clones from this study, to determine if they are fitter than lower frequency genotypes. Despite the presence of clones, all nine *S. citrulli* populations from southeastern United States were genotypically diverse, likely due to recombination through outcrossing, which was previously supported for *S. citrulli* by the four-gamete test (Brewer et al. 2015).

The dominant species causing GSB in the southeastern United States, *S. citrulli*, is not a single, panmictic population. Population genetic structure was identified at a regional level based on both Φ -statistics and analysis of variance of principal components of field populations. However, after clone correction, the magnitude of population differentiation between fields decreased or was no longer significant. Therefore, gene flow among populations from different fields occurs but is limited in some cases and clones within fields contribute to population structure. When population structure was studied without a priori assignment to populations (DAPC), we found that populations were not geographically structured unless

Fig. 4. Regression of standardized pairwise genetic distance, $\Phi_{PT}/(1 - \Phi_{PT})$, plotted against the logarithm of geographic distance in meters between nine *Stagonosporopsis citrulli* populations from the southeastern United States. The nine populations include Tift County, Georgia (GAT12, GAT13), Cook County, Georgia (GAC12, GAC13, GAC14), Suwannee County, Florida (FL12, FL13), and Colleton County, South Carolina (SC12, SC13). The filled diamonds (◆) and solid line are based on the complete dataset and the open squares (□) and dashed line are based on the clone-corrected dataset.

local, high frequency clones dominated the field population, further supporting the idea that clonal reproduction contributes to population structure of *S. citrulli*. The population structure detected among fields after clone correction could be due to limited dispersal of resident populations initiating the epidemics or to structure in the source populations introduced on seed or seedlings. Dispersal among fields could be via human-mediated movement of infected seed

Fig. 5. Correlograms showing spatial autocorrelation of *Stagonosporopsis citrulli* populations in **A**, Tift County, Georgia, sampled in 2013 (GAT13); **B**, Cook County, Georgia, sampled in 2013 (GAC13); and **C**, Cook County, Georgia, sampled in 2014 (GAC14). The thick solid line shows genetic similarity based on the autocorrelation coefficient (r) of isolates at the specified distance class. Thin dashed lines show the 95% confidence intervals for the null hypothesis of random genetic structure based on 1,000 permutations of the data.

and transplant seedlings, by ascospores, or a combination of these mechanisms. Fungi with high levels of gene flow mediated by long-distance dispersal of spores usually show IBD at a regional level (Barrès et al. 2008; Parks et al. 2008), where populations that are closer are more genetically similar than those that are geographically distant (Slatkin 1987; Wright 1943). However, we did not detect IBD for *S. citrulli*, indicating that populations in the southeastern U.S may not be solely structured by long-distance ascospore dispersal.

Temporal population genetic structure was observed for *S. citrulli* on watermelon for total and clone-corrected datasets. Between 2012 and 2013, Φ_{PT} for the four populations combined was significant showing that the populations in these years were structured. Population structure was also detected for the three *S. citrulli* populations from Cook County, GA in 2012, 2013, and 2014 that were adjacent or sampled less than 2 km apart from each other. However, this structure was no longer significant after clone correction, suggesting that local inoculum contributed to epidemics or that the same source population was introduced. Epidemics are often worse in fields adjacent to fields recently planted with cucurbits, suggesting local inoculum plays an important role in epidemics (Keinath 2002).

Spatial aggregation of *S. citrulli* was detected within two of the three field populations. Aggregation may not have been detected in one of the fields (GAC13) as the result of a reduction in statistical power at closer distances, due to a limited number of comparisons for this field. Alternatively, the field may have been sampled later in the GSB epidemic, when spatial genetic patterns were obscured by extensive local dispersal. For GAT13 and GAC14, *S. citrulli* isolates were genetically aggregated within 6- and 2-m clusters, respectively, which were the smallest sampling intervals within each field. The aggregation of clonal genotypes suggests short-distance dispersal of conidia mediated by rain splash or irrigation water splash during the growing season. Abundant pycnidia were observed on watermelon plants, whereas pseudothecia were not readily observed and, usually, only at the end of the growing season. Artificial inoculation studies conducted in Brazil showed that the spatial pattern of disease was aggregated with infection foci consistent with splash-dispersed pycnidial fungi (Café-Filho et al. 2010), which support our findings. However, ascospore production and dispersal during epidemics has been observed in cucurbit fields (Café-Filho et al. 2010; Schenck 1968) and could contribute to epidemics in the southeastern United States.

In conclusion, *S. citrulli* continues to be the dominant species causing GSB in the southeastern United States, but *S. caricae* has recently emerged in watermelon fields and may be increasing in frequency as the result of a fitness advantage. Population genetic structure indicates that *S. citrulli* is not panmictic in the southeastern United States, yet clones occurred at high frequency and were widespread at both regional and temporal scales. This suggests that long-distance dispersal is occurring, and the lack of IBD further indicates that this dispersal is likely human-mediated by infested or infected seed or transplant seedlings rather than by ascospores alone. Spatial aggregation of *S. citrulli* suggests that dispersal within fields is limited to distances expected by rainsplash. Future work on the epidemiological and fitness differences between *S. citrulli* and *S. caricae* and of prevalent and widespread clones will provide insight into the population structure and dynamics observed in GSB epidemics, leading to strategies for improved disease management in cucurbit production.

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