Genetic Diversity and Population Structure of Cucurbit Gummy Stem Blight Fungi Based on Microsatellite Markers

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ABSTRACT

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Combining population genetics with epidemiology provides insight into the population biology of pathogens, which could lead to improved management of plant diseases. Gummy stem blight, caused by three closely related species of *Stagonosporopsis*—*Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*), *S. citrulli*, and *S. caricae*—is a devastating disease of cucurbits worldwide. Sources of inoculum for epidemics, mechanisms of dispersal, and the mating system of these species are not well understood. To improve our knowledge of gummy stem blight epidemiology, we developed 18 polymorphic microsatellite markers by combining microsatellite motif enrichment with next-generation sequencing. When tested on 46 isolates from diverse cucurbit hosts and regions, the markers were robust for the

dominant and widely distributed *S. citrulli*. Within this species, we found no population structure based on broad-scale geographic region or host of origin. Using the microsatellites, a rapid polymerase chain reaction-based method was developed to distinguish the three morphologically similar species causing gummy stem blight. To better understand dispersal, reproduction, and fine-scale genetic diversity of *S. citrulli* within and among watermelon fields, 155 isolates from two field populations in Georgia, United States were genotyped with the 18 microsatellite loci. Although dominant and widespread clones were detected, we found relatively high genotypic diversity and recombinant genotypes consistent with outcrossing. Significant population genetic structure between the two field populations demonstrated that there is regional geographic structure and limited dispersal among fields. This study provides insight into the fine-scale genetic diversity and reproductive biology of the gummy stem blight pathogen *S. citrulli* in the field.

A thorough understanding of plant disease epidemics includes identifying sources of inoculum (Archie et al. 2009; Xhaard et al. 2012), knowledge of the pathogen's reproductive biology (Maciel et al. 2014; Yuen and Andersson 2013), and uncovering genetic structure of the pathogen populations (Rieux et al. 2011). Population genetic analyses can be used to track the dispersal and spatial distribution of genotypes in order to reveal inoculum sources (Prussin et al. 2014; Zwankhuizen et al. 2000) and to identify population subdivision resulting from restricted gene flow due to geographic structure (Brewer et al. 2012; Brewer and Milgroom 2010), host specialization (Fournier and Giraud 2008; Frenkel et al. 2012), and ecological adaptation among populations (Capelle and Neema 2005). Additionally, measures of genotypic diversity and recombination can be used to infer whether pathogen populations are clonal, sexual, or have mixed modes of reproduction (Maciel et al. 2014; Rieux et al. 2011). Combining population genetics with epidemiology provides insight into the population biology of pathogens (Milgroom and Peever 2003; Xhaard et al. 2012), which may lead to improved management of plant diseases, particularly if we can target inoculum sources, limit pathogen reproduction, or uncover distinct populations that may require unique management strategies.

We are interested in uncovering the population structure and both the broad- and fine-scale genetic diversity of the cucurbit gummy stem blight pathogen to better understand the epidemiology of this disease. The pathogen can overwinter in plant debris (Keinath 2002), be detected at very low incidence in seed, and infect transplant seedlings in the greenhouse (Keinath 2011); however, the major source of inoculum that initiates epidemics in the field is unclear.

It has recently been determined that gummy stem blight is caused by three closely related fungal species: *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae* and *Phoma cucurbitacearum*), *S. caricae* (syn. *P. caricae-papayae*), and *S. citrulli* (Stewart et al. 2015). Gummy stem blight is a devastating and worldwide disease of the Cucurbitaceae family, including watermelon, cucumber, cantaloupe, muskmelon, squash, pumpkin, and gourds (Chiu and Walker 1949; Keinath 2011), especially in humid environments. However, no specialization to cucurbit host species has been detected, regardless of the host of origin (Chiu and Walker 1949; Keinath et al. 1995; Kothera et al. 2003; Lee et al. 1984) (Stewart et al. 2015). Symptoms of gummy stem blight include leaf spots and necrotic, water-soaked lesions on stems and fruit. The fungus can cause symptoms on all aboveground plant parts and is referred to as black rot when it affects the fruit. Pumpkin and winter squash are particularly susceptible to black rot. After primary infection, abundant conidia are produced within pycnidia produced on vines, leaves, and other aboveground plant parts. The conidia are splash dispersed short distances to nearby plants, which can lead to rapid epidemics under wet conditions (Café-Filho et al. 2010; Wiant 1945). Pseudothecia containing ascospores are produced simultaneously (Café-Filho et al. 2010) or later in the growing season, usually on vines (Schenck 1968). Dispersal distance by ascospores has not been studied (Keinath 2011). Seed may become infested or infected by way of flower and fruit infection (de Neergard 1989).

Previous studies on the population biology of the causal pathogens have been conducted on a broad geographic scale. Genetic diversity was examined by using a single nuclear gene sequence, random amplified polymorphic DNA (RAPD) (Somai et al. 2002), and amplified fragment length polymorphism (AFLP) (Kothera et al. 2003). These studies showed that gummy stem blight is caused by at least two genetically distinct groups that are morphologically indistinguishable, with overlapping geographic and host ranges (dos Santos et al. 2009;

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Keinath et al. 1995; Kothera et al. 2003; Somai et al. 2002;). Recently, a multilocus sequencing study of diverse isolates from different cucurbit hosts around the world showed that gummy stem blight is caused by three distinct but closely related and morphologically similar species of *Stagonosporopsis* (Stewart et al. 2015), one of which is the papaya pathogen *S. caricae*. This pathogen was previously described as affecting only papaya (Boerema et al. 2004); therefore, some isolates of *S. caricae* from cucurbits had been previously misidentified as *S. cucurbitacearum* (*D. bryoniae*) due to the host species of origin and similar morphology. The two genetic groups were determined to be two phylogenetically distinct species; therefore, the name *S. citrulli* was assigned to the new species (Stewart et al. 2015) while the other species retained the name *S. cucurbitacearum*. The three species affect cucurbits similarly but only *S. caricae* is pathogenic to papaya (Stewart et al. 2015). *S. citrulli* is the most abundant, genetically diverse, and geographically widespread of the three species (Stewart et al. 2015). Although previous studies have demonstrated that there is genetic diversity among gummy stem blight isolates, diversity within and among populations at the field level needs to be addressed to uncover sources of inoculum, track pathogen dispersal, and better understand the reproductive biology of these fungi, especially the dominant species *S. citrulli*, in the field.

Previous studies on gummy stem blight isolates indicate that the fungus is self compatible, or homothallic (Chiu and Walker 1949), but some level of outcrossing, which would result in recombinant genotypes and contribute to increased diversity, may be possible because many self-compatible fungi are capable of outcrossing (Atallah et al. 2004; Debuchy and Turgeon 2006). The fungus reproduces asexually and sexually by the production of conidia and ascospores, respectively. The asexual and sexual reproductive states form in culture (Corlett 1981; Wiant 1945) and are observed on a variety of cucurbit hosts (Keinath 2014; Wiant 1945). However, it is not possible to determine which of the three *Stagonosporopsis* spp.—all of which could have possibly been identified as *D. bryoniae*—was observed in previous studies. Fine-scale studies on the diversity and population structure of the gummy stem blight pathogens will improve our understanding of the reproductive mode and mating system of this fungus and the capacity of populations to evolve resistance to multiple fungicides or overcome resistance to different host genotypes, knowledge that is critical for effective disease management (McDonald and Linde 2002; Milgroom 1996).

In order to answer these questions about gummy stem blight epidemics, we need molecular markers that can be used to consistently compare and study populations. Microsatellite markers, also known as simple sequence repeats (SSR), are ideal for our purposes because they are highly polymorphic, codominant, easy to amplify by polymerase chain reaction (PCR), work well with small quantities of DNA, and allow us to answer fine-scale questions about populations (Selkoe and Toonen 2006). Additionally, microsatellites are easy to use and interpret, can be used on very few to numerous samples, and are relatively inexpensive, especially when multiplexed (Schoebel et al. 2013), which keeps them very popular for ecological, evolutionary, and epidemiological studies (Atallah et al. 2004; Prussin et al. 2014; Rieux et al. 2011; Selkoe and Toonen 2006). Unlike RAPDs and AFLPS, microsatellite markers are species or genus specific, preventing amplification of DNA from nontarget organisms, and can be consistently compared over multiple years and among different labs (Santana et al. 2009). Next-generation sequencing methods have made development of these markers much easier, providing an abundance of putative markers at a relatively low cost (Frenkel et al. 2012; Malausa et al. 2011; Schoebel et al. 2013). Our aim is to use population genetics to provide insight about the genetic diversity and population structure of the gummy stem blight pathogens on geographically broad and fine scales to enhance epidemiological studies. The specific objectives of this study were to (i) develop microsatellite markers for the gummy stem blight pathogens, (ii) determine whether the markers can be used to distinguish the three *Stagonosporopsis* spp. causing gummy stem blight, (iii) determine

whether worldwide populations are structured by geographic region or host, and (iv) analyze fine-scale genetic diversity and population structure of *S. citrulli* within watermelon fields in Georgia, United States to better understand dispersal and reproduction.

MATERIALS AND METHODS

Isolate maintenance and DNA extraction. Gummy stem blight isolates from diverse geographic locations and hosts within the Cucurbitaceae family, including watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), cantaloupe and muskmelon (*Cucumis melo*), acorn squash (*Cucurbita pepo*), butternut squash (*Cucurbita moschata*), pumpkin (*Cucurbita* sp.), and gourd (*Cucurbita* sp.), were collected for this study (Table 1). Isolates representing each of the two previously detected genetic groups (Somai et al. 2002) and three *Stagonosporopsis* spp. (Stewart et al. 2015) were included. An isolate of *S. caricae* (ATCC52577; American Type Culture Collection, Manassas, VA) from papaya (*Carica papaya*) was included in this study. Isolates from the United States were grown on one-quarter strength potato dextrose agar (qPDA) at 23°C. DNA was obtained for isolates originally collected from outside the United States (Table 1). Isolates were maintained for long-term storage on filter paper at −20°C.

DNA was initially purified from fresh mycelium using the DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions, and later purified by using a simplified fresh tissue extraction protocol (Milgroom et al. 2008). Briefly, a plug of mycelium was transferred to PDA overlaid with sterile cellophane. After 3 days of growth in the dark at room temperature, approximately 50 mg fresh mycelium was harvested and added to 1 ml of lysis buffer (50 mM EDTA [pH 8], 100 mM Tris [pH 8], 3.5% sodium dodecyl sulfate, proteinase K at 250 µg/ml, and 1% sodium bisulfite), vortexed for 1 min, and incubated at 65°C for 15 min. Samples were vortexed briefly and centrifuged at 14,000 × *g* for 5 min. The supernatant was removed and added to 200 µl of 7.5 M sodium acetate, vortexed for 10 s, placed on ice for 15 min, and centrifuged at 14,000 × *g* for 3 min. The supernatant was added to 700 µl of isopropanol, gently mixed to precipitate the DNA, and centrifuged at 14,000 × *g* for 5 min. The supernatant was discarded and the pellet was rinsed twice with 70% ethanol, dried, and resuspended in 100 µl of sterile H₂O.

Development of microsatellite markers. Microsatellite sequences were identified using a combined microsatellite motif enrichment and next-generation sequencing approach (Malausa et al. 2011). A genomic library enriched for microsatellite motifs was developed from *S. citrulli* isolate C5-5 by hybridization to 12 biotinylated oligonucleotide repeat probes consisting of (GT)₈, (TC)_{9.5}, (TTC)₇, (GTA)_{8.33}, (GTG)_{4.67}, (TCC)₅, (GTT)_{6.33}, (TTTC)₆, (GATA)₇, (TTAC)_{6.75}, (GATG)_{4.25}, and (TTGG)_{5.25}. The library was pooled with other libraries, each with unique adapter sequences, and submitted for Roche 454 GS-FLX Titanium sequencing at the Sequencing and Genotyping Facility at the Cornell Life Sciences Core Facility (Ithaca, NY). MID-sorted 454 reads were trimmed of adapter sequences and assembled in SeqMan Pro (DNASTAR, Madison, WI). Contigs and singletons were searched for at least five perfect repeats of trimeric, tetrameric, pentameric, and hexameric microsatellites in msatcommander (Faircloth 2008). Sequences with at least 40-bp flanking sequence on each side of the repeat were considered acceptable for primer design. Primers for amplification of microsatellite loci were designed with Primer3 (Rozen and Skaletsky 2000).

Candidate microsatellite loci were evaluated on a panel of 13 isolates (marked in Table 1 by an asterisk) from the southeastern United States to ensure polymorphism in our target populations. A three-primer method (Schuelke 2000) was used in the first round of marker selection. The sequence 5'-CAGTCGGGCGTCATCA-3' (CAG tag) (Hauswaldt and Glenn 2003) was added to the 5' end of the forward primer for each of the candidate microsatellite markers. The third primer consisted of the CAG tag 5' labeled with the

fluorescent dye HEX (Integrated DNA Technologies, Coralville, IA). PCR was carried out in 12 µl with 1.2 µl of 10× ExTaq buffer (Takara Bio, Inc., Mountain View, CA), 1.2 µl of 2.5 mM dNTPs, 0.05 µl of 10 µM 5' CAG tag-labeled forward primer, 0.5 µl of 10 µM reverse primer, 0.5 µl of 10 µM 5' HEX-labeled CAG tag primer, 0.5 U of ExTaq (Takara Bio, Inc.), and 0.5 µl of DNA template (10 to 100 ng/µl). Thermal cycling conditions were 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; followed by 72°C for 5 min. Amplification of single PCR products within the expected size range was confirmed by electrophoresis on a 1% (wt/vol) agarose gel. A 1:10 dilution of the PCR products (1 µl) was mixed with 0.5 µl of ROX-labeled size standard (Georgia Genomics Facility, Athens, GA) and 9.5 µl of Hi-Di formamide (Applied Biosystems, Foster City, CA). Products were denatured by incubation at 95°C for 5 min and immediately placed on ice. Fragment analysis was conducted at the Georgia Genomics Facility on an Applied Biosystems 3730xl 96-capillary

DNA Analyzer. GeneMapper (v.4.0; Applied Biosystems) was used to determine allele sizes. Only primer sets that produced single peaks in the expected size range and showed polymorphism in our panel of 13 isolates were optimized for multiplex PCR. Microsatellite marker sequences for isolate C5-5 were deposited in GenBank under accession numbers KM364537 to KM364556 for *Db01* to *Db20*, respectively.

The forward primers of the microsatellite markers selected for multiplex PCR (Table 2) were labeled at the 5' end with one of the following fluorescent dyes: 6FAM (Integrated DNA Technologies), VIC, PET, or NED (Applied Biosystems). Multiplex reactions were optimized so that loci with alleles of similar size ranges were labeled with different dyes. The multiplex reactions were tested on the panel of 13 isolates as well as 33 additional isolates from the United States, Canada, Sweden, The Netherlands, Greece, Israel, and China. Multiplex PCR was carried out using a Type-it Microsatellite PCR Kit (Qiagen) following the manufacturer's

TABLE 1. Origin and species of gummy stem blight isolates

Region ^a	Location	Original host species	Isolate name ^b	Source ^c	<i>Stagonosporopsis</i> spp. ^d
SE USA	Tift County, GA	<i>Citrullus lanatus</i> (watermelon)	C5-1*	K.S.	<i>citrulli</i>
SE USA	Tift County, GA	<i>C. lanatus</i> (watermelon)	C5-5*	K.S.	<i>citrulli</i>
SE USA	Tattnall County, GA	<i>C. lanatus</i> (watermelon)	UR4-1*	K.S.	<i>citrulli</i>
SE USA	Collier County, FL	<i>C. lanatus</i> (watermelon)	09-090-1*	K.S.	<i>citrulli</i>
SE USA	Suwanee County, FL	<i>C. lanatus</i> (watermelon)	PDC-6*	K.S.	<i>citrulli</i>
SE USA	Suwanee County, FL	<i>C. lanatus</i> (watermelon)	PDC-2	K.S.	<i>citrulli</i>
SE USA	Sampson County, NC	<i>C. lanatus</i> (watermelon)	NCA-4*	K.S.	<i>citrulli</i>
SE USA	Sampson County, NC	<i>C. lanatus</i> (watermelon)	NC10-7*	K.S.	<i>citrulli</i>
SE USA	Sampson County, NC	<i>C. lanatus</i> (watermelon)	NC10-15*	K.S.	<i>citrulli</i>
SE USA	Charleston County, SC	<i>C. lanatus</i> (watermelon)	SC4-3*	K.S.	<i>citrulli</i>
SE USA	Charleston County, SC	<i>C. lanatus</i> (watermelon)	SC4-5	K.S.	<i>citrulli</i>
SE USA	Charleston County, SC	<i>C. lanatus</i> (watermelon)	SC4-10	K.S.	<i>citrulli</i>
SE USA	Alamo, TX	<i>C. lanatus</i> (watermelon)	RD7	A.K.	<i>citrulli</i>
SE USA	Colquitt County, GA	<i>Cucumis sativus</i> (cucumber)	GC-7*	K.S.	<i>citrulli</i>
SE USA	Colquitt County, GA	<i>C. sativus</i> (cucumber)	GC-14*	K.S.	<i>citrulli</i>
SE USA	Edisto, SC	<i>C. sativus</i> (cucumber)	C68	A.K.	<i>citrulli</i>
SE USA	Tift County, GA	<i>C. melo</i> (cantaloupe)	GS5-17*	K.S.	<i>citrulli</i>
SE USA	Tift County, GA	<i>C. melo</i> (cantaloupe)	GS7-1*	K.S.	<i>citrulli</i>
SE USA	Oklahoma	<i>C. melo</i> (cantaloupe)	RB2	A.K.	<i>citrulli</i>
SE USA	Pharr, TX	<i>C. melo</i> (cantaloupe)	RD6	A.K.	<i>citrulli</i>
SE USA	Johnson County, NC	<i>Cucurbita pepo</i> (acorn squash)	AcSq5	A.K.	<i>citrulli</i>
NE USA	Sussex County, DE	<i>Citrullus lanatus</i> (watermelon)	RJ6	A.K.	<i>citrulli</i>
NE USA	Knox County, IN	<i>C. lanatus</i> (watermelon)	RT1	A.K.	<i>citrulli</i>
NE USA	Wicimico, MD	<i>C. lanatus</i> (watermelon)	RJ1	A.K.	<i>citrulli</i>
NE USA	New York	<i>C. lanatus</i> (watermelon)	GSB29	A.K.	<i>cucurbitacearum</i>
NE USA	Michigan	<i>Cucumis sativus</i> (cucumber)	C166	A.K.	<i>caricae</i>
NE USA	Tompkins County, NY	<i>C. sativus</i> (cucumber)	GSB19	A.K.	<i>cucurbitacearum</i>
NE USA	Onondaga County, NY	<i>C. melo</i> (muskmelon)	DIDYNY1	A.K.	<i>citrulli</i>
NE USA	Knox County, IN	<i>C. melo</i> (muskmelon)	RT4	A.K.	<i>citrulli</i>
NE USA	Tompkins County NY	<i>C. melo</i> (muskmelon)	GSB26	A.K.	<i>cucurbitacearum</i>
NE USA	Michigan	<i>Cucurbita moschata</i> (butternut squash)	RT2	A.K.	<i>cucurbitacearum</i>
NE USA	Montgomery County, NY	<i>C. moschata</i> (butternut squash)	GSB5	A.K.	<i>cucurbitacearum</i>
NE USA	Ontario County, NY	<i>Cucurbita</i> sp. (pumpkin)	GSB21	A.K.	<i>cucurbitacearum</i>
NE USA	Indiana	<i>Cucurbita</i> sp. (decorative gourd)	RU1	A.K.	<i>cucurbitacearum</i>
California	Greenhouse	<i>Citrullus lanatus</i> (watermelon)	RG3	A.K.	<i>caricae</i>
Other region	China	<i>C. lanatus</i> (watermelon)	W85	A.K.	<i>citrulli</i>
Other region	China	<i>C. lanatus</i> (watermelon)	W87	A.K.	<i>citrulli</i>
Other region	The Netherlands	<i>Cucumis sativus</i> (cucumber)	RV1	A.K.	<i>citrulli</i>
Other region	Sweden	<i>C. sativus</i> (cucumber)	RV2	A.K.	<i>citrulli</i>
Other region	Canada	<i>C. sativus</i> (cucumber)	RV16	A.K.	<i>caricae</i>
Other region	Israel	<i>C. melo</i> (muskmelon)	ID1	A.K.	<i>citrulli</i>
Other region	Greece	<i>C. sativus</i> (cucumber)	RV3	A.K.	<i>citrulli</i>
Other region	Greece	<i>C. sativus</i> (cucumber)	RV7	A.K.	<i>citrulli</i>
Other region	Greece	<i>C. sativus</i> (cucumber)	RV8	A.K.	<i>citrulli</i>
Other region	Greece	<i>C. sativus</i> (cucumber)	RV9	A.K.	<i>citrulli</i>
Other region	Greece	<i>C. sativus</i> (cucumber)	RV10	A.K.	<i>citrulli</i>

^a Regional designations used to group isolates by the geographic location of original collection include southeastern United States (SE USA), northeastern United States (NE USA), California, or a region outside of the United States (other region).

^b DNA was provided for isolates from regions outside of the United States. Isolates followed by an asterisk (*) were included in the original panel used to evaluate candidate microsatellite loci.

^c Isolates or DNA were kindly provided by Dr. Katherine Stevenson (K.S.), Department of Plant Pathology, University of Georgia, Tifton; or Dr. Anthony Keinath (A.K.), Coastal Research and Education Center, Clemson University, Charleston, SC.

^d Isolate assignment to one of the three *Stagonosporopsis* spp. that cause gummy stem blight as described previously (Stewart et al. 2015). The species epithet is listed for each isolate.

instructions, except that reactions were scaled to 10 µl. Each of the three multiplex reactions consisted of 5 µl of 2× Type-it Master Mix, 1 µl of 10× primer mix (which consisted of 2 µM each primer in the multiplex), 1 µl of DNA template (10 to 100 ng/µl), and RNase-free water. Thermal cycling conditions were 95°C for 5 min; 28 cycles of 95°C for 30 s, 57°C for 90 s, and 72°C for 30 s; followed by 60°C for 30 min. PCR products were diluted 1:15 in double-distilled H₂O and 1 µl of diluted PCR product was combined with 0.1 µl of the internal size standard Genescan-500 Liz (Applied Biosystems) and 9.9 µl of Hi-Di formamide (Applied Biosystems). Samples were incubated at 95°C for 5 min and placed immediately on ice, and fragment analysis was carried out at the Georgia Genomics Facility, as described above. Genemapper v.4.0 was used to score alleles. Loci were distinguished by fluorescent dye and allele size range.

The number of alleles and unbiased gene diversity (h) for each microsatellite locus was estimated with Genalex v.6.41 (Peakall and Smouse 2006). Genetic differentiation (Φ_{PT} , an analog of G_{ST} measured via analysis of molecular variance [AMOVA]) (Peakall et al. 1995) among geographic regions and host species was estimated using GenAlEx v.6.41 on clone-corrected data sets,

which included only one representative of each unique multilocus genotype. Significant differentiation between populations was determined by comparing observed values to the distribution of 1,000 randomizations of the data. In order to visualize major patterns of variation within and among populations from different geographic regions and hosts, principal coordinates analysis (PCA) was conducted with GenAlEx v.6.41 on pairwise genetic distances of all genotypes. Genotypic diversity ($\hat{G}$) (the probability that two individuals taken at random have unique multilocus genotypes) or $[N(N-1)](1-\sum p_i^2)$, where p_i is the frequency of the i th multilocus genotype and N is the sample size for each population, was estimated in MultiLocus v.1.3b (Agapow and Burt 2001). Multi-locus linkage disequilibrium was determined for the clone-corrected dataset using the index of association (I_A) (Smith et al. 1993) estimated with MultiLocus v.1.3b: $I_A = s_k^2/\sigma_k^2 - 1$, where k is the number of loci at which pairs of individuals have different alleles, s_k^2 is the observed variance of k , and σ_k^2 is the expected variance of k under linkage equilibrium. I_A is not significantly different from zero when the population is in linkage equilibrium. P values were estimated by 1,000 random permutations of the data.

TABLE 2. Repeat motif, primer sequences, multiplexing primers and reactions, allele sizes and number, and gene diversity (h) for *Stagonosporopsis citrulli* microsatellite loci

Locus	Repeat motif	Primer sequence (5'→3')	Fluorescent label for F primer ^a	Multiplex reaction	Range of allele sizes (bp)	Number of observed alleles ^b	Gene diversity ^c
<i>Db01</i>	(ACC) ₉	F: CGGTCCGGTCAACCTACTAC R: CACGCCAGCAAATCTCACTA	6FAM	1	356–364	4	0.532
<i>Db02</i>	(ACTGG) ₅	F: GAGGGTGGTGGTTTGGTAG R: ACATGGACGAGGAGAACGAG	6FAM	1	186	1	0
<i>Db03</i>	(AATGC) ₇	F: TCTATCTGTTGTCTCGCTGGAG R: ACATCCATCCATCCATCCAT	NED	1	294–303	2	0.475
<i>Db04</i>	(ACTGG) ₅	F: GTGGGGTTGATGGTGACTTT R: GCTGCAGATGTAATGCCTGA	NED	1	224	1*	0
<i>Db05</i>	(AATG) ₆	F: TATGACGTTGGGCAAGTGAG R: TTTGCTGGGATGGTGTGTGA	PET	1	216–224	2*	0.508
<i>Db06</i>	(AGC) ₈	F: GGTGACATCTGCGTGAATG R: TGGTTGTTTGGTTGTTTGA	VIC	1	283–289	3	0.675
<i>Db07</i>	(AAAG) ₇	F: ATTTGAACCTTGGGCGTTTG R: ATGATAATGGCGACGGAGAT	VIC	1	213–225	2	0.322
<i>Db08</i>	(AAC) ₁₀	F: AAAATCCACATGCGAAGAGG R: GTGGCAGTGGTAGTGGTCCT	6FAM	2	311–367	11	0.838
<i>Db09</i>	(AATC) ₉	F: AGACTCAACCAACCCACAC R: CGAAATCAATGAACCAACGA	6FAM	2	216–232	3	0.160
<i>Db10</i>	(ACCT) ₅	F: GACGACAAGGCAGAGAAAGG R: CGCTGAGGTTTTTGGATTTG	NED	2	330–334	2	0.513
<i>Db11</i>	(AAGG) ₅	F: AAATCAGGCGGTGGAATATG R: AGGTTTCTGGCATGTTACAG	NED	2	215–227	3	0.652
<i>Db12</i>	(ATCC) ₅	F: TCAGTCCATCCAATCCAACA R: CGCAAAGGATAAGAGCGAAG	PET	2	215–227	4	0.598
<i>Db13</i>	(AGC) ₇	F: ATATTGAGTCTGGGTGGA R: GTGGCGGTGGTAGTGGTAGT	VIC	2	370–376	2	0.489
<i>Db14</i>	(ACAT) ₇	F: ATGAGCAGCGAATTGGATTG R: CCGGGGTAGTTTGTGTTGTT	VIC	2	217–229	3	0.570
<i>Db15</i>	(ACT) ₆	F: ATGGGAGTCGTGGTGAAAG R: TGAGTTTAGGGACTGGAATGC	6FAM	3	245–257	3	0.294
<i>Db16</i>	(ATC) ₉	F: GCCCTTTTCCCATCTTCTC R: TTTCTTTTCTCCGAGAGTCG	6FAM	3	148–154	3	0.252
<i>Db17</i>	(ATCC) ₉	F: TCGCGGAGATCTGTTCTTCT R: GAGTTGTGTCGGTGGATGTG	NED	3	198–202	2	0.386
<i>Db18</i>	(AAC) ₁₅	F: GAGCACGCATATAGCAGCAA R: AGCCGTAGCCGTAGTCGTTA	PET	3	280–305	6	0.637
<i>Db19</i>	(ACGC) ₅	F: TCTCCCATCCTCCAGAACAC R: TCTCTTCCAACCGTCCAATC	VIC	3	312–316	2	0.356
<i>Db20</i>	(AGG) ₈	F: AAGATGGCTAGCGCACAGAG R: TGACGAGGCAAAGGTAAGG	VIC	3	147–154	3	0.332

^a Fluorescent primers allowed for visualization on ABI3730 DNA Analyzer and cost-effective, efficient polymerase chain reaction (PCR) multiplexing.

^b Number of alleles listed is for *S. citrulli*. The asterisks indicate where additional alleles were consistently observed for all three species. For *Db04*, alleles of 196, 199, and 202 bp were detected for *S. cucurbitacearum* isolates and an allele of 191 bp was detected for *S. caricae* isolates. For *Db05*, alleles of 216 and 220 bp were detected for *S. cucurbitacearum* isolates and alleles of 216 and 218 bp were detected for *S. caricae* isolates. *Db06* and *Db07* could be PCR amplified for *S. cucurbitacearum* (allele sizes of 191 bp and 218 or 216 bp, respectively) and *Db11* could be PCR amplified for *S. caricae* (allele sizes of 221, 226, or 232 bp).

^c Unbiased gene diversity estimated by GenAlEx v.6.41 (Peakall and Smouse 2006).

Bayesian cluster analysis, a powerful method for identifying population structure and assigning individuals to populations (Pritchard et al. 2000), was not used in this study because linkage disequilibrium among loci within populations, likely due to nonrandom mating of *S. citrulli* from selfing, violates the assumptions of this type of analysis.

Comparison of microsatellite loci across lineages.

Although all microsatellite loci were consistently amplified from isolates of *S. citrulli*, only 2 of the 20 microsatellite loci were amplified in isolates from all three closely related *Stagonosporopsis* spp. that cause gummy stem blight. To compare sequences among isolates and determine whether homologous regions were amplified across the three species, alleles of the two loci (*Db04* and *Db05*) were sequenced for 17 isolates, including 9 isolates from *S. citrulli*, 5 isolates from *S. cucurbitacearum*, and 3 isolates from *S. caricae*. The PCRs were carried out in a total volume of 25 μ l. Reaction components included 2.5 μ l of 10 $\times$ PCR buffer (Takara Bio, Inc.), 2.5 μ l of dNTPs, 1.25 μ l of 10 μ M forward and reverse primers, 0.15 μ l of ExTaq (Takara Bio, Inc.), and 1 μ l of DNA template. Cycling conditions included an initial denaturation step at 94°C for 2 min; followed by 35 cycles with a denaturation step at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; followed by a final extension at 72°C for 5 min. Amplification of single PCR products within the expected size range was confirmed by electrophoresis on a 1% (wt/vol) agarose gel. PCR products were purified with QIAquick spin columns (Qiagen). Sequencing was conducted at the Georgia Genomics Facility of the University of Georgia using the Applied Biosystems Automated 3730 DNA Analyzer with Big Dye Terminator chemistry and Ampli-Taq-FS DNA Polymerase. All gene regions were sequenced in both directions with forward and reverse primers. Sequences were aligned and manually edited as needed in Geneious (v.6; Biomatters Ltd., Auckland, New Zealand). Alignments were deposited in TreeBASE under accession numbers 16262 and 16261 for *Db04* and *Db05*, respectively. Phylogenies were reconstructed by maximum likelihood in MEGA5 (Tamura et al. 2011). The Hasegawa-Kishino-Yano and Kimura 2-parameter models of evolution for *Db04* and *Db05*, respectively, as determined most appropriate in MEGA5, were implemented. Support for internal branches was determined by 500 bootstrap replicates.

PCR-based marker for detecting lineages. A rapid PCR-based assay for distinguishing the three morphologically similar species was developed using three microsatellite markers in a single reaction, including *Db01* that amplifies a fragment only from isolates of *S. citrulli*, *Db06* that amplifies a fragment only from isolates of *S. citrulli* or *S. cucurbitacearum*, and *Db05* that amplifies a fragment from isolates from all three species. Three isolates from each species were used to test the marker, including C5-5, UR4-1, and DIDYNY from *S. citrulli*; RT2, GSB26, and GSB5 from *S. cucurbitacearum*; and RG3, C166, and ATCC52577 from *S. caricae*. Two additional isolates from *Alternaria* and *Phoma* spp. (Keinath et al. 1995), which are similar in colony morphology to gummy stem blight isolates and are frequently isolated from symptomatic cucurbits, were included to determine whether bands were specific for the *Stagonosporopsis* isolates. The PCR reactions were carried out in a total volume of 25 μ l. Reaction components included 2.5 μ l of 10 $\times$ PCR buffer (Takara Bio, Inc.), 2.5 μ l of dNTPs, 1.25 μ l of 10 μ M forward and reverse primers for each of the three loci (*Db01*, *Db05*, and *Db06*) (Table 2), 0.15 μ l of ExTaq (Takara Bio, Inc.), and 1 μ l of DNA template. Cycling conditions were the same as for the microsatellite sequencing reaction. Electrophoresis of PCR products was conducted on a 2.5% (wt/vol) agarose gel run at 95 V (4.75 V/cm) for 3.5 h. DNA was visualized with 500 $\times$ GelRed (Biotium, Hayward, CA) added prior to gel casting.

Diversity and population structure in field populations.

To understand fine-scale population genetic structure and diversity of *S. citrulli* and to test the utility of the microsatellite markers in south-eastern United States field populations, 200 leaves from watermelon plants with lesions characteristic of gummy stem blight were collected

arbitrarily at distances of 5 to 20 m apart in July 2012 from two fields in Tift County, GA and Cook County, GA. The two fields were separated by 20 km. Isolates of *S. citrulli* were obtained from surface-disinfested leaves by soaking a 1-cm² segment of a leaf containing the margin of a lesion for 1.5 min in 0.6% sodium hypochlorite and rinsing twice with sterile distilled water. Three cuttings per leaf were placed on a 100-mm petri dish of qPDA. After 3 days of growth at 25°C, a 4-mm² agar segment containing hyphal tips was transferred to a fresh dish of qPDA. Identity as a *Stagonosporopsis* sp. was initially confirmed by growth rate and colony morphology. Isolates were maintained for long-term storage on filter paper at –20°C. DNA was extracted using the protocol described above (in the section “Isolate maintenance and DNA extraction”) (Milgroom et al. 2008). All isolates were genotyped with the 20 microsatellite markers using three multiplex reactions carried out with the Type-it Microsatellite PCR Kit (Qiagen), as described above.

For each of the two populations, genotypic diversity ($\hat{G}$) was estimated in MultiLocus v.1.3b (Agapow and Burt 2001). Multilocus linkage disequilibrium, a measure of nonrandom mating, was determined for the clone-corrected dataset using the I_A (Smith et al. 1993) estimated with MultiLocus v.1.3b. P values were estimated by 1,000 random permutations of the data. Recombination events were detected using the four-gamete test (Hudson and Kaplan 1985) on pairs of microsatellite loci. Measured via AMOVA, Φ_{PT} Peakall et al. 1995) between the field populations was estimated using GenAlEx v.6.41 on full and clone-corrected data sets. Significant differentiation between field populations was determined by comparing observed values to the distribution of 1,000 randomizations of the data. PCA was conducted with GenAlEx v.6.41 on pairwise genetic distances of all genotypes in order to visualize major patterns of variation within and between the two populations. The power of the microsatellite loci to resolve genotypes was tested by plotting the proportion of genotypes recovered against the number of loci analyzed with 1,000 randomizations of the data in MultiLocus v.1.3b.

RESULTS

Microsatellite loci. In total, 102,497 contigs and singletons were obtained from 210,507 reads, resulting in 34 Mb of unique sequence. In all, 22,705 of the contigs fit our criteria for development as microsatellite markers. Sixty-eight sets of primers were designed and tested for PCR amplification. Fifty-four sets (80%) amplified a single product and were visible on an agarose gel. In all, 29 sets consistently produced a single, well-defined peak during fragment analysis on the ABI DNA Analyzer, and 18 sets were polymorphic across the panel of 13 isolates. Although they were not polymorphic among the 13 isolates tested, two additional loci were added to the reactions because they appeared to be polymorphic among the other *Stagonosporopsis* spp. The robust primer sets were organized into three multiplex reactions by using four different dyes and combining loci of distinct allele sizes with the same dye (Table 2). Multiplex reactions 1 and 2 each consist of seven loci and reaction 3 consists of six loci. The multiplex reactions worked consistently for template DNA, ranging from 10 to 100 ng/ μ l.

For the loci that were polymorphic within *S. citrulli*, unbiased gene diversity (h) ranged from 0.160 to 0.838, and 2, 3, or 4 alleles were detected per locus, except for *Db08* and *Db18*, which had 11 and 6 alleles, respectively. The locus *Db02* was not polymorphic among isolates in this study (Table 2), and *Db04* was not polymorphic within *S. citrulli* isolates. Only *Db04* and *Db05* could be PCR amplified across all three *Stagonosporopsis* spp. causing gummy stem blight. For *Db04*, alleles of 196, 199, and 202 bp were detected for *S. cucurbitacearum* isolates and an allele of 191 bp was detected for *S. caricae* isolates. For *Db05*, alleles of 216 and 220 bp were detected for *S. cucurbitacearum* isolates, and alleles of 216 and 218 bp were detected for *S. caricae* isolates. *Db06* and *Db07* could be PCR amplified for *S. cucurbitacearum* (allele sizes of 191 bp and 218 or 216 bp, respectively) and *Db11* could be PCR amplified for *S. caricae* (allele sizes of 221, 226, or 232 bp).

Of the 46 isolates from diverse geographic regions and host plants, and the three *Stagonosporopsis* spp. (Stewart et al. 2015), only the 36 isolates that belonged to *S. citrulli* consistently amplified across all of the loci; therefore, population genetic analyses could only be conducted on isolates from this species. Among the 36 isolates, there were 30 unique multilocus genotypes, 3 of which were represented by 2 or more isolates. The three repeated genotypes included one consisting of three isolates from watermelon from South Carolina (SC4-3, SC4-5, and SC4-10), one consisting of two isolates from watermelon from China (W85 and W87), and one consisting of four isolates from Europe (three from Greece and one from the Netherlands) on cucumber (RV1, RV8, RV9, and RV10). All other isolates had unique genotypes. No clustering by hosts or region was observed by PCA (Fig. 1). Although sample sizes were small, there was no partitioning of genetic diversity by host (watermelon [$n = 15$] × cucumber

[$n = 7$] × muskmelon/cantaloupe [$n = 7$], $\Phi_{PT} = 0.009$, $P = 0.325$) or region (southeastern United States [$n = 19$] × northeastern United States [$n = 5$] × other region [$n = 6$], $\Phi_{PT} = 0.009$, $P = 0.327$) on the clone-corrected dataset. Although three clonal genotypes were detected, genotypic diversity was relatively high ($G = 0.984$). However, nonrandom mating was detected even after clone correction for repeated genotypes ($I_A = 0.694$, $P < 0.001$).

To compare the two microsatellite loci that amplified for all three species to determine whether allele size differences were due to variation in repeat length and gain insight into variation within and among the three *Stagonosporopsis* spp. at these loci, we sequenced *Db04* and *Db05* for a select set of 17 isolates (Fig. 2). For both loci, the sequences were identical or nearly identical within species yet they were extremely divergent among species, providing 99 to 100% bootstrap support for each species. For the 205-nucleotide (nt) alignment of *Db05* (Fig. 2A), *S. cucurbitacearum* differed from

Fig. 1. Principal coordinates analysis of pairwise genetic distances of multilocus genotypes of 36 isolates of *Stagonosporopsis citrulli* from diverse cucurbit hosts and geographic regions. The first two principal coordinates, PC1 and PC2, are shown. Multilocus genotypes were based on alleles at 18 polymorphic microsatellite loci (*Db01*, *Db03*, and *Db05* to *Db20*). Population assignment of each isolate based on geographic origin (southeast United States [SE USA], northeastern United States [NE USA], or other region) is indicated by the color of the data point, whereas original host (*Citrullus lanatus* [watermelon], *Cucumis sativus* [cucumber], *C. melo* [cantaloupe or muskmelon], or *Cucurbita pepo* [squash or gourd]) is indicated by the shape of the data point, as shown in the key.

Fig. 2. Maximum likelihood trees of the loci **A**, *Db05* and **B**, *Db04*. Both loci were sequenced and aligned for 17 isolates representing the three species of *Stagonosporopsis* causing gummy stem blight. All isolates were originally collected from cucurbit hosts, except ATCC52577, which was originally isolated from papaya in Hawaii. The original host and geographic origin of each cucurbit isolate is listed in Table 1. Bootstrap support for each clad is shown.

S. citrulli by 23 single nucleotide polymorphisms (SNPs) and 5 insertion/deletions (indels) at regions other than the microsatellite region, and *S. caricae* differed from *S. citrulli* by 18 SNPs and 6 indels at regions other than the microsatellite repeat. There was one SNP and an indel in the microsatellite repeat region within *S. citrulli* isolates, one indel within *S. cucurbitacearum* isolates, and no variation within *S. caricae* isolates. For the 206-nt alignment of *Db04* (Fig. 2B), *S. cucurbitacearum* differed from *S. citrulli* by 6 SNPs and one large indel, and *S. caricae* differed from *S. citrulli* by 14 SNPs and the same large indel. There was no variation within *S. citrulli* isolates, two SNPs and one indel within *S. cucurbitacearum* isolates, and one indel within *S. caricae* isolates.

PCR-based marker for distinguishing the three species causing gummy stem blight. The marker (Fig. 3) was designed using three sets of primers, including *Db05* that produces a 216- to 224-bp fragment in all three species, *Db06* that produces a 283- to 289-bp fragment in *S. citrulli* and a 268-bp and slightly fainter fragment in *S. cucurbitacearum*, and *Db01* that produces a 356- to 364-bp fragment in *S. citrulli*. The size and number of fragments correlated perfectly with the previously determined species (Stewart et al. 2015). The isolates of *Alternaria* and *Phoma* spp. did not amplify.

Diversity and population structure in field populations. To evaluate diversity and population structure and to test the microsatellite markers on field populations in the southeastern United States, we genotyped 83 and 72 isolates from two watermelon fields in Tift County, GA and Cook County, GA, respectively (Table 3). One hundred symptomatic leaves were collected from each field but gummy stem blight isolates were not recovered from all leaves due to secondary fungi and contamination. All *Stagonosporopsis* isolates were identified as *S. citrulli* based on amplification and allele size of microsatellite loci. Genotypic diversity of 0.904 and 0.926 for Tift County and Cook County, respectively, was relatively high, even though 47 and 51% of the isolates did not have unique genotypes. Twelve repeated genotypes, considered as clones, were present in the Tift County field and nine clones were present in the Cook County field. Each field contained a different dominant clone that included 19 and 17 individuals for Tift County and Cook County, respectively. Four clones were present in both of the fields, including one clone represented by a single isolate from each field, two clones with

a single isolate from Tift County and 3 or 5 isolates from Cook County, and one clone represented by 2 isolates from Tift County and 17 isolates from Cook County. Nonrandom mating (I_A) was detected within each field, even after clone correction. However, recombination events were detected using the four-gamete test (Hudson and Kaplan 1985) on the loci *Db01*, *Db03*, and *Db05*, which each contained two alleles (*Db01*: 356/364 bp, *Db03*: 294/303 bp, and *Db05*: 224/216 bp) within the field populations. All four combinations of alleles for *Db01* and *Db03*, *Db03* and *Db05*, and *Db01* and *Db05* were detected among isolates within the populations.

Although they shared clones, populations of *S. citrulli* from the two watermelon fields in Georgia were genetically structured. There was overlap among the genotypic profiles of isolates from the two fields (Fig. 4); however, subtle differences in the clustering of isolates can be observed and values of PC1 and PC2 are significantly different between fields (equal variance, two-tailed *t* test, $P < 0.001$ for both PC1 and PC2). Genetic differentiation between fields was detected on both the entire ($\Phi_{PT} = 0.086$, $P < 0.001$) and clone-corrected ($\Phi_{PT} = 0.019$, $P = 0.024$) datasets.

To determine whether fewer than 18 loci would be sufficient for distinguishing genotypes from the two field populations, we plotted the proportion of genotypes identified against the number of loci analyzed (Fig. 5). With 7 loci, 50% of the genotypes could be identified; yet, with 15 loci, 95% of the genotypes could be identified. Eighteen loci appear to be sufficient for resolution of the total number of unique genotypes because the data are nearing a plateau; however, one or two additional polymorphic loci could lead to the identification of additional genotypes in these populations.

DISCUSSION

To characterize the genetic diversity and population structure of gummy stem blight fungi, particularly in the southeastern United States, to provide insight into the epidemiology of this disease, we developed 20 microsatellite markers using a method that combines microsatellite repeat enrichment and next-generation sequencing (Malaua et al. 2011). During the development of these markers, it was discovered that gummy stem blight of cucurbits is caused by three distinct species: *S. caricae*, *S. cucurbitacearum*, and *S. citrulli* (Stewart et al. 2015). *S. caricae* had been described as

Fig. 3. Polymerase chain reaction (PCR)-based marker for distinguishing *Stagonosporopsis citrulli*, *S. cucurbitacearum*, and *S. caricae*. The leftmost lane (L) is a 100-bp ladder with sizes of visible fragments indicated. Three isolates from each species are shown (*S. citrulli*: C5-5, UR4-1, and DIDYNY; *S. cucurbitacearum*: RT2, GSB26, and GSB5; and *S. caricae*: RG3, C166, and ATCC52577). The PCR-based marker uses three sets of primers in a single reaction: *Db05* produces an approximately 220-bp fragment in all three lineages, *Db06* produces an approximately 285-bp fragment in lineage 1 and approximately 270-bp fragment in lineage 2, and *Db01* that produces an approximately 360-bp fragment in lineage 1. No fragments were visible for *Phoma* spp. (P) and *Alternaria* spp. (A), which are vaguely similar to *Stagonosporopsis* spp. in colony morphology and are frequently recovered from symptomatic plants.

TABLE 3. Genetic diversity and linkage disequilibrium in field populations of *Stagonosporopsis citrulli* causing gummy stem blight

Population	Sample size (<i>N</i>)	Number of genotypes (<i>g</i>)	Repeated fraction ^a	Genotypic diversity ^b	Number of repeated genotypes	Number of isolates in each repeated genotype	I_A (<i>P</i> value) ^c
Tift County, GA	83	44	0.47	0.904	12	19, 5, 4, 4, 3, 3, 3, 2, 2, 2, 2, 2	0.881 (<0.001)
Cook County, GA	72	40	0.51	0.926	9	17, 5, 4, 4, 3, 2, 2, 2, 2	0.271 (0.024)

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

^b Genotypic diversity = $[N/(N - 1)](1 - \sum p_i^2)$, where p_i is the frequency of the *i*th genotype.

^c Index of association (I_A) was estimated with MultiLocus v.1.3b (Agapow and Burt 2001) on the clone-corrected dataset, which included a single representative of each multilocus genotype. *P* values were determined by 1,000 randomizations.

a papaya pathogen and *S. cucurbitacearum* (syn. *D. bryoniae*) was found to consist of two morphologically similar but phylogenetically distinct lineages; therefore, *S. citrulli* was erected to encompass one of the species. Of the 20 loci developed, 18 were polymorphic within *S. citrulli*, which is the most common and widespread of the three *Stagonosporopsis* spp. that cause gummy stem blight (Stewart et al. 2015). It is suggested for future studies not to add two markers, *Db02* and *Db04*, to the multiplex reactions, because they are not polymorphic; however, *Db04* is useful for comparisons across species. Although it would be advantageous to have molecular markers that PCR amplify for all three species, cross-amplification of microsatellites across species is often not possible (Barbara et al. 2007; Njambere et al. 2010; Selkoe and Toonen 2006), especially for fungi (Dutech et al. 2007). When microsatellite loci do amplify in congeneric species, they are often invariable or contain null alleles (Selkoe and Toonen 2006), both of which were issues that we experienced when using the markers on isolates from *S. cucurbitacearum* and *S. caricae*. Only two of the loci, *Db04* and *Db05*, amplified across all three species. Except for these two loci and *Db06* and *Db07* that could be PCR amplified for *S. cucurbitacearum* and *Db11* that could be PCR amplified for *S. caricae*, the other loci did not amplify in *S. cucurbitacearum* or

S. caricae, which was the result of either mutation in the flanking sequence that prevented annealing of the primers or the complete absence of these loci or either flanking sequence within the other closely related species. The two loci that did PCR amplify across species were variable in allele size as a result of indels rather than changes in the number of microsatellite repeats. The loci also had numerous SNPs among lineages which, when combined with the differences in size due to indels, leads to them being problematic markers for population genetic studies due to the assumption that fragments of the same size are alleles that are identical by descent (Selkoe and Toonen 2006). The markers were robust, especially for populations within the southeastern United States, where 99% of the 600 cucurbit gummy stem blight isolates we genotyped to date (data not shown) belong to *S. citrulli*. The other 1% (four isolates) of isolates we genotyped from the southeastern United States belong to *S. caricae* and were all obtained from a single watermelon field in Tift County, GA in 2013. Overall, these new microsatellite markers were reproducible and inexpensive, especially when multiplexed, and will be used in future studies aimed at answering epidemiological questions.

When tested on 46 isolates from diverse cucurbit hosts and regions around the world, the markers were consistent and repeatable for

Fig. 4. Principal coordinates analysis of pairwise genetic distances of multilocus genotypes of 155 isolates of *Stagonosporopsis citrulli* sampled from symptomatic watermelon (*Citrullus lanatus*) leaves from two field sites in Georgia. The first two principal coordinates, PC1 and PC2, are shown. Multilocus genotypes were based on alleles at 18 polymorphic microsatellite loci (*Db01*, *Db03*, and *Db05* to *Db20*). The field site of each isolate (Tift County, GA or Cook County, GA) is indicated by the color and shape of the data point, as shown in the key. Overlapping of isolates from the two fields results in various shades of gray.

Fig. 5. Power of the microsatellite loci to resolve genotypes of *Stagonosporopsis citrulli* sampled from two Georgia watermelon fields in July 2012. The proportion of genotypes identified is plotted against the number of loci analyzed with 1,000 randomizations of the data in MultiLocus v.1.3b (Agapow and Burt 2001).

S. citrulli. Within *S. citrulli*, we found no population structure based on broad-scale geographic region or host of origin, suggesting widespread dispersal around the world. This is in contrast to another homothallic fungal plant pathogen, *Sclerotinia sclerotiorum*, which is quite distinct on canola in different regions of the world (Attanayake et al. 2013). In our study, three clones of *Stagonosporopsis citrulli* were detected but each came from the same host. One clone was from watermelon in a single field in South Carolina. The second clone was represented by three isolates from watermelon in China, and the third clone was represented by four isolates from cucumber in Europe (Greece, $n = 3$, and The Netherlands, $n = 1$). It is probable that the clones in Europe were dispersed on seed. Future studies aimed at determining worldwide patterns of migration by tracking genotypes of isolates collected from cucurbit production regions using the microsatellite markers would be informative for identifying sources of inoculum for greenhouse and field epidemics.

Although differences in aggressiveness among the three species to cucurbit hosts have not been detected, they do vary in pathogenicity to papaya (Stewart et al. 2015) and could vary in fungicide resistance or reproductive biology. Therefore, it is important to be able to rapidly detect and distinguish the species to determine which are present in a particular host, field, or region. Using the microsatellite markers, we developed a PCR-based assay that discriminates the three species causing gummy stem blight of cucurbits, something that we were not able to do based on simple morphological features. This method is much simpler, cheaper, and faster than a sequence-based approach. The marker also works for *S. caricae* isolates from papaya. In future studies, the PCR-based marker can be used to determine the frequency and distribution of the three *Stagonosporopsis* spp. causing gummy stem blight, which is an area in need of further investigation, because the regional and worldwide distribution of the species is not well known. If *S. citrulli* represents the majority of isolates in a population of interest, the 18 polymorphic microsatellite markers would be useful for population genetic analyses.

To better understand dispersal, reproduction, and fine-scale genetic diversity of *S. citrulli* within and among watermelon fields and test the utility of the microsatellite markers on populations causing gummy stem blight within the southeastern United States, 155 isolates from two field populations in Georgia were genotyped with the 20 microsatellite loci. To our knowledge, this is the first study to investigate within-field genetic diversity of the gummy stem blight pathogen. All of the isolates from Georgia belonged to *S. citrulli*. Eighteen of the loci were polymorphic in these populations and were used in the population genetic analyses. Although dominant and widespread clones—inferred by identical multilocus genotypes—were detected, we found relatively high genotypic diversity and recombinant genotypes consistent with recurrent recombination. It is not clear whether the isolates with clonal genotypes resulted from homothallic sexual reproduction (selfing), asexual reproduction, or a combination of both. Abundant pycnidia (asexual reproductive structures) full of asexual spores are produced by *S. citrulli* in the field; therefore, asexual reproduction is assumed to contribute to the production of clonal genotypes. Multilocus linkage disequilibrium (I_A) within both populations even after clone correction suggests nonrandom mating that results from selfing, although other mechanisms such as epistasis or population admixture (Milgroom 1996) cannot be ruled out completely. Many self-compatible fungi are capable of outcrossing (Atallah et al. 2004; Debuchy and Turgeon 2006), which leads to increased diversity through recombination and the production of novel genotypes. To confirm that *S. citrulli* can self and outcross in nature (i.e., it has a mixed mating system), we would need to collect and genotype ascospore progeny from the pseudothecia (sexual reproductive structures) and demonstrate that some structures contain progeny with identical genotypes and some contain progeny with recombinant genotypes. This is important for understanding the epidemiology and management of gummy stem blight, because a mixed mode of reproduction with sexual recombination leads to pathogens with a high evolutionary potential (McDonald and Linde

2002), with outcrossing producing new combinations of alleles and clonal amplification by asexual reproduction or selfing propagating successful combinations (Milgroom 1996). This potential for rapid evolution could explain why isolates causing gummy stem blight are able to rapidly develop fungicide resistance (Avenot et al. 2012; Keinath 2012; Stevenson et al. 2004). The evolution of quantitative resistance likely involves multiple genes, which will evolve much faster in recombining populations where advantageous alleles can be brought together by sexual reproduction that involves some level of outcrossing. Additionally, multigene resistance to several fungicides is enhanced in a sexually recombining population.

Significant population genetic structure between the two field populations of *S. citrulli* demonstrates that this is not a panmictic population at the regional level and dispersal among fields is limited, even though structure was not detected on a worldwide scale. This difference could be from the increased statistical power at the field scale from larger sample sizes. The detected population structure could result from several factors, including different selective pressures from different management practices within the two fields, genetic drift within each population with few migrants between them, or different source populations arriving on transplant seedlings. Potential sources of primary inoculum for gummy stem blight epidemics, including infected seed, transplants, and plant debris, have been identified (Keinath 2002; Koike 1997; Lee et al. 1984). However, it is not entirely clear which of these sources most contributes to primary inoculum that initiates epidemics in greenhouse production of transplants or in the field. Whether epidemics in the field are initiated by infected transplants, infected seed when fields are directly seeded, airborne ascospores from debris, or ascospores or conidia from wild cucurbit species that serve as reservoirs or a combination of factors has yet to be elucidated; however, this study provides insight into the fine-scale genetic diversity and reproductive biology of *S. citrulli* in the field.

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