POPULATION BIOLOGY, FUNGICIDE RESISTANCE, AND MATING SYSTEM OF GUMMY STEM BLIGHT FUNGI IN THE SOUTHEASTERN UNITED STATES

by

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ABSTRACT

Over half of U.S. cucurbits are produced in the southeastern states; however, crops in this region are exposed to a destructive foliar disease called gummy stem blight (GSB). A recent study revealed that GSB is caused by three morphologically similar, but genetically distinct *Stagonosporopsis* species: *S. citrulli*, *S. cucurbitacearum*, and *S. caricae*. In order to understand the spatial genetic structure of GSB fungi in the southeastern U.S., 528 isolates collected from nine fields over three years were genotyped with 16 microsatellite markers. *S. caricae* was reported for the first time in the southeastern U.S. Populations of the dominant species *S. citrulli* showed high genetic diversity and significant genetic structure, along with widespread and prevalent clones. Genetically similar isolates were aggregated within fields at a fine scale, indicating local splash dispersal of spores. Of the genotyped isolates, 132 were selected and tested for sensitivity to four fungicides commonly used in GSB management: azoxystrobin, tebuconazole, boscalid, and fluopyram. Diverse responses were discovered; however, the fungicide resistance profiles of isolates were not associated with the genetic similarity of *S. citrulli* isolates. All *S. caricae* isolates were resistant to

tebuconazole, while all the *S. citrulli* isolates were sensitive to this fungicide. Sequencing of the target gene *Cyp51* and quantification of the expression of *Cyp51* and *ScAtrG* (an ABC transporter) by real-time PCR did not reveal the molecular mechanisms of tebuconazole resistance in *S. caricae.* In addition, the mating-type locus (*MAT1*) that controls sexual reproduction of filamentous Ascomycota was identified from the draft genomes of the three species of GSB fungi. Both mating-type idiomorphs were found within each species supporting homothallism, or self-compatibility, of all three *Stagonosporopsis* species causing GSB. Positive selection was detected for the genes within the *MAT1* locus, which may have contributed to divergence among the GSB species. This project provides much needed information on the biology of GSB fungi, particularly on the population biology, mating system, and variation in fungicide resistance profiles, which helps us to better understand epidemics and provide suggestions on better disease management of GSB of cucurbits in the southeastern U.S.

INDEX WORDS: Cucurbits, Gummy stem blight, *Stagonosporopsis citrulli*, *Stagonosporopsis cucurbitacearum*, *Stagonosporopsis caricae*, microsatellites, population genetics, azoxystrobin, tebuconazole, boscalid, and fluopyram, *MAT1*, positive selection

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

JUSTIFICATION

Plant disease epidemiology is the field that interprets the dynamics of disease over time and space, as affected by host plants and environmental conditions (Jeger 2000). It overlaps with and is complemented by population genetics of plant pathogen, which focuses on the evolutionary factors maintaining or altering the spatial and temporal genetic structure within and among populations (McDonald 2004). Integrated within a comprehensive field known as population biology (Milgroom and Peever 2003), many key concepts are shared between epidemiology and population genetics. For example, sources of inoculum for disease epidemics contribute to population structure of the pathogen. Gene flow, or migration, between pathogen populations is by way of dispersing propagative materials across different geographic scales. The mating system of pathogens influences the genetic diversity of populations, as sexual reproduction and outcrossing result in genetic recombination. Fungicides applied to pathogen populations in the field are one of the main artificial selection pressures for fungicide-resistant individuals conferring higher fitness when exposed to fungicide. Currently, molecular markers are widely used to track genetic variation, population structure, and associated biological characters of pathogens, such as aggressiveness on host cultivars and resistance to fungicides. Therefore, information about pathogen population biology could add to our knowledge of the evolutionary biology of the pathogens that causes disease, and also provide novel insights

into disease management, where effective strategies are expected to limit disease epidemics (McDonald 2015; McDonald and Mundt 2016).

GUMMY STEM BLIGHT OF CUCURBITS

Cucurbits are plants in the family Cucurbitaceae that are characterized by hairy, tendril-bearing climbers with relatively large, fleshy fruits (Robinson and Decker-Walters 1997). Examples include: pumpkins, squash, melons, watermelons, cucumbers, and various types of gourds. Cucurbit species are widely distributed in subtropical and tropical areas with hot and sunny climates; however, they also grow in temperate regions. Cucurbit plants produce juicy, fleshed fruits called pepos, which contain high concentrations of water-soluble vitamins and are commonly served in cuisines from cultures all over the world. The United States is one of the world's top cucurbit producers (FAOSTAT 2014), with Georgia, Florida, South Carolina and North Carolina producing more than half of the commercial cucurbit crop in the U.S., contributing approximately $400 million annually to the economy (NASS 2015). High cucurbit production in the southeastern U.S. is an outcome of the fertile, sandy soils in the subtropical region of the Atlantic coastal plain, with mostly flat terrain and a sufficient supply of irrigation water from rivers and creeks.

The yield of cucurbits can be significantly reduced by diseases such as gummy stem blight (GSB), which has been the most devastating foliar disease in the southeastern U.S., in both fields and greenhouses, since the 1970s (Keinath et al., 1995; Schenck, 1968; St. Amand and Wehner, 1995). Pathogens of GSB are necrotrophic fungi in the phylum Ascomycota with a wide host range among cucurbits (Chiu and Walker, 1949b; Keinath, 2011). The causal agent of GSB used to be known as *Didymella bryoniae* (Fuckel) Rehm

(Corlett, 1981), and its anamorph *Stagonosporopsis cucurbitacearum* (Aveskamp et al. 2010). Recent study based on multilocus sequencing determined that GSB is caused by three morphologically similar, but genetically distinct *Stagonospropsis* species (Stewart et al. 2015). The type strain of *D. bryoniae* belongs to *S. cucurbitacearum*; *S. caricae* is a pathogen previously known only to cause papaya fruit rot; and *S. citrulli* is a newly described species.

On cucurbit plants, GSB fungi can affect any vegetative structures above the soil at any growth stage after seed germination (Wiant 1945). Water-soaked lesions develop on leaf petioles and main stems of transplant seedlings in greenhouses (Koike 1997). The cankers on cucurbit stems are always in the cortical tissues, making the stems wilt, girdle, and eventually crack causing the plant to exude an amber-colored gummy substance. The gummy stem exudate is where the name of the disease arose (Agrios 2005). The initial symptoms on cucurbit leaves are chlorosis at the margins that gradually develop into dark brown, circular necrotic spots. Black rot of the fruit rind is typically irregular shaped and yellowish, and more frequently occurs in pumpkins and squash (Wiant 1945). In 1891, these were the first identified symptoms of infection by GSB in the U.S. (Chiu and Walker 1949a; 1949b). Favorable conditions for GSB, such as high humidity, may cause seedling damping-off in the greenhouse and epidemics on cucurbit crops in the field (Schenck 1968). GSB not only decreases the yield of cucurbit crops, but also reduces the market quality of the cucurbit fruits (Keinath 2000).

GSB fungi are distributed globally (Jiang et al. 2015; Lee et al. 1984; van Steekelenburg 1983), but the disease is most common in tropical and subtropical regions of the world, including the southeastern U.S. due to the biology of the pathogen (Sitterly and

Keinath 1996). Growth of GSB fungi occur only within a temperature range of 7 to 35°C, with the most rapid growth at 27°C (Wiant 1945) and optimal spore germination at 28°C (Chiu and Walker 1949b). With artificial point-source inoculation of GSB fungi into commercial rain-fed watermelon fields in Brazil, the temporal disease development was described by a Gompertz model and spatial autocorrelation showed an aggregated pattern of decreasing disease with increasing distance from the inoculum source (Café-Filho et al. 2010). These studies provide some understanding of dispersal (Gusmini et al. 2005); however, the source of inoculum of GSB epidemics, especially the major sources of inoculum in the field, is not well understood. GSB fungi may be introduced into fields on seed or infected transplant seedlings. On cucurbit seed samples from East Asia, Southern Africa and South Europe, GSB fungi was isolated not only on the surface of seed coat surfaces, but also from the inner part, including perisperm and cotyledon tissues (Lee et al. 1984). In a California greenhouse, GSB fungi isolated from symptomatic tissues of watermelon transplant seedlings without any fruiting bodies, provided indirect evidence of GSB infection originating from seedborne inoculum (Koike 1997). One possible explanation of the route by which GSB fungi entered into the cucurbit seeds was through maturing fruits with black rot symptoms (Lee et al. 1984). On greenhouse cucumbers in southern England, there was conflicting evidence with very few observed GSB fungi infections during early seed germination and seedling stages, even after artificial inoculation of the pathogen onto the seeds (Brown and Preece 1968). Therefore, seedborne inoculum has not been verified as the only source of epidemics. Local survival of GSB fungi on cucurbit plant debris in the soil may be another possible source of inoculum for field epidemics. In an early over-winter trial in Wisconsin, GSB fungi isolated from cucurbit stem tissues were

inoculated with agar plugs and left on the soil surface for an entire winter (Chiu and Walker 1949b). In another test in the Netherlands, the ascospores and conidia produced on infested cucumber stem pieces developing from the previous season rarely survived the harsh winter weather, no matter if they were stored in a greenhouse or outdoors (van Steekelenburg 1983). From the two previous described cases, the dormant mycelia were thought to be the survival materials because both the pseudothecia and pycnidia were empty in early spring. It was also observed that one-year intervals without a cucurbit crop were effective for GSB eradication in watermelon fields (Keinath 1996). A later study reported a significantly shorter survival time of GSB fungi on muskmelon crown tissues buried in the soil compared to fungi in debris placed on the soil surface (Keinath 2008). These results verified that inoculum could also originate from plant debris, underlining the importance of improved sanitation by way of crop debris incorporation into the soil after harvesting.

LIFE CYCLE AND MATING OF GUMMY STEM BLIGHT FUNGI

Filamentous fungi in the phylum Ascomycota, including GSB fungi, exist as haploids for most of their life cycle. For *Stagonosporopsis* species, sexual reproduction begins when two compatible haploid individuals fuse with one another. Fusion, or plasmogamy, results in dikaryotic (N+N) hyphae, which develop into the sexual fruiting body, called a pseudothecium. Alternatively, because it is homothallic, a single individual can produce pseudothecia. Karyogamy occurs within the pseudothecia, making a diploid zygote, which is quickly followed by meiosis, resulting in four haploid nuclei. Subsequently, a mitotic division occurs producing eight ascospores in each ascus. The characterized centrum

development of pseudothecium was also observed in GSB fungi (Skarshaug 1981). The mature pseudothecium of GSB fungi is dark in color and globular in shape, and embedded within the substrate, which could be either host plant tissue or artificial media (Punithalingam and Holliday 1972). The ascospores are forcibly discharged from each ascus and disseminated out of the pseudothecium through the ostiole (Chiu and Walker 1949a). When conditions are suitable, the ascospores germinate to produce hyphae. Asexual spores, or conidia, are clonally produced mitotically in structure called pycnidia. The conidia also germinate to produce hyphae. Abundant pycnidia produced on infected plant tissue contribute to GSB epidemics (Schenck 1968).

GSB fungi are considered as a homothallic, or self-compatible, fungus (Chiu and Walkers 1949a; Keinath 2014b), but the structure of the mating-type locus that controls and regulates the mating process in these species is still unknown. Additionally, the mating system of the three *Stagonosporopsis* species may differ. It is not clear which of the three species was used for prior reproductive studies of GSB fungi since they were all considered as one species prior to 2015. In general, the genes controlling fungal mating ability in Ascomycota are collectively called mating-type genes. The proteins encoded by mating-type genes are transcription factors responsible for regulating cell identity and controlling the mating ability of the fungus. There is a single mating-type locus in the filamentous fungi of Ascomycota called *MAT1*. *MAT1* has two possible forms: *MAT1-1* and *MAT1-2*. These are defined as idiomorphs rather than alleles, because of their dissimilar sequences and having descended from unrelated ancestors (Butler 2006). Individuals can mate if they are different idiomorphs. Homothallic fungi studied to date have all the necessary mating-type genes of both idiomorphs within their genomes. For this reason they can complete sexual

reproduction with themselves and potentially outcross with different individuals. In homothallic species, the organization of mating-type genes within the genome is diverse. The genes can be adjacent or distantly located from one another. The direction of open reading frames (ORFs) varies among species (Debuchy and Paret 2006).

The architecture of the *MAT1* locus in the genome of *Stagonosporopsis* species could provide insight into the mating system within this species complex. If a mixed mating system consisting of both selfing and outcrossing occurs in *Stagonosporopsis* species, it could impact the genetic diversity within and among populations since frequent outcrossing would contribute to increased levels of genetic diversity due to recombination. A diverse population can evolve resistance to fungicides much faster than a clonal or asexual population (Peever and Milgroom 1994). In another homothallic species *Aspergillus nidulans*, outcrossing occurs preferentially between genetically different individuals (Billiard et al. 2012). Understanding the mating system and underlying genetic structure of mating-type genes in GSB fungi is crucial for better understanding disease epidemics. Previous studies on the genetic diversity of GSB fungi have been conducted on a broad geographic scale. With random amplified polymorphic DNA (RAPD) analyses, sequences of internal transcribed spacer (ITS) regions of rRNA genes, GSB fungi isolates could be distinguished from closely related and morphologically similar *Phoma* species that frequently cause similar symptoms on cucurbit crops (Somai et al. 2002b). RAPD markers show that GSB fungi isolates from the U.S. have high genetic diversity. They were categorized into two distinct RAPD groups (RGs) (Somai et al. 2002a). Using the same RAPD strategies, isolates of GSB fungi from Brazil also revealed a high level of genetic diversity, but fell into one of the RGs (dos Santos et al. 2009). A later study of a worldwide

collection of GSB fungi isolates using amplified fragment length polymorphism (AFLP) analysis showed high levels of diversity and two distinct genetic groups (Kothera et al. 2003).

GUMMY STEM BLIGHT MANAGEMENT AND FUNGICIDE RESISTANCE

The management of GSB requires both cultural and chemical control practices, as no available cultivars of watermelon, cucumber or cantaloupe have shown resistance to GSB fungi, despite reported differences in susceptibility among commonly cultivated cucurbit species in the U.S. (Keinath 2014a; McGrath et al. 1993). It is recommended that only certified pathogen-free commercial seeds and greenhouse-produced transplant seedlings be used (Sitterly and Keinath 1996). In order to limit local survival of GSB fungi, it is suggested that growers practice a minimum two-year rotation with non-cucurbit crops (Keinath 1996; 2000), such as cotton or peanut, which are recommended in southern Georgia. Other cultural practices include deep plowing to prevent survival of GSB fungi on plant debris (Sitterly and Keinath 1996), avoidance of overhead irrigation to reduce periods of leaf wetness (Thomas et al. 2012b), and soil fumigation with cabbage residue amendments (Keinath 1996). Application of fungicides is by far the most effective option for GSB management (Avenot et al. 2012; Keinath 2000; 2012; Thomas et al. 2012b). The protectant fungicide chlorothalonil is one option, but should only be applied early in the growing season, as it can cause phytotoxicity on mature cucurbit rinds (Keinath, 2000). Six major classes of systemic fungicides with different modes of action (MOAs) have been used for management of GSB: 1) methyl benzimidazole carbamates (MBC) fungicides, 2) quinone-outside inhibitor (QoI) fungicides, 3) succinate dehydrogenase inhibitor (SDHI)

fungicides, 4) demethylation inhibitor (DMI) fungicides, 5) anilino-pyrimidines (AP) fungicides, and 6) phenylpyrroles (PP) fungicides. The efficacy of AP fungicide cyprodinil and PP fungicide fludioxonil remain effective (Keinath 2015); however, resistance to MBC, QoI, and SDHI fungicides evolved within a few years after introduction due to single-site mutations in GSB fungi.

In the mid-1990s, thiophanate-methyl, a fungicide in the MBC class was deemed less effective on GSB due to development of resistance in GSB fungi populations. The resistance of GSB fungi to MBC fungicide was later confirmed, both in South Carolina and New York (Keinath and Zitter, 1998). The MBC fungicides inhibit mitosis and cell division. Microtubules, one of the essential cytoskeleton molecules for cellular function, are composed of two elements: α- and β-tubulin. MBC fungicides target the β1-tubulin assembly, specifically, during mitosis (Zhou et al. 2016). This significantly blocks cell division of fungal hyphae. Single-site mutations in the β-tubulin gene that are responsible for resistant to MBC fungicides were reported in *Cercospora beticola* (Trkulja et al. 2013), *Monilinia fructicola* (Fan et al. 2014), and *Venturia inaequalis* (Koenraadt et al. 1992). The resistance in GSB fungi resulted in sharply reduced use of MBC fungicides. Similarly, the QoI fungicide azoxystrobin initially provided excellent control of GSB, but by the end of the 1990s, cases of ineffectiveness appeared in Georgia (Stevenson et al. 2004) and South Carolina (Keinath 2009). The high efficacy of boscalid, an SDHI fungicide, to GSB lasted until 2007 (Seebold and Langston 2003), when widespread resistance developed in Georgia (Thomas et al. 2012a) and South Carolina (Keinath 2012). The target of QoI and SDHI fungicides is the electron transport chain on inner membranes, or cristae, of mitochondria. The proton gradients within inter-membrane space generated by electron

transportation through four molecular complexes serve oxidative purposes, which is the major source of ATP synthesis by all eukaryotes. The action sites of azoxystrobin and boscalid are cytochrome b (Cytb) of Complex III and succinate dehydrogenase (SDH) of Complex II. The later one is also part of the citric acid cycle. Based on the information from the analogous genes in *Magnaporthe grisea* (Avila-Adame and Köller 2003) and *Alternaria alternata* (Karaoglanidis et al. 2011), the substitution of alanine for glycine at amino acid position 143 (G143A) was also observed on Cytb of GSB fungi (Finger 2014). Similar to *A. alternata*, the histidine residue on the subunit B of SDH, are replaced by tyrosine (H277Y) or arginine (H277R) by mutation in the resistance strains of GSB fungi (Avenot et al. 2008; 2012). The inhibition of the electron transport chain halts the production of ATP and eventually shuts down the energy source of fungal hyphae metabolism and growth (Bartlett et al. 2002).

GSB fungi remained sensitive to triazole fungicides in both Georgia (Thomas et al. 2012b) and South Carolina (Keinath and Hansen 2013) since the first one was labeled for cucurbits in 2008. Triazoles belong to the fungicide class of sterol biosynthesis inhibitors (SBI), which are used against a broad spectrum of true fungi. Fungal sterol, or ergosterol, plays important structural and signaling roles in fungal cell membranes. In a fungal cell, 20 steps are required to synthesize ergosterol from the substrate acetyl-CoA. Triazoles target cytochrome P450 lanosterol 14α-demethylase (Kuck et al. 2002), which is also known as ERG11 or Cyp51. Triazole fungicides function as demethylation inhibitors (DMIs) that block the ergosterol synthesis in GSB fungi. According to the recent extension recommendations in Georgia (Langston 2014), South Carolina (Keinath and Miler 2014), and Florida (Dufault and Paret 2014), GSB management should be conducted primarily

with triazole fungicides. The possible development of resistance to triazoles by GSB fungi is a serious concern for researchers and growers of cucurbits in the southeastern U.S. Isolates resistant to DMI fungicide have been observed in other fungal species in both medical and agricultural settings (Sierotzki and Gisi 2003). In addition to the increase of unspecific efflux of fungicide via ATP binding cassette (ABC) transporters, the mode of action of fungicides against plant-pathogenic fungi were classified into two major categories (Ma and Michailides 2005). The first category is qualitative resistance to triazoles due to single-site mutation in the *Cyp51* gene. It varies from one candidate site (Y136F) in *Blumeria graminis* f.sp. *tritici* (Yan et al. 2009), to multiple possible mutation sites in *Aspergillus fumigatus* (Gisi 2014) and *Phakopsora pachyrhizi* (Schmitz et al. 2014). The second category is quantitative triazole-resistance as a result of overexpression of *Cyp51*. In *Penicillium digitatum*, tandem repeats in promoter regions were observed (Hamamoto et al. 2000). Genetic mechanisms of induced overexpression by exposure of DMI fungicides in *Sclerotinia homoeocarpa* (Ma and Tredway 2013), *Cercospora beticola* (Bolton et al. 2012), and *Mycosphaerella graminicola* (Selim et al. 2014) was not clear. Some triazole-resistant isolates of GSB fungi have been recently detected in Georgia (Li et al. 2016), but the molecular basis of triazole resistance in GSB fungi needs to be investigated.

With the development of fungicide resistance within populations of GSB fungi, the profiles of resistance to fungicides in different classes has not been studied. It is not known if GSB fungi have lost resistance to fungicides that are no longer used, since fungicide resistance may come with a fitness cost (Pringle and Taylor, 2002). Under the selection pressure of fungicides, resistant isolates have a higher fitness and their frequency increases in the population. On the other hand, possible fitness costs of fungicide resistance would

select against resistant isolates when exposure to that fungicides ceases, resulting in fungicide sensitivity within the population after the abandonment of that fungicide or other fungicides with same mode of action. It is not clear if this occurs in GSB fungi populations in the southeastern U.S. For GSB fungi isolates that are resistant to multiple fungicides, it is not known if the acquirement of multiple fungicide resistances has been randomly or stepwise accumulated (Li et al. 2014).

RESEARCH OBJECTIVES

The purpose of this research project is to understand the spatial genetic structure, profile of resistance to multiple fungicides, and mating system of GSB fungi. Additional insight will be provided from investigation of the spatial genetic structure of this pathogen within and among fields in the southeastern U.S., and the epidemiology of the disease at regional and fine-scale levels. Resistance profiles of resistance to multiple fungicides would provide more information on the evolutionary biology of the pathogen, and facilitate improvement of disease management strategies. Knowledge of mating-type genes of GSB fungi will broaden our insight on the mating system of a homothallic fungus, and the divergence of closely related fungal species. In order to accumulate knowledge of the population biology of pathogenic fungus, and to help provide effective GSB management strategies, objectives of this research project are to: 1) explore the spatial genetic structure and population dynamics of GSB fungi within and among watermelon fields; 2) investigate the fungicide resistance profiles of unique and clonal genotypes of GSB fungi; 3) detect differences in sensitivity to a triazole fungicide among *Stagonosporopsis* species; and 4) identify and measure evolution of mating-type genes in three *Stagonosporopsis* species.

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CHAPTER 2

SPATIAL GENETIC STRUCTURE AND POPULATION DYNAMICS OF GUMMY STEM BLIGHT FUNGI WITHIN AND AMONG WATERMELON FIELDS[1]

[1] Li, H. and M. T. Brewer. 2016. *Phytopathology*. 106: 562-603.
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ABSTRACT

The epidemiology of gummy stem blight 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 U.S. 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 U.S.; however, *S. citrulli* remained the dominant species, representing 96.4% of the isolates. Principal coordinates analysis, discriminant analysis of principal 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 where 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.

INTRODUCTION

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 (Maynard

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 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; Keinath 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 the major source(s) 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 subdivion. If local inoculum is the sole source of field epidemics we expect to see isolation-by-distance, where increasing genetic differentiation would occur with increasing geographic 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 U.S., 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 2001). It is a major threat to cucurbit production in the southeastern U.S., 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 U.S. economy (NASS 2014). Within the southeastern U.S., 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 a homothallic fungi based on different molecular markers, including AFLP (Kothera et al., 2003), RAPD (Somai et al. 2002), and ITS 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 at a regional scale across the southeastern U.S. 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 U.S., (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 U.S.

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 U.S. (Fig. 2.1): 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 larger, unless otherwise indicated. Twenty-two single-conidia isolates recovered from a field in Colleton County, South Carolina in 2013 were kindly provided by Anthony 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 Suwanee 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, ten transects along watermelon rows were placed at approximately 5.5 m intervals (Fig. 2.2). A single symptomatic leaf was collected every 5.5 m for a total of ten leaves along each of the ten transects. The sampling for GAC14 was carried out within a smaller area with ten transects along watermelon rows placed at intervals of approximately 2 m (Fig. 2.2). A symptomatic leaf was collected every 2 m along each transect for a total of ten leaves along each of the ten transects.

A 1-cm^2 lesion from each symptomatic leaf was surface-sterilized in 0.6% NaClO for one min, followed by two rinses with sterile distilled H_2O. Three 0.25-cm^2 segments from the lesion margin were excised and placed onto quarter-strength potato dextrose agar (qPDA) in a 100-mm Petri dish. A 4-mm^2 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 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 d on qPDA covered with sterile cellophane using a modified method (Lee et al. 1988). Approximately 100 mg mycelium was incubated in 1 ml lysis buffer (50 mM EDTA pH 8, 100 mM Tris pH 8, 3.5% SDS, 500 µg/ml proteinase K, 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 ammonium acetate (7.5 M) was added to the supernatant. The mixture was vortexed 10 s, placed on ice for 15 min, and then centrifuged at 14,000 *g* for 3 min. The supernatant was precipitated with an equal volume of isopropanol and centrifuged at 14,000 *g* for 5 min. The pellet was rinsed twice with 70% ethanol, air-dried, and resuspended in 100 µl sterile H_2O. The DNA quality was measured using a Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA). The DNA was stored at -20°C.

Multilocus genotyping and identification of *Stagonosporopsis* species

All isolates were genotyped with 20 previously developed microsatellites markers in three multiplex reactions (*Db01–Db20*; Brewer et al. 2015). Each of the reactions was carried out in a 10 µl volume containing: 1 µl genomic DNA (3–20 ng), 5 µl 2× Type-it Multiplex PCR Master Mix (Qiagen, Germantown, MD), and 1 µl 10× 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 PCR products was added to 9.9 µl Hi-Di formamide (Life Technology, Grand Island, NY) and 0.1 µl 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 Georgia

Genomics Facility (GGF, 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

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 data set 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).

Linkage disequilibrium (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 1000, with 100 batches each iterated 1000 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; Maynard 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; Peakall and Smouse 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 multilocus genotypes 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 U.S. 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 (BIC) increases or decreases by a negligible amount.

Analyses of spatial genetic structure

To investigate an isolation-by-distance 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}).

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) where 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 linkage disequilibrium

From the 800 leaves with lesions symptomatic of GSB, 506 *Stagonosporopsis* isolates were recovered. Isolation frequency was very low in some populations (Table 2.1), 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, GA sampled in 2013 (4% of the GAT13 population) and 15 were from the Cook County, GA field sampled in 2014 (20% of the population GAC14).

For population genetic analyses of *S. citrulli* in the southeastern U.S., four of the microsatellite loci were discarded because they were monomorphic (*Db02, Db03*, *and Db04*), or did amplify for the majority of isolates (*Db01*). Thus, analyses were based on multilocus genotypes 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 2.2) 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 2 alleles. GAT12 and FL12 both had more private alleles, which are alleles detected in one population, than the other populations (Table 2.1) with richness of 0.18 and 0.46, respectively, compared to a range of 0.02 to 0.06 for the other populations.

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 2.2). There were 66 repeated MLG (g_2) across the entire southeastern

U.S. 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 (labeled as *ns* in Table 2.2), 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 (labeled as $y_1, y_2, \ldots, y_{13}$, in Table 2.2) and 15 MLG were shared among three or more populations (labeled as $x_1, x_2, \ldots, x_{15}$, in Table 2.2). 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 9 members across 5, 7, 6, 5, and 5 populations, respectively (Table 2.2).

All *S. citrulli* populations in the southeastern U.S. were in multilocus linkage disequilibrium based on the index of association (I_A) and the modified index of association ($\overline{r_d}$) (Table 2.3). Even after clone correction, all *S. citrulli* populations were in multilocus linkage disequilibrium based on the modified index of association ($\overline{r_d}$). 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

2.4). 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 (Φ_{PT} = 0.073, P < 0.001) and the clone-corrected dataset (Φ_{PT} = 0.015, P < 0.001), although there was 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. GAC 14, and FL12 v. FL13).

Principal coordinates analysis (PCoA) showed an overlap of genotypes in both 2012 and 2013 (Fig. 2.3). In 2012 (Fig. 2.3A), however, there was clustering of isolates within populations along the principal coordinates. This was 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. 2.3B), 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).

Discriminant analysis of principal components (DAPC) supported K = 10 optimal clusters. Clusters 1, 3, 5, 7, and 8 overlapped while the remaining five clusters were isolated (Fig. 2.4A). 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 2.2). Cluster 2 contained the other dominant clone with 19 members from FL13 and 39 total members among 7 of the populations. These two clusters that contained the dominant clones from FL13 are represented by orange (cluster 2) and magenta (cluster 10; Fig. 2.4B). 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. 2.5), 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. 2.6). For GAT13 (Fig. 2.6A), isolates within 6-meter clusters were more genetically similar than expected at random. For GAC13 (Fig. 2.6B), isolates

were not aggregated by genetic similarity. For GAC14 (Fig. 2.6C), isolates within 2-meter 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 U.S. (Stewart et al. 2015). Our results show that *S. citrulli* continues to be the dominant species in the southeastern U.S. representing 96.4% of the isolates identified in this study. Interestingly, we first identified *S. caricae* in the southeastern U.S. 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 U.S., and if it this increase is *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 U.S. 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 U.S. 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 U.S. 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 U.S. (Avenot et al. 2012; Keinath 2012; Thomas et al. 2012). An 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 U.S. we found clones that occurred at high frequency and were widespread at both regional and temporal scales. In seven out of the nine *S. citrulli* populations clones represented over 40% of the isolates (Table 2.1). Five populations had at least one high frequency clone with more than ten members (Table 2.2). 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, where 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 U.S. 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 U.S., *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 local, high frequency clones dominated the field population further supporting 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 and/or to structure in the source populations introduced on seed or seedlings. Dispersal among fields could be via human-mediated movement of infected seed 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 isolation-by-distance (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-meter and 2-meter 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 U.S.

In conclusion, *S. citrulli* continues to be the dominant species causing GSB in the southeastern U.S., 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 U.S., 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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Table 2.1. Estimates of genetic diversity for *Stagonosporopsis citrulli* populations from the southeastern U.S. sampled in 2012, 2013 and 2014

Population[a]	Number of Isolates (N)	Number of Multilocus Genotypes (g)	Repeated Fraction[b]	$\hat{G}$[c]	Allelic[d] Richness	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 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.
[b]Repeated fraction = $1 - (g/N)$.
[c]Genotypic diversity ($\hat{G}$), which is the probability that two randomly selected individuals have unique multilocus genotypes, 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).

Table 2.2. Clonal composition of *Stagonosporopsis citrulli* populations from the southeastern U.S. sampled in 2012, 2013 and 2014

Population[a]	*N*	*g*[b]	g_2[c]	Number of isolates in each repeated multilocus genotype[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, 3^{ns}, 2^{y3}, 2^{y4}, 2^{y5}, 2^{y7}, 2^{y8}, 2^{y12}, 2^{y13}, 2, 2, 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 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.

[b]Number of multilocus genotypes in each population.

[c]Number of multilocus genotypes represented by more than one member.

[d]Superscripts of 'x' or 'y' indicate multilocus genotypes (MLGs) 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 non-significant 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.

Table 2.3. Estimates of multilocus linkage disequilibrium for *Stagonosporpsis citrulli* populations in the southeastern U.S.

Population [a]	*N*	I_A[b]	$\overline{r_d}$[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 MLG unless the repeated MLG showed a high and non-significant 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 1984, Maynard 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 1000 permutations.

[c]Modified index of association, 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 1000 permutations of the data.

[d]Clone corrected for the total population.

Table 2.4. Geographic and temporal population structure measured by Φ_{PT} between *Stagonosporopsis citrulli* populations in the southeastern U.S. sampled in 2012, 2013, and 2014.

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

[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 dataset comparisons (in parentheses) include a single representative of each MLG unless the repeated MLG showed a high and non-significant 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 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 1000 permutations of the data.

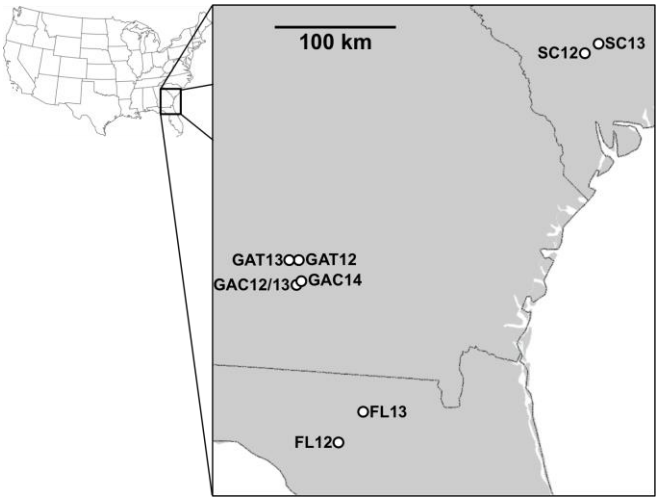

Fig. 2.1 Map of watermelon fields in the southeastern U.S. sampled for gummy stem blight fungi. The nine populations include: Tift County, GA (GAT12, GAT13); Cook County, GA (GAC12, GAC13, GAC14); Suwannee County, FL (FL12, FL13); and Colleton County, SC (SC12, SC13). The digits in the population name indicate the sampling year.

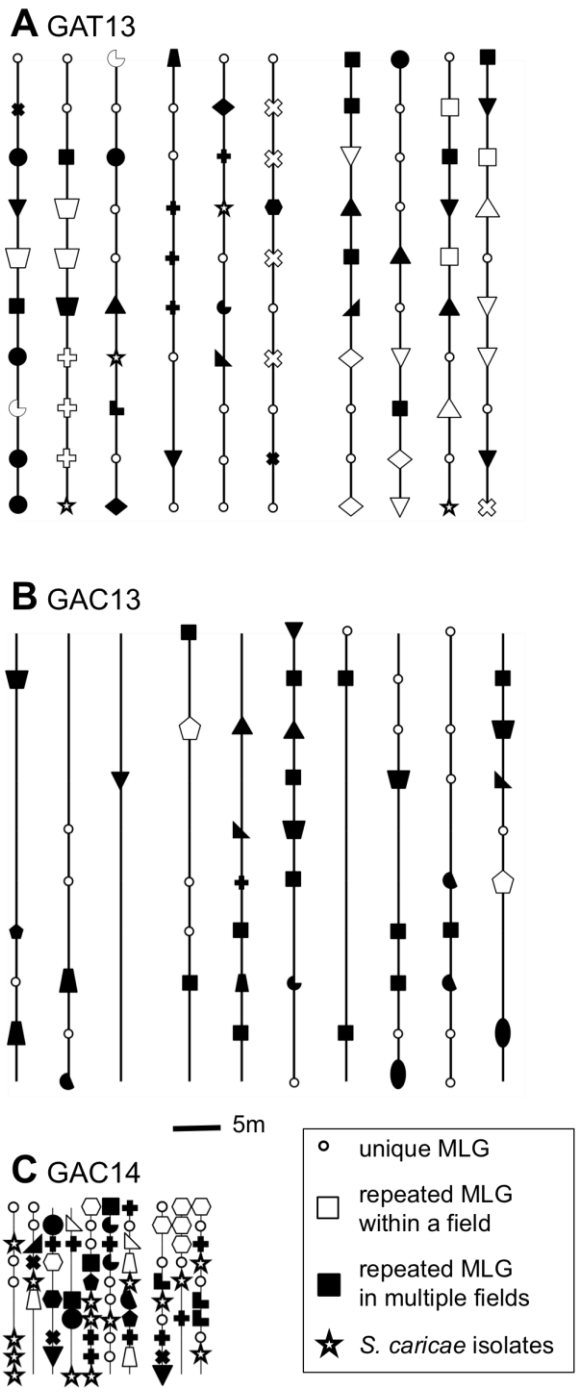
A GAT13
B GAC13
5m
C GAC14
unique MLG
repeated MLG within a field
repeated MLG in multiple fields
S. caricae isolates

Fig. 2.2. Within-field sampling location of *Stagonosporopsis citrulli* and *S. caricae* isolates recovered from symptomatic watermelon leaves in **A**, Tift County, GA sampled in 2013 (GAT13), **B,** Cook County, GA sampled in 2013 (GAC13), and **C**, Cook County, GA sampled in 2014 (GAC14). Vertical lines represent planting rows of watermelon from which leaves were collected. Unique multilocus genotypes (MLG) or repeated MLG that could have arisen through recombination are represented by small, unfilled circles. Repeated MLG that likely arose clonally are represented by symbols as described in the figure. GAT13 and GAC13 fields were sampled at approximately 5.5 m intervals along rows that were 5.5 m apart and GAC14 was sampled at approximately 2 m intervals along rows that were 2 m apart.

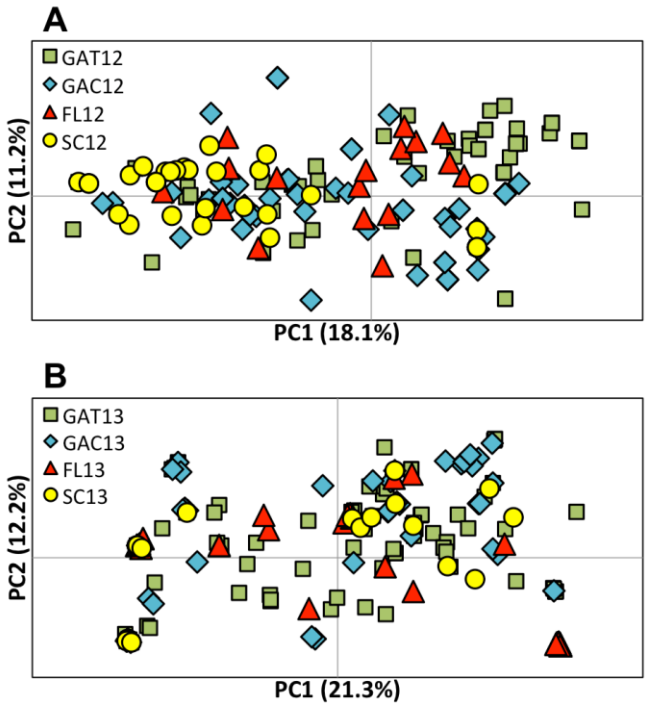

Fig. 2.3. Principal coordinates analysis (PCoA) of *Stagonosporopsis citrulli* isolates collected from watermelon fields in the southeastern U.S. in **A,** 2012 and **B,** 2013. Sampling locations include: Tift County, Georgia (GAT12, GAT13); Cook County, Georgia (GAC12, GAC13); Suwannee County, Florida (FL12, FL13); and Colleton County, South Carolina (SC12, SC13).

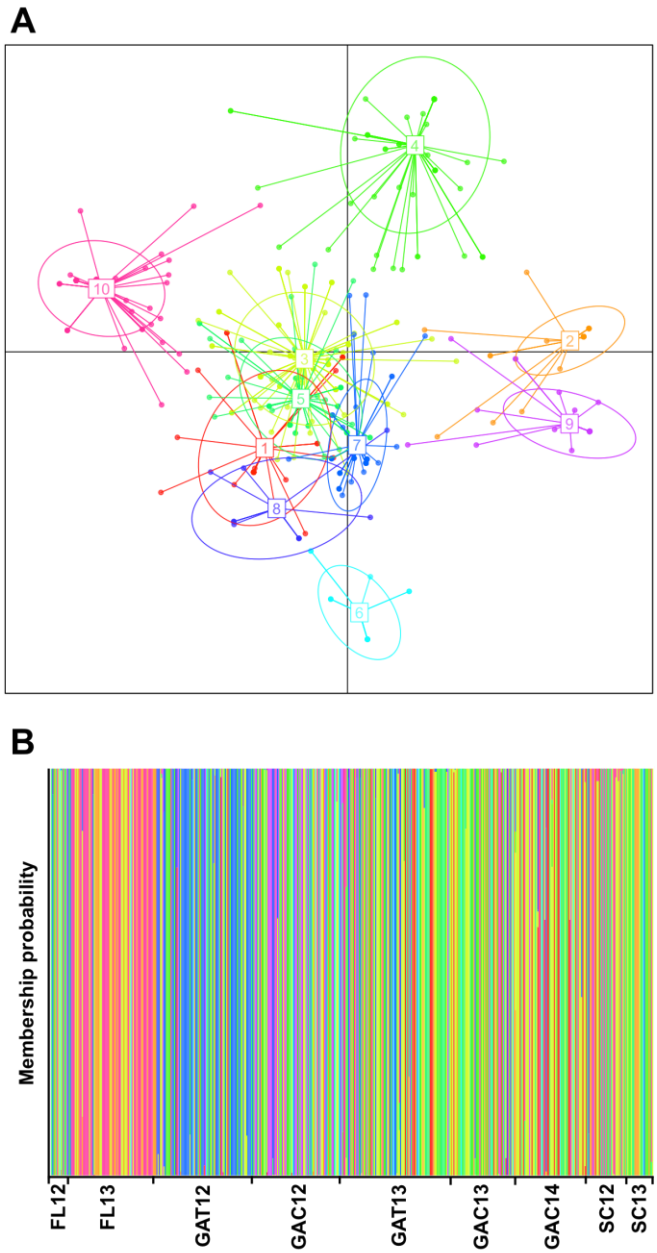

Fig. 2.4. Discriminant analysis of principal components for *Stagonosporopsis citrulli* from the southeastern U.S. with **A**, Scatterplot of the ten assigned clusters based on Bayesian information criterion; and **B**, histogram of assignment probability of 509 *S. citrulli* isolates from nine field populations from the southeastern U.S. into the 10 genetic clusters. The nine populations include: 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.

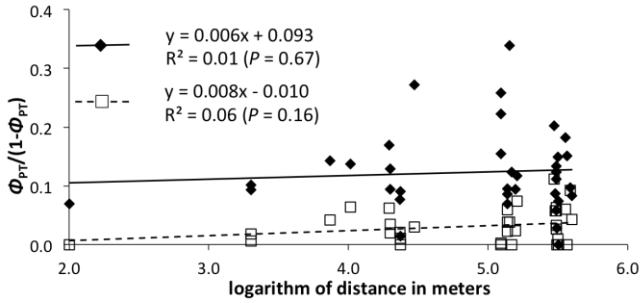

Fig. 2.5. Regression of standardized pairwise genetic distance, $\Phi_{PT}/(1-\Phi_{PT})$, plotted against the logarithm of geographic distance in meters between nine *Stagonosporopsis citrulli* populations from the southeastern U.S. The nine populations include: Tift County, GA (GAT12, GAT13); Cook County, GA (GAC12, GAC13, GAC14); Suwannee County, FL (FL12, FL13); and Colleton County, SC (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.

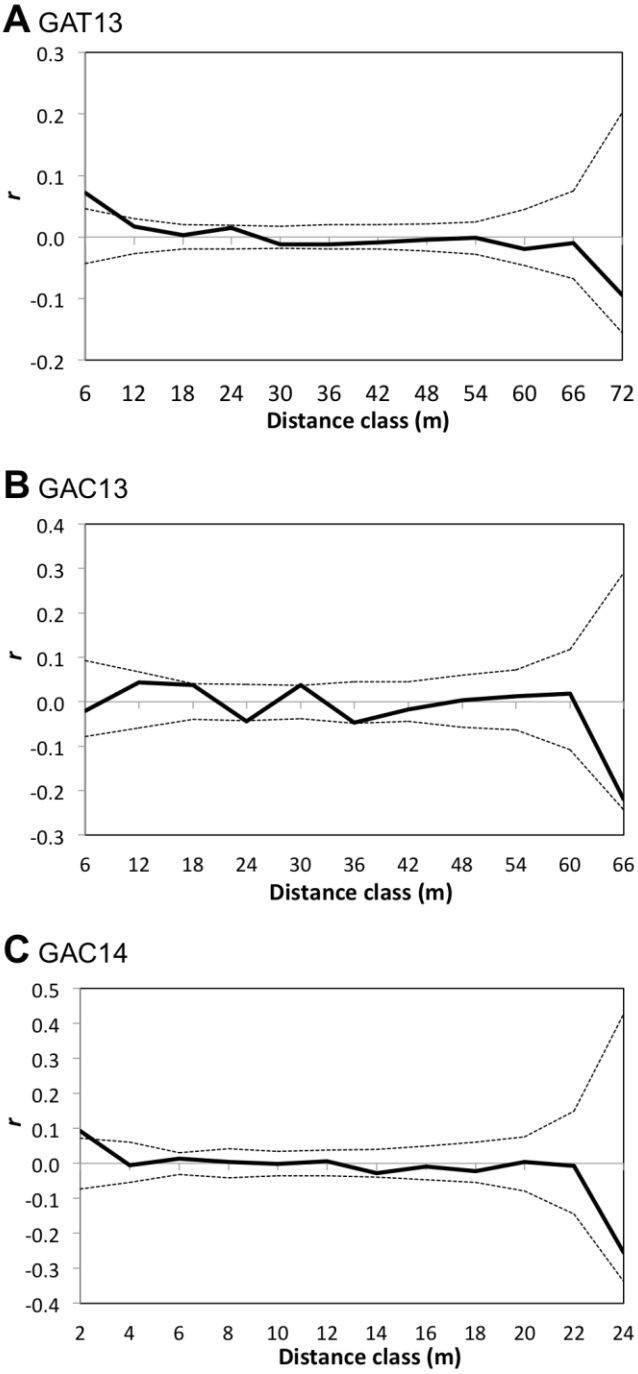

Fig. 2.6. Correlograms showing spatial autocorrelation of *Stagonosporopsis citrulli* populations in **A**, Tift County, GA sampled in 2013 (GAT13); **B**, Cook County, GA sampled in 2013 (GAC13); and **C**, Cook County, GA sampled in 2014 (GAC14). The thick solid line shows genetic similarity based 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.

CHAPTER 3

FUNGICIDE RESISTANCE PROFILES OF UNIQUE AND CLONAL GENOTYPES OF GUMMY STEM BLIGHT FUNGI[1]

[1] Li, H., T. A. Nuckols., D. Harris., K. L. Stevenson., and M. T. Brewer. To be submitted to *Pest Management Science*

ABSTRACT

Gummy stem blight (GSB) is a devastating disease of cucurbits that has been effectively managed with fungicide applications. However, GSB fungi have rapidly evolved resistance to multiple classes of fungicides. To better understand the evolution and persistence of fungicide resistance in field populations, we studied resistance profiles associated with unique and clonal genotypes of GSB fungi. We examined 113 *Stagonosporopsis citrulli* isolates from watermelon fields in the southeastern U.S. previously shown to be polymorphic at 16 microsatellite loci, as well as 19 *S. caricae* isolates. Each isolate was genotyped with the marker (*cytb)* for azoxystrobin (QoI) resistance, and phenotyped for sensitivity to tebuconazole (DMI), boscalid (SDHI), and fluopyram (SDHI). *Cyp51* and *SdhB* genes of selected isolates with varying sensitivity to DMI and SDHI fungicides, respectively, were sequenced. All *S. caricae* isolates were resistant to tebuconazole and azoxystrobin, and sensitive to boscalid and fluopyram. All *S. citrulli* isolates were sensitive to tebuconazole, and sensitive to fluopyram with one exception. And all but two isolates were resistant to azoxystrobin. Phenotypic differences in response to boscalid were detected among *S. citrulli* isolates, but the phenotype was not associated with multilocus genotypes (MLG), suggesting independent evolutionary events to this fungicide or frequent genetic recombination within population. This study provides critical information for effectively managing both species of GSB fungi present in the southeastern U.S., while providing novel insights into the evolution of fungicide resistance in plant-pathogenic fungi.

INTRODUCTION

Gummy stem blight (GSB) is a devastating disease of cucurbits in the southeastern United States. GSB is caused by three closely related but genetically distinct species: *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryonia*e), *S. citrulli*, and *S. caricae*. Application of fungicides has by far been the most effective option for management of GSB (Avenot et al. 2012; Keinath 2012; Thomas et al. 2012b). Three classes of systemic fungicides with different modes of action (MOAs) have been widely used for managing GSB: quinone-outside inhibitor (QoI) fungicides, succinate dehydrogenase inhibitor (SDHI) fungicides, and demethylation inhibitor (DMI) fungicides. The targets of both QoI and SDHI fungicides are the electron transport chains on the inner membranes, or cristae, of mitochondria. The action sites of QoI and SDHI fungicides are cytochrome b (Cytb) of Complex III and succinate dehydrogenase (SDH) of Complex II, respectively. The inhibition of the electron transport chain halts the production of ATP and eventually shuts down the energy source of fungal hyphae metabolism and growth (Bartlett et al. 2002). The QoI fungicide azoxystrobin provided excellent control of GSB until the late 1990s when resistance appeared in Georgia (Stevenson et al. 2004) and South Carolina (Keinath 2009). Similar to orthologous genes in fungicide-resistant *Magnaporthe grisea* (Avila-Adame and Köller 2003) and *Alternaria alternata* (Karaoglanidis et al. 2011), the substitution of alanine for glycine at amino acid position 143 (G143A) was observed in Cytb of QoI-resistant *S. citrulli* (Finger 2014). GSB fungi were sensitive to boscalid, an SDHI fungicide, until 2007 (Seebold and Langston 2003), when widespread resistance developed in Georgia (Thomas et al. 2012a) and South Carolina (Keinath 2012). Similar to boscalid-

resistant *A. alternata*, the histidine residue on the subunit B of SDH, is replaced by tyrosine (H277Y) or arginine (H277R) in resistant strains of *S. citrulli* (Avenot et al. 2008; 2012).

Unlike the quantitative, rapid evolution of resistance to the single-site mode of action QoI and SDHI fungicides, resistance to DMI fungicides is quantitative and more complex. DMI fungicides belong to the sterol biosynthesis inhibitor (SBI) class of fungicides, which are used against a broad spectrum of true fungi. Fungal sterol, or ergosterol, plays important structural and signaling roles in fungal cell membranes. DMI fungicides block ergosterol biosynthesis functioning as demethylation inhibitors that target cytochrome P450 lanosterol 14α-demethylase (Kuck et al. 2002), known as ERG11 or Cyp51. GSB fungi have remained sensitive to DMI fungicides in both Georgia (Thomas et al. 2012b) and South Carolina (Keinath and Hansen 2013) since 2008, when they were first labeled for use on cucurbits.

Early work on the diversity and species composition of GSB fungi in the southeastern U.S. revealed that *S. citrulli* continued to be the dominant species, while *S. caricae* emerged in Georgia only in the last few years (Brewer et al. 2015). Moreover, populations of *S. citrulli* showed high genetic diversity with prevalent and widespread clones (Li and Brewer 2016; Chapter 2). Some clonal genotypes may be widespread due to fitness associated with resistance to commonly used fungicides. Additionally, GSB fungi, particularly the different *Stagonosporopsis* species, may differ in their underlying resistance to different classes of fungicides. Identification of differences in response to fungicides between the two species found in the southeastern U.S. is crucial for managing GSB. In addition, we are interested the evolutionary mechanisms for acquiring resistance to multiple fungicides. Therefore, the objectives of this study were to: 1) identify fungicide

resistance profiles of GSB fungi collected from the southeastern U.S to QoI, SDHI, and DMI fungicides, and 2) assess the association of fungicide resistance profiles with genetic similarity of isolates.

MATERIALS AND METHODS

GSB isolates

A total of 132 GSB isolates collected from multiple watermelon fields in the southeastern U.S. that were previously genotyped with microsatellite markers (Li and Brewer 2016; Chapter 2) were selected for fungicide-resistance profiling: 113 *S. citrulli* isolates and 19 *S. caricae* isolates (Table 3.1). The *S. citrulli* isolates were collected in 2013 from fields in three counties in the southeastern U.S.: 40 from Cook County, Georgia (GAC13); 16 from Tift County, Georgia (GAT13); and 20 from Suwannee County, FL (FL13). In 2013, the field in Cook County, Georgia (GAC13) was adjacent to the field (GAC12) where 18 *S. citrulli* isolates were collected in 2012; and 2.0 km from the other field (GAC14) where 19 isolates were collected in 2014. Isolates of *S. citrulli* with both unique genotypes and shared genotypes based on 16 microsatellite loci (Li and Brewer 2016) were included. Among *S. citrulli*, there were 23 isolates shared among 7 repeatedly sampled multilocus genotypes (MLG) within the same field, and 39 isolates among three repeatedly sampled MLG across different fields. These widespread MLG, or clones were denoted as X1, X4, and X5 (Chapter 2), and include 11, 12, and 16 isolates, respectively. In addition to *S. citrulli*, nineteen isolates of *S. caricae* were included. Four isolates were sampled from field GAT13 and 15 isolates were from the field GAC14.

DNA extraction

Using a method modified from Lee et al. (1988), genomic DNA from each of the 132 GSB isolates was extracted from fresh mycelium, incubated over 4 days at 25°C in the dark. Approximately 100 mg of mycelium from each isolate was harvested from quarter-strength PDA covered with sterile cellophane, then mixed with 1 ml mini-prep lysis buffer (50 mM EDTA, pH 8; 100 mM Tris, pH 8; 3.5% SDS; 500 µg/ml proteinase K; and 1% sodium bisulfite). The mixture was vortexed for 1 min, then incubated for 15 min at 65°C. After centrifugation at 14,000 *g* for 5 min, the supernatant was added to 200 µl ammonium acetate (7.5 M), and mixed by vortexing for 10 s. The mixture was then place on ice for 15 min. Following centrifugation at 14,000 *g* for 5 min, the supernatant was precipitated with an equal volume of isopropanol. After another at 14,000 *g* for 5 min, the pellet was rinsed twice with 70% ethanol and air-dried. The DNA pellet was dissolved in 100 µl sterilized deionized H_2O and stored at -20°C.

Genotyping with *CytB* for QoI resistance

Genotypes of all 132 GSB isolates that were resistant or sensitive to QoI fungicide were characterized by allele-specific PCR targeting a partial fragment of *CytB* where the G143A mutational site responsible for QoI resistance was located (Finger et al. 2014). Reactions containing each of the reverse primers in separate reactions, DB-RR or DB-RS (Table 3.2), were used to amplify a 165-bp fragment of *CytB* from genomic DNA of QoI-resistant and -sensitive *S. citrulli* isolates, respectively. Amplification is *S. citrulli* was accomplished along with the forward primer DB-F (Table 3.2). The primer DB-F did not amplify from *S. caricae*, so a new forward primer was developed from the sequence

identified in our *S. caricae* draft genomes to assess potential QoI resistance in this species. The forward primer DB-Fca (Table 3.2) was used to amplify a 236-bp fragment of *CytB* of *S. caricae* when used with the same reverse primers, DB-RR or DB-RS.

In vitro mycelial growth assays

The *in vitro* mycelial growth assays (Thomas et al. 2012) that compare relative mycelial growth (RG) on fungicide-amended medium with mycelial growth on control medium were conducted to detect resistant phenotypes. Sensitivity to SDHI and DMI fungicides was assayed for all the 132 GSB isolates, and sensitivity of *S. caricae* isolates to a QoI fungicide was assayed to confirm the genotypes based on the *Cytb* marker. Technical formulations of two SDHI fungicides, boscalid (80.6% active ingredient; BASF Corp., Research Triangle Park, NC) and fluopyram (98.13% a. i.; Bayer CropScience, Durham, NC), and the DMI fungicide tebuconazole (97.5% a. i.; Bayer CropScience, Kansas City, MO) were dissolved in acetone to obtain a stock solution of 30 mg/ml. A 10× dilution of each stock solution was then prepared in 1 ml acetone. Azoxystrobin in analytical standard (99.4% a. i.; Sigma-Aldrich, Co., St. Louis, MO) was dissolved in acetone to obtain a stock solution of 10 mg/ml. The diluted solutions of each fungicide were added to 1 L autoclaved and cooled PDA to make the concentration of 3.0 µg/ml for boscalid, fluopyram, and tebuconazole, and 10.0 µg/ml for azoxystrobin. A control medium for each assay was prepared by adding acetone to PDA to obtain final acetone concentration of 0.1% by volume. The azoxystrobin-amended medium, as well as the control medium for QoI assays, contained 0.1 mg/ml SHAM (Salicylhydroxamic acid) to inhibit the alternative respiratory pathway that some fungi are able to utilize when growing on artificial medium (Ziogas et al. 1997).

For each of the 132 GSB isolates, 5-mm diameter mycelial plugs were removed with a cork borer from the edge of 1-week-old colonies and placed on media amended with boscalid, fluopyram, or tebuconazole, as well as control medium. After incubating in the dark at 25°C for 4 days, the RG for each isolate was calculated as the ratio of the mean diameter of the colony on fungicide-amended medium to the mycelial growth on the control medium, corrected by subtracting 5 mm for the initial mycelial plug. For each of the 19 *S. caricae* isolates, 5-mm diameter mycelial plugs were placed on medium amended with azoxystrobin and SHAM, as well as control medium, then incubated in the dark at 25°C for 7 days. Phenotypes for each type of fungicide were assigned to each isolate based on RG values: isolates with RG greater than 25% were considered as resistant and isolates with RG values equals to or less than 25% were considered sensitive (Thomas et al. 2012). Assays for each fungicide were conducted twice.

EC_{50} of *S. caricae* towards SDHI fungicide

The concentrations of two SDHI fungicides, boscalid and fluopyram, required to inhibit mycelial growth by 50% (EC_{50}) for each of the 19 *S. caricae* isolates were determined. Each SDHI fungicide was dissolved in acetone to obtain a stock solution of 30 mg/ml. Each stock solution was diluted with acetone and added to 1 L autoclaved cooled PDA, in order to obtain desired concentrations of 3.0, 1.0, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, 0.0003 μg/ml, as well as a control medium with same amount of acetone but 0 μg/ml fungicide. Mycelial plugs in 5-mm diameter were removed from the edge of 1-week-old colonies of each of the 19 *S. caricae* isolates using a cork borer and placed on fungicide-amended and control medium. After incubation in the dark at 25°C for 4 days, relative

inhibition for each *S. caricae* isolate on each fungicide concentration was calculated as the ratio of inhibited mycelial growth on fungicide-amended medium to the mycelial growth on the control medium. Assays for each SDHI fungicide were conducted twice. The EC_{50} value for each replication was calculated by linear regression of probit-transformed relative inhibition on a common logarithm of fungicide concentration. Normality of EC_{50} values was detected by a Shapiro-Wilk test on the logarithm of EC_{50} values from both experimental replications of each SDHI fungicide. A paired Student's *t*-test was used to compare the differences of EC_{50} values calculated from each experiment replications. All statistical analyses were conducted in JMP Pro 11.0.0 (SAS Institute Inc. Cary, NC). EC_{50} values of every *S. caricae* isolates were calculated from data from both replications.

Sequencing partial *SdhB* gene

In order to determine the underlying genotype of isolates determined to be resistant to two SDHI fungicides in the mycelial growth assays, we amplified and sequenced a 569 bp fragment of the succinate dehydrogenase subunit b (*SdhB*) gene, containing the H277R/Y sites responsible for resistance to SDHI fungicides (Avenot et al. 2012). PCR was conducted using a 25-µl volume reaction, prepared with reagents from TaKara Bio Inc. (Otsu, Shiga, Japan): 3.5 µl 10× buffer, 2.0 µl dNTP mixture (2.5 mM), 0.5 µl TaKaRa ExTaq (5U/µl), 1.5 µl of each primer pair (10 µM; Table 3.2), and 1 µl template DNA (3–20 ng). The thermal cycling conditions were: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min, with a final extension step at 72°C for 10 min. PCR products were prepared for sequencing using USB ExoSAP-IT (Affymetrix, Inc., Cleveland, OH) according to the manufacturer's

protocol. Sequencing reactions for each isolate were prepared by mixing 1 µl of prepared PCR products and 1 µl of each sequencing primer (3.3 µM). Products were submitted to the Georgia Genomics Facility where sequencing reactions were conducted using Big Dye Terminator chemistry and Ampli-Taq-FS DNA polymerase, followed by electrophoresis on a 3730xl 96-capillary DNA Analyzer (Applied Biosystems, Foster City, CA). The partial sequences of *SdhB* genes and upstream regions were aligned using Geneious 7.0.6 (Biomatters Ltd., Auckland, New Zealand).

Association of multiple fungicide resistance profiles with genetic similarity

Fungicide resistance profiles of all 113 *S. citrulli* and 19 *S. caricae* isolates were determined by genotype of *Cytb* for azoxystrobin (QoI fungicide), and phenotypes of resistant or sensitive to boscalid (SDHI fungicide), fluopyram (SDHI fungicide), and tebuconazole (DMI fungicide). Based on multilocus genotype data from 16 microsatellite loci (Li and Brewer 2016, Chapter 2), minimum spanning networks were built in the program poppr 2.1.1 (Kamvar et al. 2014) to illustrate the genetic dissimilarity and Bruvo's genetic distance between each pair of MLG out of the 113 *S. citrulli* isolates. Bruvo's genetic distances between MLG were calculated by a stepwise mutation model.

RESULTS

Responses to QoI and DMI fungicides

The previous allele-specific PCR for detecting azoxystrobin resistance (Finger et al. 2014) is only able to amplify fragment of *CytB* of *S. citrulli*. The mutational site G143A responsible for azoxystrobin resistance, where the reverse the reverse primers (either DB-

RR or DB-RS) target, is located on a 45-bp exon. However, The forward primer DB-F is located upstream in an intron region that is not identical between *S. citrulli* and *S. caricae* (data not shown). The newly developed forward primer Db-Fca is located in the upstream intron region of *S. caricae* and able to amplify a 236-bp fragment when used in PCR with the reverse primers: DB-RR or DB-RS.

Based on the allele-specific PCR, only two *S. citrulli* isolates had amplification with reverse primer DB-RS, indicating genotypes of sensitivity to azoxystrobin. They were from Tift County, GA, 2013, and Suwannee County, FL, 2013. The remaining 111 isolates had amplification with reverse primer DB-RR, showing genotypes of resistance to azoxystrobin. All 19 *S. caricae* isolates also had amplification with reverse primer DB-RR, indicating genotypes of resistance to azoxystrobin. Since resistance to azoxystrobin in *S. caricae* had not been previously tested or reported, phenotypes of isolates with sequences coding for alanine (A) at position 143 were verified using *in vitro* mycelial growth assay. Growth on medium amended with azoxystrobin (10.0 µg/ml) and SHAM (0.1 mg/ml) relative to growth on the control medium amended only with SHAM was greater than 80% for all 19 *S. caricae* isolates.

Based on results of mycelial growth assays, all 113 *S. citrulli* isolates were sensitive to DMI fungicide tebuconazole, while all 19 *S. caricae* isolates were resistant to tebuconazole.

Responses to the two SDHI fungicides

Based on results of the *in vitro* mycelial growth assays at a discriminatory concentration of 3.0 µg/ml. *S. citrulli* isolates showed diverse responses to the SDHI

fungicide boscalid. Among the 113 isolates, 68 were resistant to boscalid, while 45 were sensitive (Table 3.3). For the fluopyram, only one isolate collected from Cook County, Georgia, 2014, showed a resistant phenotype to fluopyram (relative growth rate of 60%). This isolate was among the 45 *S. citrulli* isolates that were sensitive to boscalid and had a unique MLG.

Seven MLG shared by isolates collected from the same field showed the same SDHI sensitivity phenotype. Four of them contained 15 isolates that were resistant to boscalid; the remaining three contained 8 isolates that were all sensitive to boscalid. For the three MLG shared by isolates collected from different fields, the phenotypes to SDHI fungicide varied within two of them. The genotypes were highly diverse based on partial sequences of the *SdhB* gene, particularly at amino acid locus 277. All 12 isolates of MLG X4 were resistant to boscalid and has sequences coding for amino acid Y (tyrosine) at locus 277. Six out of 11 isolates in MLG X1 were resistant to boscalid with sequences coding for Y at locus 277. The other five isolates were sensitive to boscalid, with sequences coding for H (histidine) at locus 277. In MLG X5, nine out of 16 isolates were sensitive to boscalid with sequences coding for H at locus 277. Seven isolates were resistant to boscalid, but four had sequences coding for Y at position 277 and three has sequences coding for R (arginine).

Based on the *in vitro* mycelial growth assay all 19 *S. caricae* isolates that were sensitive to both boscalid and fluopyram. Mycelial growth assays of the 19 *S. caricae* were conducted on media amended with serial concentrations of each SDHI fungicide. Based on the Shapiro-Wilk test, EC_{50} values of each replication of both fungicides were normally distributed after logarithm transformation. No significant differences ($P < 0.05$) of EC_{50} values were detected for paired Student's *t*-test between the two experimental repeats for

each fungicide. With *F*-statistics, significant coefficients ($P < 0.05$) of regression were shown for all the isolates from two SDHI fungicides. EC_{50} values of every isolate for each fungicide were plotted in a histogram (Fig. 3.1). The EC_{50} values of boscalid varied from 0.03 to 0.07 μg/ml, with an average of 0.04 μg/ml; while the EC_{50} values of fluopyram ranged from 0.06 to 0.20 μg/ml, with an average 0.12 μg/ml.

Profiles to multiple fungicides were not associated with genetic similarity

Six different profiles of resistance to multiple fungicides were identified among the GSB fungi (Table 3.3). All 19 *S. caricae* isolates had the same profile (Type VI), which was characterized by resistance to tebuconazole and azoxystrobin, and sensitivity to boscalid and fluopyram. All the 113 *S. citrulli* isolates were sensitive to tebuconazole, but were classified into five different profile types. A majority of the *S. citrulli* islates (Type I; $n = 67$) was resistant to boscalid and a minority of them (Type III; $n = 43$) was sensitive to this fungicide. Two unique isolates were sensitive to azoxystrobin, but one was resistant to boscalid (Type II) and the other was sensitive (Type IV). Another unique isolate was resistant to fluopyram, but sensitive to boscalid (Type V).

Genetic relationship among all 113 *S. citrulli* isolates were determined based on multilocus genotypes (MLG) of 16 microsatellite loci analyzed by minimum spanning network by genetic dissimilarity (Fig 3.2A) and Bruvo's genetic distance (Fig. 3.2B). In the networks, each circle represents an MLG. Larger circles are MLG shared by more than one isolate. The thickness of the lines indicates the genetic dissimilarity or Bruvo's genetic distance: the darker the line, the closer genetic relationship between two MLG. Three widespread MLG shared by isolates from different populations are labeled X1, X4, and X5.

All MLG are shaded based on fungicide resistance profiles. Both networks indicate that the three profiles (II, IV, and V) with a single isolate also had unique MLG and did not show a close genetic relationship among each other. Isolates from more common profile types I and III, which were different in responses to boscalid, were distributed throughout the networks, without any clustering pattern at any section or branch of the networks. Seven MLG shared by *S. citrulli* isolates collected from the same population all had the same type of profile. Three were Type III and four were Type I. MLG X4 shared by 12 isolates collected from different populations were Type I, as well. However, isolates from two MLG (X1 and X5) did not share the same fungicide resistance profile, particularly to boscalid. Within MLG X1, six of 11 isolates were Type I, while the other five were in Type III. Of the 16 isolates in MLG X5, seven were Type I and nine were in Type III.

Partial sequences of *SdhB*, the target of SDHI fungicides (boscalid, fluopyram), were obtained from 39 isolates belonging to MLG X1, X4, and X5. All 14 isolates in Type III had sequences coding for the amino acid H (histidine) at site 227. Genotypes varied among Type I isolates. All six Type I isolates in MLG X1 and all 14 isolates in MLG X4 had sequences coding for Y(tyrosine) at site 227. Four of seven Type I isolates in MLG X5 also had sequences coding for Y at this site, but sequences of the other three coded for R(arginine). Overall, there were no clear relationships among MLG and fungicide resistance profiles. Additionally, some MLG showed multiple profiles and different genotypes responsible for boscalid resistance.

DISCUSSION

The application of fungicides has been the most effective way to manage GSB (Avenot et al. 2012; Keinath 2012; Thomas et al. 2012b). In order to understand the evolution of fungicide resistance in GSB fungi in the southeastern U.S., resistance profiles to four types of fungicides belonging to three different chemical classes: QoI (azoxystrobin); SDHI (boscalid and fluopyram); and DMI (tebuconazole), were assessed for 113 isolates of *S. citrulli*, as well as 19 isolates of *S. caricae*. The efficacies of different classes of fungicides to GSB fungi have changed significantly over the past 20 years. The QoI fungicide azoxystrobin was highly efficacious in managing GSB until the late 1990sm but only a few years after its introduction, resistance was reported in GSB fungi from Georgia (Stevenson et al. 2004) and South Carolina (Keinath 2009). After that time, use of QoI fungicide was no longer recommended by extension agencies in these states. However, resistance to azoxystrobin has been maintained in populations of the *S. citrulli*, which is the dominant GSB species in the southeastern U.S. Only two of 113 isolates in this study were sensitive to this fungicide. The allele-specific PCR developed by Finger et al. (2014) required modification of the forward primer in order to apply this methodology to the recently emerging *S. caricae* species and is a useful tool to detect the genotype associated with resistance to QoI fungicides. All 19 isolates of *S. caricae* were also resistant to azoxystrobin. Therefore, QoI fungicides remain ineffective in managing GSB in the southeastern U.S. and GSB fungi do not show evidence of suffering a fitness to cost to maintaining this resistance.

Diverse responses to the SDHI fungicide boscalid were observed in our study. Previously, boscalid-resistant phenotypes of GSB fungi were reported in Georgia (Thomas et al. 2012a) and South Carolina (Keinath 2012). In our study, a majority of the *S. citrulli*

isolates (68 out of 113) were found resistant to boscalid, while a minority of isolates (45 out of 113) were still sensitive to this fungicide. On the other hand, all 19 isolates of *S. caricae* were sensitive to boscalid. The other SDHI fungicide, fluopyram, was recently labelled for GSB management in 2014. Based on results, all isolates of GSB fungi tested, except for one resistant *S. citrulli* isolate, were sensitive to fluopyram. However, the EC_{50} values of *S. caricae* for fluopyram (mean of 0.12 μg/ml) were higher than boscalid (mean of 0.04 μg/ml).

Sensitivity to the DMI fungicide tebuconazole differed between *S. citrulli* and *S. caricae*. All 113 *S. citrulli* isolates were sensitive to tebuconazole, while all 19 *S. caricae* isolates were resistant to this fungicide. DMI fungicides have been effective in managing GSB in Georgia (Thomas et al. 2012b) and South Carolina (Keinath and Hansen 2013), since the first one was labeled for cucurbit use in 2008. The tebuconazole resistance in these 19 isolates of *S. caricae* is the first observation of DMI fungicide resistance among GSB fungi. However, the molecular basis for DMI resistance in *S. caricae* is not yet clear (Chapter 4). Mechanisms that have been excluded are mutations in *Cyp51*, changes to the *Cyp51* promote region, and overexpression of *Cyp51* or the ABC transporter *ScAtrG* (Chapter 4). Suggestions to help minimize fungicide resistance development include designing proper application time, dose, and number, and alternation or mixtures of modes of actions (van den Bosch et al. 2014). Therefore, fluopyram or a mixture of tebuconazole and boscalid would target both *S. citrulli* and *S. caricae* to effectively manage GSB in the southeastern U.S.

Six types of fungicide resistance profiles to a QoI fungicide, two SDHI fungicides, and a DMI fungicide were found in these GSB fungi. All *S. caricae* isolates were characterized as

one type, which was resistant to tebuconazole but sensitive to boscalid. On the other hand, all five types of *S. citrulli* were sensitive to tebuconazole. Among all 113 *S. citrulli* isolates, 45 (Type III, IV and V) were also sensitive to boscalid, while the remaining 68 (Type I and II) were resistant. Type II and IV contained single isolates that were sensitive to the QoI fungicide azxosytrobin. The only isolate detected as resistant to fluopyram was Type V, while all other isolates were still sensitive to this fungicide. Sensitivity to boscalid served as the major differentiating factor among *S. citrulli* populations. The stepwise pattern (Li et al. 2014) of resistance acquisition to multiple fungicides was not observed in GSB fungi.

Associations of phenotypes of interest and genetic structure of pathogen populations are very useful in revealing evolutionary patterns and processes. Phenotypes of interest often include host specificity, virulence, and fungicide resistance. Populations of the soil-borne pathogen *Phytophthora nicotianae* collected worldwide showed a strong association between the genetic clustering of microsatellite MLG and host of origin (Biasi et al. 2016). Isolates of the foliar pathogen *Pseudocercosporella capsellae* collected from different *Brassica* hosts showed similarities based on ITS sequences and virulence (Gunasinghe et al. 2016). Resistance to metalaxyl-M, a phenylamide fungicide, was linked with specific microsatellite alleles of *Phytophthora infestans* (Montes et al. 2016). Although multiple fungicide resistance profiles of GSB fungi in the southeastern U.S. were associated with the species *S. citrulli* or *S. caricae*, within the dominant species *S. citrulli*, resistance profiles were not associated with genetic similarities based on 16 microsatellite loci. The three isolates of *S. citrulli* with unique profiles had unique genotypes. Thus, the response to boscalid was the only variable that could be compared, and it showed no clustering based on genetic distance. This is likely due to genetic recombination or independent assortment

of microsatellite loci and the *SdhB* locus during sexual reproduction of *S. citrulli*. Two MLG (X1 and X5) shared by isolates collected from different populations did not share the same resistance profile. One (X5) had a different genotype for amino acid site 227 of *SdhB* that is responsible for boscalid resistance. This was likely the result of multiple mutation events and convergent evolution for boscalid resistance. In a population of *Pestalotiopsis longiseta* collected in Japan, azoxystrobin resistance was not associated with the phylogenetic clades based on inter simple sequence repeat (ISSR) data (Yamada et al. 2016). This trade-off model was introduced to illustrate the evolution of virulence of *Phytophthora infestans*, where fitness of highly aggressive isolates was measured throughout the season (Andrivon et al. 2013). In the agroecosystem, fungicide applications produce strong selection pressures on pathogen populations. However, the fitness of resistant isolates of *Phytophthora infestans* as not high when the selection pressure was removed. Trade-offs in fitness for fungicide resistance vary for different fungicides and could explain the lack of association between fungicide resistance profile and genetic relationship of GSB fungi in the southeastern U.S.

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Table 3.1. Sampling year and location of 113 *Stagonosporopsis citrulli* isolates selected for resistance to multiple fungicides, with multilocus genotype (MLG) assignment

Population	Sampling location and year	No. isolates	Unique MLG	No. sharing MLG within population [a]	No. sharing MLG among populations [b]
GAC12	Cook County, GA; 2012	18	8	2	8 (4, 0, 4)
GAC13	Cook County, GA; 2013	40	18	9 (5, 4, 2)	13 (2, 2, 9)
GAC14	Cook County, GA; 2013	19	9	5 (3, 2)	5 (2, 0, 3)
GAT13	Tift County, GA; 2013	16	9	0	7 (3, 4, 0)
FL13	Suwannee County, FL; 2013	20	7	7	6 (0, 6, 0)
Total		113	51	23	39

[a] Number of isolates in MLG X1, X4, and X5, respectively in parentheses

[b] Number of isolates in each MLG was in parentheses

Table 3.2. Nucleotide sequence, purpose, and source of primers used in this study

Primer	Sequence	Purpose	Source
DB-RR	5'-GCAGATGTCATTATGAGG-3'	Reverse primer for azoxystrobin-resistant allele of *S. citrulli* and *S. caricae*	Finger et al. 2014
DB-RS	5'-GCAGATGTCATTATGAGC-3'	Reverse primer for azoxystrobin-sensitive allele of *S. citrulli* and *S. caricae*	Finger et al. 2014
DB-F	5'-CGTATAGTCTCTGAGGAACC-3'	Forward primer for genotype to azoxystrobin response of *S. citrulli*	Finger et al. 2014
Sc-Fa	5'-AAGAAAGAAAAGAAGAAGGT-3'	Forward primer for genotype to azoxystrobin response of *S. caricae*	This study
Sc-SdhBF	5'-GCTGGATGCGCTCATTCG-3'	Forward primer for partial sequence of succinate dehydrogenase subunit B	This study
Sc-SdhBR	5'-CGATAGTACGTGCATACAACGA-3'	Reverse primer for partial sequence of succinate dehydrogenase subunit B	This study

Table 3.3. Number of isolates and responses (R-resistant, or S-sensitive) to QoI (azoxystrobin), SDHI (boscalid, and fluopyram), and DMI (tebuconazole) fungicides producing six resistance profile types among 132 isolates of gummy stem blight fungi

Type	GSB species	No. of isolates	Azoxystrobin (QoI)	Boscalid (SDHI)	Fluopyram (SDHI)	Tebuconazole (DMI)
I	*S. citrulli*	67	R	R	S	S
II	*S. citrulli*	1	S	R	S	S
III	*S. citrulli*	43	R	S	S	S
IV	*S. citrulli*	1	R	S	R	S
V	*S. citrulli*	1	S	S	S	S
VI	*S. caricae*	19	R	S	S	R

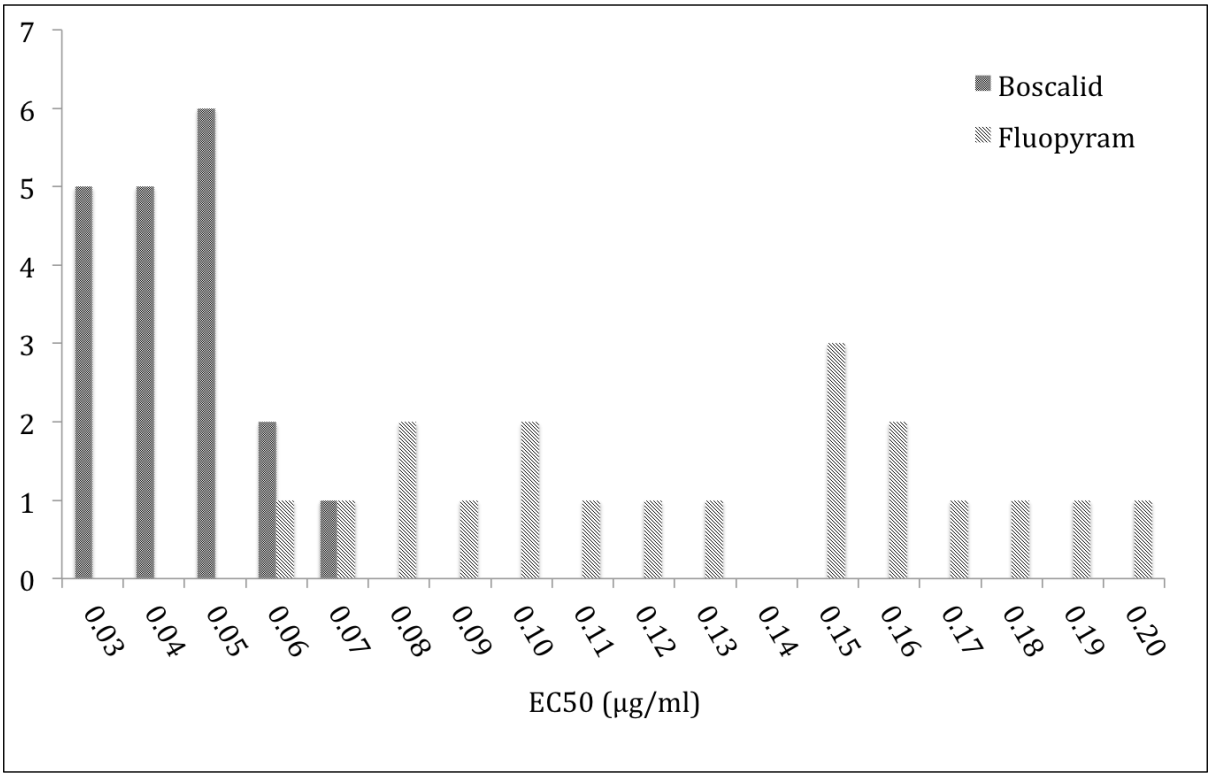
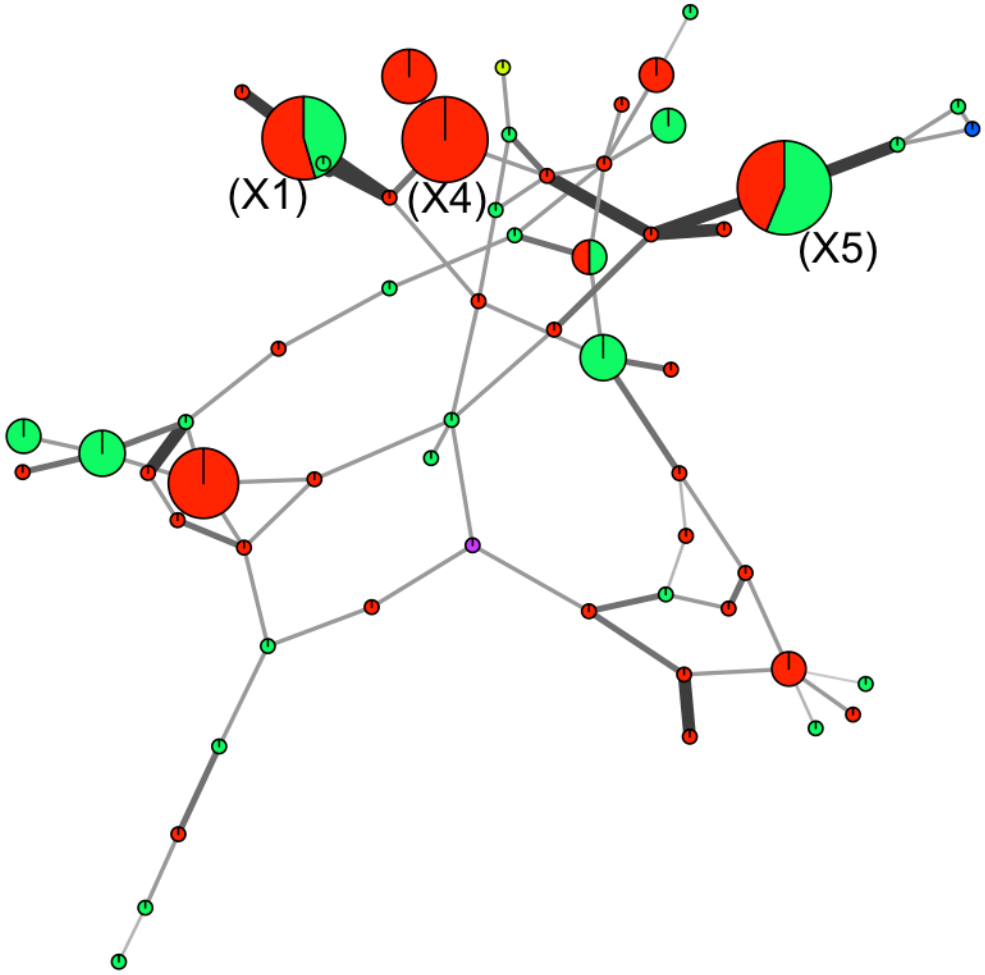


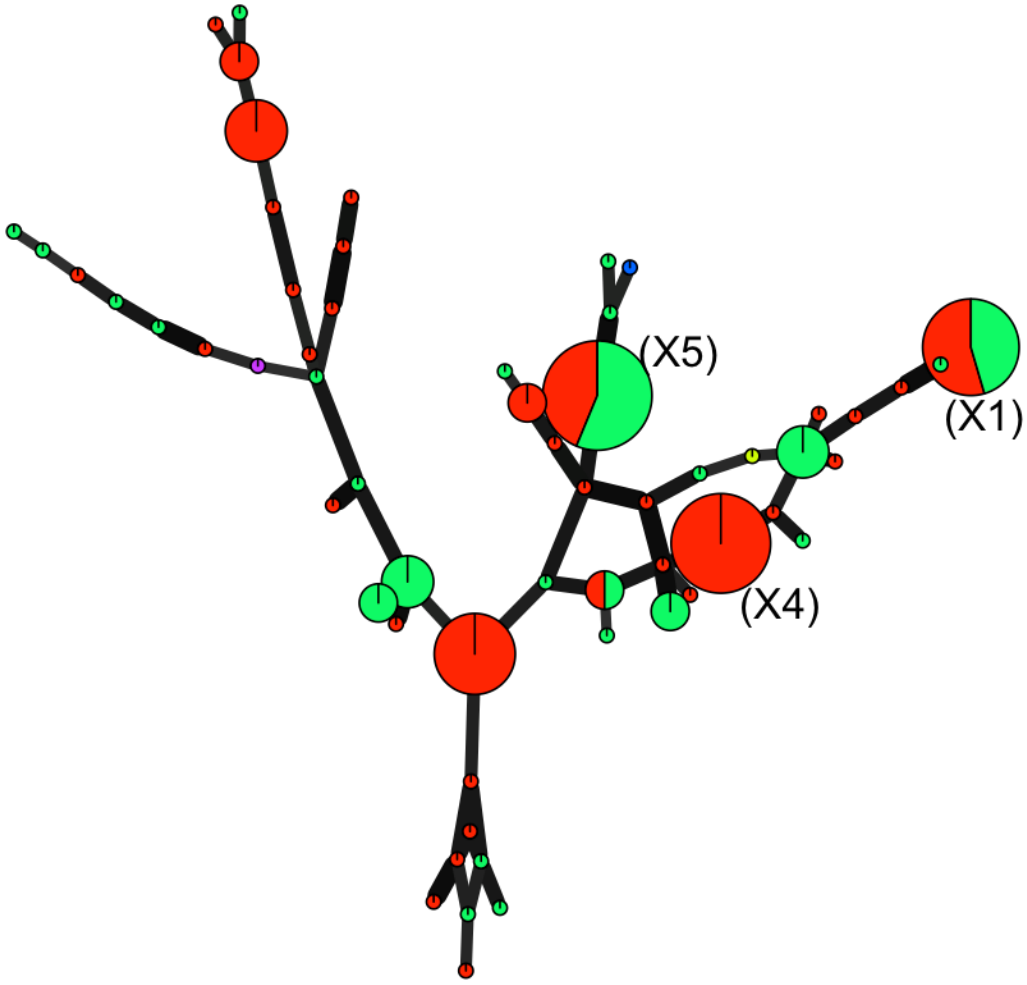
Fig. 3.1. Histogram of EC_{50} (fungicide concentration inhibiting 50% of the mycelial growth) of two SDHI fungicides: boscalid of fluopyram for the 19 *S. caricae* isolates collected from Georgia

A



I – $A^{R}T^{S}B^{R}F^{S}$: 67
II – $A^{S}T^{S}B^{R}F^{S}$: 1
III – $A^{R}T^{S}B^{S}F^{S}$: 43
IV – $A^{R}T^{S}B^{S}F^{R}$: 1
V – $A^{S}T^{S}B^{S}F^{S}$: 1

B



S. citrulli (I) $A^RT^SB^RF^S$: 67
S. citrulli (II) $A^ST^SB^RF^S$: 1
S. citrulli (III) $A^RT^SB^SF^S$: 43
S. citrulli (IV) $A^RT^SB^SF^R$: 1
S. citrulli (V): $A^ST^SB^SF^S$: 1

Fig. 3.2. Minimum spanning network – **A**) by genetic dissimilarity; and **B**) by Bruvo's distance – of the 113 *S. citrulli* isolates from the southeastern U.S. based on multilocus genotypes (MLG) from 16 microsatellite loci. In the networks, each circle represents an MLG. Larger circles are MLG shared by more than one isolate. The thickness of the lines indicates the genetic dissimilarity or Bruvo's genetic distance: the darker the line, the closer genetic relationship between two MLG. Three widespread MLG shared by isolates from different populations are labeled X1, X4, and X5. All MLG are colored based on fungicide resistance profiles (R-resistant and S-sensitive) to: A-azoxystrobin, T-tebuconazole, B-boscalid, and F-fluopyram, as shown in the legend.

CHAPTER 4

DIFFERENCES IN SENSITIVITY TO A TRIAZOLE FUNGICIDE AMONG *STAGONOSPOROPSIS* SPECIES[1]

[1] Li, H., K. L. Stevenson., and M. T. Brewer. 2016. *Plant Disease*. 100: 2106-2112.
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ABSTRACT

Gummy stem blight (GSB) is a destructive disease of cucurbits caused by three closely related *Stagonosporopsis* species. In the southeastern U.S., GSB management relies heavily on triazole fungicides. Our objectives were to determine if resistance to triazoles has developed in populations of GSB fungi in the southeastern U.S., and, if so, to investigate the molecular basis of resistance. A tebuconazole sensitivity assay was conducted on 303 *Stagonosporopsis citrulli* and 19 *S. caricae* isolates collected from the southeastern U.S. in 2013 and 2014, as well as three *S. citrulli*, three *S. cucurbitacearum*, and six *S. caricae* isolates from other regions or years. Tebuconazole resistance was detected for all 19 *S. caricae* isolates from the southeastern U.S., and one *S. caricae* isolate from Brazil. All *S. citrulli* and *S. cucurbitacearum* isolates were sensitive to tebuconazole. For resistant and sensitive isolates of *S. caricae*, coding and promoter regions of the target gene, *Cyp51*, were sequenced, and expression levels of *Cyp51* and *ScAtrG* (an ABC transporter) were measured. Tebuconazole resistance was not associated with mutations within *Cyp51*, multiple copies of *Cyp51*, changes in the promoter region, or increased expression of *Cyp51* or *ScAtrG*. Tebuconazole resistance may explain the increase in frequency of *S. caricae* isolates recovered from GSB-infected cucurbits in Georgia.

INTRODUCTION

Cucurbits are susceptible to gummy stem blight (GSB), a destructive foliar disease that has caused severe epidemics in the southeastern U.S. over the past several decades (Gusmini et al. 2005; Keinath et al. 1995; Schenck 1968; St. Amand and Wehner 1995). These epidemics can have major economic impacts because more than one third of the

cucurbits produced in the U.S. are from southeastern states, including Georgia, Florida, and South Carolina, which alone contribute approximately $356 million to the local economy (NASS 2015). GSB not only decreases yield, but also reduces fruit quality (Keinath 2000). GSB is caused by three morphologically similar, but genetically distinct *Stagonosporopsis* species: *S. cucurbitacearum* (syn. *Didymella bryoniae*), *S. citrulli*, and *S. caricae* (Stewart et al. 2015). All three fungal species are necrotrophs with wide host ranges among cucurbits (Chiu and Walker 1949; Keinath 2011; Stewart et al. 2015) affecting aboveground, vegetative structures at any growth stage after seed germination (Wiant 1945). The three *Stagonosporopsis* species are pathogenic to cucurbits with similar virulence; however, *S. caricae* is also pathogenic to papaya (Stewart et al. 2015) causing fruit and stem rot and leaf spot of papaya. Until recently, only *S. citrulli* was recovered from GSB-infected cucurbits in the southeastern U.S. (Stewart et al. 2015). In 2013, four of 246 GSB fungi sampled from four watermelon fields in the southeastern U.S. were identified as *S. caricae* (Li and Brewer, 2016). The four *S. caricae* isolates came from a single field in Tift County, GA. In 2014, 15 of 76 GSB fungi sampled from a single watermelon field in Cook County, GA were identified as *S. caricae* (Li and Brewer, 2016).

Cultivars of watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*) or muskmelon (*Cucumis melo*) resistant to *Stagonosporopsis* species are not currently available (Keinath 2014), so management of GSB requires both cultural and chemical control strategies. It is recommended that growers use only certified pathogen-free seed and seedlings (Sitterly and Keinath 1996). To limit local survival of GSB fungi on debris, a minimum 2-year rotation with non-cucurbit crops is recommended (Keinath 1996; Keinath 2000). Other cultural practices include incorporation of infested residue to reduce survival

on plant debris (Sitterly and Keinath 1996), avoidance of overhead irrigation to reduce periods of leaf wetness (Thomas et al. 2012b), and soil fumigation with cabbage residue amendments (Keinath 1996). Nonetheless, fungicide application is by far the most effective option for GSB management (Keinath 2000; Keinath 2012; Thomas 2012b). Unfortunately, GSB fungi in the southeastern U.S. have repeatedly and rapidly evolved resistance to several classes of fungicides. In the mid-1990s, thiophanate-methyl, a methyl benzimidazole carbamate (MBC) fungicide (Fungicide Resistance Action Committee, FRAC #1) (FRAC 2016), was deemed ineffective against GSB due to the development of resistance in pathogen populations (Keinath and Zitter 1998). Initially, azoxystrobin, a quinone outside inhibitor (QoI) fungicide (FRAC #11), provided excellent control of GSB, but by the end of 1990s cases of resistance appeared in Georgia (Stevenson et al. 2004) and South Carolina (Keinath 2009). Boscalid, an SDHI fungicide (FRAC #7), provided control for GSB until 2007 (Seebold and Langston 2003) when widespread resistance developed in Georgia (Thomas et al. 2012a) and South Carolina (Keinath 2012). GSB fungi remain sensitive to triazoles, the largest subgroup of demethylation inhibitors (DMI, FRAC #3) (Keinath and Hansen 2013; Thomas et al. 2012b). DMI fungicides inhibit ergosterol biosynthesis by targeting lanosterol 14α-demethylase, which is encoded by the gene *Cyp51* (Kuck et al. 2002). Triazoles are used against a broad spectrum of fungi in medical and agricultural settings (Moore et al. 2000; Price et al. 2015; van den Bossche et al. 2003).

In Georgia (Langston 2014), Florida (Dufault and Paret 2014), and South Carolina (Keinath and Miller 2014), management of GSB relies heavily on triazoles, which is concerning, because resistance to triazoles has been observed for many other plant-pathogenic fungi (Cools et al. 2013; Price et al. 2015). However, unlike the qualitative

resistance observed for single-site fungicides, such as azoxystrobin, resistance to triazoles is quantitative, often resulting in development of a continuum of resistance phenotypes (Cools and Fraaije, 2013). Mechanisms of resistance to triazoles include: (1) single-site mutations in the coding region of *Cyp51* leading to changes in the amino acid sequence of the protein making it unrecognizable by the fungicide (Cools et al. 2011; Frenkel et al. 2015; Schmitz et al. 2014); (2) overexpression of *Cyp51* due to changes in the promoter region (Cools et al. 2012; Schnabel and Jones 2001) or other unknown enhancers of transcription (Bolton et al. 2012; Ma and Tredway 2013); (3) multiple copies of *Cyp51* genes (Brunner et al. 2016); and (4) overexpression of transporters that control the efflux of fungicides (Kretschmet et al. 2009). Common transporter groups include ATP binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters (Cools et al. 2013). The objectives of this study were to determine if resistance to triazole fungicides has developed in field populations of GSB fungi in the southeastern U.S., and, if so, to investigate the molecular basis of triazole resistance in GSB fungi.

MATERIALS AND METHODS

Isolates of gummy stem blight fungi

A total of 322 fungal isolates was collected from five watermelon (*Citrullus lanatus*) fields in Georgia, Florida and South Carolina in July 2013 and 2014 (Table 1). There were 53 isolates sampled from a field in Cook County, GA 2013; 76 isolates sampled from Cook County, GA in 2014; 99 isolates sampled from Tift County, GA in 2013; 72 isolates sampled from Suwannee County, FL in 2013; and 22 isolates sampled from Colleton County, SC in 2013 (Table 1). The detailed sampling methods and isolation procedures for these fungi

have been described previously (Li and Brewer, 2016). Nineteen of the field isolates were previously identified as *S. caricae*, while the remaining 303 isolates were identified as *S. citrulli* (Brewer et al. 2015; Li and Brewer, 2016). In addition, twelve GSB or papaya fruit rot isolates, including three *S. citrulli*, three *S. cucurbitacearum*, and six *S. caricae* isolates were screened for resistance (Table 1). These isolates were collected from different cucurbit growing regions across the U.S., from South America, and from additional hosts including cucumber (*Cucumis sativus*), muskmelon (*Cucumis melo*), butternut squash (*Cucurbita moschata*), and papaya (*Carica papaya*) (Stewart et al. 2015). All isolates were recovered on quarter-strength potato dextrose agar (qPDA) from filter paper maintained at -20°C. In total, 306 *S. citrulli* isolates, three *S. cucurbitacearum* isolates, and 25 *S. caricae* isolates were tested for tebuconazole sensitivity.

Fungicide sensitivity assay

Tebuconazole was selected to determine triazole sensitivity because it is widely used for GSB management in the southeastern U.S. We used an *in vitro* mycelial growth assay developed by Thomas et al. (2012a) that compares mycelial growth on a tebuconazole-amended medium (3.0 µg/ml) with mycelial growth on a control medium. Technical grade tebuconazole (97.5% active ingredient; Bayer CropScience, Kansas City, MO) was dissolved in acetone to obtain a stock solution of 30 mg/ml. A 10× dilution of the stock solution was prepared in 1 ml acetone and added to 1 L autoclaved, cooled PDA to obtain a concentration of 3.0 µg/ml tebuconazole. A control medium was prepared by adjusting PDA to a final acetone concentration of 0.1% by volume.

For each isolate, 5-mm diameter mycelial plugs were obtained from the edge of a 1-week-old colony using a cork borer and placed in petri dishes of tebuconazole-amended and control media. Isolates were incubated in the dark at 25°C for 4 days. Mycelial growth was estimated as the average diameter of the colony less 5 mm for the mycelial plug. The relative growth (RG) for each isolate was calculated as the ratio of mycelial growth on the tebuconazole-amended medium to the mycelial growth on the control medium. Based on the previously developed assay (Thomas et al., 2012a) a phenotype of resistant to tebuconazole was assigned to GSB isolates with RG values greater than 25% and sensitive to tebuconazole (S) was assigned to isolates with RG values less than 25%. We observed that resistant isolates could be further divided into two phenotypic categories, which consisted of highly resistant isolates (HR) with RG values greater than 75% and moderately resistant isolates (MR) with RG from 25% to 75%. The mycelial growth assay was repeated for isolates with HR and MR phenotypes.

Identification of *Cyp51* in draft genomes of *Stagonosporopsis* species

Draft genomes of nine GSB or papaya fruit rot isolates, including three *S. citrulli*, three *S. cucurbitacearum*, and three *S. caricae*, were assembled. A high quality DNA extraction method (Fulton et al. 1995) was used. Cetyl trimethylammonium bromide (CTAB) lysis buffer was prepared by mixing 6.5 ml of Buffer A (0.35 M sorbitol; 0.1 M Tris-HCl, pH 9; and 5 mM EDTA, pH 8), 6.5 ml of Buffer B (0.2 M Tris-HCl, pH 9; 50 mM EDTA, pH 8; 2 M NaCl; 2% CTAB), 2.6 ml of Buffer C (5% Sarkosyl), 1.75 ml PVP (0.1%), and 1.25 µl Proteinase K. Approximately 500 mg mycelium growing at 25°C in the dark for 7 days on qPDA covered with sterile cellophane was removed and added to CTAB lysis buffer. The

mixture was agitated with two 5-mm glass beads (VWR Soda Lime, Radnor, PA, USA) at 1,750 RPM for 2 min in a 2010 Geno/Grinder (SPEX SamplePrep, Metuchen, NJ, USA). The mixture was then inverted ten times after adding 5.75 ml potassium acetate (5 M), and incubated on ice for 30 min, followed by centrifugation for 20 min at 14,000 *g*. The supernatant was added to one volume of chloroform-isoamylalcohol (v/v 24:1), followed by centrifugation for 10 min at 14,000 *g*. The supernatant was added to 100 µl RNase A (10 mg/ml) and incubated at 37°C for 120 min. An equal volume of isopropanol and 1/10 volume of sodium acetate was added, then the mixture was incubated at 25°C for 5 min, followed by centrifugation at 14,000 *g* for 30 min. The supernatant was discarded and the pellet was dissolved in 100 µl deionized H_2O, after being rinsed twice with 70% ethanol and air-dried.

Genomic DNA for each isolate was submitted to the Georgia Genomics Facility (Athens, GA) for next-generation sequencing (NGS) library preparation, followed by sequencing reactions using the Illumina MiSeq platform according to the PE300 protocol (Illumina Inc. San Diego, CA). Files of the forward and reverse reads of each isolate with optimal *k*-mer values of 64 (substrings of length *k* in DNA sequence data) were assembled *de novo* into draft genomes using ABySS v.1.3.6 (Simpson et al. 2009) on the Georgia Advanced Computing Resource Center Linux cluster.

Nucleotide sequence of the *Cyp51* gene from the annotated genome of *Didymella exigua* (Scaffold 10: 784720-786692; Nordberg et al. 2014) and paralogs in the genomes of *Gibberella zeae* (GenBank accession numbers FJ216393 and FJ216400; Yin et al. 2009) and *Aspergillus fumigatus* (GenBank accession numbers AF338659 and AF338660; Mellado et al. 2001) were used to search for homologs in each draft genome of the nine GSB isolates

using megaBLAST (Altschul et al. 1990). With pairwise megaBLAST, the *Cyp51* regions identified in GSB isolates and from the *D. exigua* genome were compared with paralogs from *G. zeae* and *A. fumigatus*.

Sequencing of Cyp51 from Stagonosporopsis caricae

The *Cyp51* genes from 19 isolates of *S. caricae* were aligned and compared. In addition to the sequences identified in the three draft genomes of tebuconazole-sensitive *S. caricae* isolates (Table 1), 16 isolates were PCR-amplified and sequenced including: 11 isolates highly resistant (HR) to tebuconazole (GA6057 and GA6081 from Tift County, GA sampled in 2013; GA8002, GA8007, GA8016, GA8036, GA8051, GA8061, GA8091 and GA8098 from Cook County, GA sampled in 2014; and 2245 from Brazil), three isolates moderately resistant (MR) to tebuconazole (GA6011 and GA6074 from Tift County, GA sampled in 2013; GA8025 from Cook County, GA sampled in 2014), and two isolates sensitive (S) to tebuconazole (CBS102399 from Brazil and CBS248.90 from Chile). Genomic DNA was extracted from fresh mycelium using a method modified from Lee et al. (1988). Approximately 100 mg of mycelium from each isolate was harvested after incubation at 25°C in the dark for 4 days by removing the mycelium from qPDA covered with sterile cellophane. The mycelium was mixed with 1 ml mini-prep lysis buffer (50 mM EDTA, pH 8; 100 mM Tris, pH 8; 3.5% SDS; 500 µg/ml proteinase K; and 1% sodium bisulfite) by vortexing for 1 min. The mixture was then incubated for 15 min at 65°C, followed by centrifugation for 5 min at 14,000 *g*. The supernatant was added to 200 µl ammonium acetate (7.5 M), and vortexed for 10 s. The mixture was placed on ice for 15 min, then centrifuged at 14,000 *g* for 3 min. The supernatant was precipitated with an equal volume

of isopropanol and centrifuged at 14,000 *g* for 5 min. The pellet was then resuspended in 100 µl sterilized deionized H_2O, after rinsing twice with 70% ethanol and air-drying. The DNA was stored at -20°C.

Based on the *Cyp51* sequence from the draft genome of *S. caricae*, two pairs of primers were designed using Primer3 (Rozen and Skaletsky 2000) to amplify the promoter and coding regions of *Cyp51* (Table 2). PCR was conducted using a 20-µl volume reaction, prepared with reagents from TaKara Bio Inc.: 3.5 µl 10× buffer, 3.0 µl dNTP mixture (2.5 mM), 5 µl TaKaRa ExTaq (5U/µl), 1.5 µl of each pair primers (10 µM), and 1 µl template DNA (3–20 ng). The thermal cycling conditions were: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 2 min, with a final extension step at 72°C for 10 min. PCR products were prepared for sequencing using USB ExoSAP-IT (Affymetrix, Inc., Cleveland, OH) according to the manufacturer's protocol. To cover the entire promoter and coding regions of *Cyp51*, six primers were designed for the sequencing reactions (Table 2). Sequencing reactions for each isolate were prepared by mixing 1 µl of prepared PCR products and 1 µl of each sequencing primer (3.3 µM). Products were submitted to the Georgia Genomics Facility where sequencing reactions were conducted using Big Dye Terminator chemistry and Ampli-Taq-FS DNA polymerase, followed by electrophoresis on a 3730xl 96-capillary DNA Analyzer (Applied Biosystems). The sequences of the *Cyp51* genes and upstream regions were aligned using Geneious 7.0.6 (Biomatters Ltd., Auckland, New Zealand). The sequences from resistant isolates and moderately resistant isolates were compared to sequences from sensitive isolates to identify polymorphic sites responsible that were

associated with tebuconazole resistance. Polymorphic sites in the coding regions were identified as synonymous (same amino acid) or non-synonymous (different amino acid).

RNA extraction and reverse transcription

Twelve isolates were selected to measure expression levels of *Cyp51* and *ScAtrG* (an ABC transporter gene). These isolates included: four *S. caricae* isolates highly resistant (HR) to tebuconazole (GA8007 and GA8016 from Cook County, GA sampled in 2014 and 2245 sampled from Brazil) or moderately resistant (MR) to tebuconazole (GA6074 from Tift County GA sampled in 2013) and four *S. caricae* isolates sensitive (S) to tebuconazole (C166, RG3, ATCC5257, and CBS102399). For comparison among GSB species, expression levels were measured in two sensitive *S. citrulli* isolates (GA8073 from Cook County, GA sampled in 2014 and C5-5) and two sensitive *S. cucurbitacearum* isolates (GSB26 and RT2).

Approximately 100 mg fresh mycelium of each isolate was harvested from PDA amended with tebuconazole (0.3 μg/ml) after incubating at 25°C in the dark for 7 days by removing the mycelium from PDA covered with sterile cellophane. Mycelium was added to PureLink lysis buffer (Life Technologies, Carlsbad, CA, USA) containing 1% 2-mercaptoethanol. Two 5-mm glass beads were added to each tube, and the mixtures were homogenized at 1,750 RPM for 2 min using a 2010 Geno/Grinder. The manufacturer's instructions for the PureLink RNA Mini Kit were followed for extraction of total RNA from each isolate. The RNA quality was determined by electrophoresis on a 1% (w/v) agarose gel and the concentration was measured using a Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA).

Reverse transcription was carried out with a PrimerScript RT reagent Kit (TaKaRa Bio Inc., Otsu, Shiga, Japan). The genomic DNA elimination reaction required a 15-µl volume mixture containing: 3.5 µl RNA extract (1-2 ng), 3 µl 5× gDNA Eraser Buffer, and 1.5 µl gDNA Eraser. The mixture was incubated at 42°C for 2 min. Reverse transcription was conducted in a 30-µl volume containing: 15 µl gDNA elimination reaction solution from the previous step, 4.5 µl 5× PrimeScript Buffer 2, 1.5 µl PrimeScript RT Enzyme Mix I, and 1.5 µl RT Primer Mix. The mixture was incubated at 37°C for 15 min, followed by 85°C for 2 seconds. The complementary DNA (cDNA) was stored at -20°C. To replicate the experiment, the same 12 isolates were grown a second time, then the RNA was extracted and reverse transcribed to cDNA as described above.

Expression level of *Cyp51* and *ScAtrG* by real-time PCR

A pair of primers (Table 2) with 100% similarity to all three species was developed for amplification of a 110 bp fragment of *Cyp51* for real-time PCR. *Actin*, a gene with a constant expression level, was used as an internal control in the real-time PCR. *Actin* was identified in the draft genomes of the nine GSB fungi by MegaBLAST with the homolog from *Didymella rabiei* (GenBank accession KM244530; Kim et al. 2015). Primers (Table 2) with 100% similarity to all three GSB species were designed to amplify a 93-bp fragment of *Actin*.

To investigate if increased fungicide efflux from the cell by a transporter was associated with tebuconazole resistance in *S. caricae*, the expression levels of a transporter gene were measured by real-time PCR. Using homologs of transporter genes in *Botrytinia fuckeliana,* the draft genomes of nine GSB isolates were searched by megaBLAST. The genes

included two ABC transporters: *BcatrB* (GenBank accession AJ006217; Schoonbeek et al., 2001) in the pleiotropic drug resistance (ABC-G) group and *BcatrD* (GenBank accession AJ272521; Hayashi et al. 2001) in the multidrug resistance (ABC-B) group; and one type of MFS transporter: *MfsM2* (GenBank accession CQ292707; Kretschner et al. 2009). Only a homolog of the pleiotropic drug resistance group (ABC-G), which we named *ScAtrG,* was identified in the draft genomes of GSB fungi. A set of primers (Table 2) with 100% similarity to the three *Stagonosporopsis* species was designed for the amplification of a 66-bp fragment of *ScAtrG* by real-time PCR.

Real-time PCR reactions were conducted to detect the expression levels of *Cyp51*, *ScAtrG* and *Actin* on 12 GSB isolates using StepOnePlus Real-Time PCR System (Life Technologies). Three technical replicate reactions were conducted for each gene for each of the 12 isolates. Each reaction was conducted in 20-µl volumes containing: 2.0 µl cDNA template, 10.0 µl Power SYBR Green PCR Master Mix (Life Technologies), 1.0 µl forward primer and 1.0 µl reverse primer. The 'two-step' cycling conditions were an initial heating at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and a final heating at 95°C for 15 s. The cycle threshold (C_t) value of each reaction was recorded as the expression level. The expression level of each gene for each isolate was calculated as the mean C_t value of the three technical replicates, when the standard error of the three C_t values was less than 0.5. The mean C_t values of *Cyp51* and *ScAtrG* were standardized by the mean C_t values of the internal control *Actin* to determine the relative expression level of *Cyp51* and *ScAtrG*. All steps from isolate growth to real-time PCR were repeated a second time. ANOVA was conducted using JMP Pro v11 (SAS Institute, Cary, NC) to identify

significant differences in expression level between tebuconazole-resistant and tebuconazole-sensitive isolates.

RESULTS

Differences in tebuconazole sensitivity of *Stagonosporopsis* species

All 303 *S. citrulli* isolates collected from the southeastern U.S. in 2013 and 2014 were found to be sensitive to tebuconazole (Table 1). All *S. caricae* isolates from the southeastern U.S. were moderately resistant (n = 3) or highly resistant (n = 16) to tebuconazole (Table 1). The *S. caricae* isolate from Brazil was highly resistant to tebuconazole. All other GSB or papaya fruit rot *Stagonosporopsis* isolates assayed were sensitive to tebuconazole, including nine *S. caricae* isolates originally collected from outside the southeastern U.S.

Cyp51 gene sequence and primer pairs

The numbers of contigs with a minimum length of 200 bp varied from 2,275 in *S. caricae* isolate RG3 to 7,991 in *S. cucurbitacearum* isolate GSB26. The *Cyp51* gene was 1,676 bp in length (Fig. 1) and contained three exons (246 bp, 198 bp, and 1,134 bp) and two introns (50 bp and 48 bp) (GenBank accessions KU847917 - KU847921). The *Cyp51* genes identified in the GSB or papaya fruit rot fungi and *D. exigua* were homologous to *Cyp51B* of *G. zeae* and *A. fumigatus*. The homologs of *Cyp51A* of *G. zeae* and *A. fumigatus* were not detected in the nine draft genomes or the complete genome of *D. exigua.* A total of 1,314 bp of the upstream promoter region was sequenced.

Cyp51 sequence variation among *S. caricae*

The *Cyp51* genes from 11 highly tebuconazole-resistant *S. caricae* isolates, three moderately resistant isolates and two tebuconazole-sensitive isolates were sequenced and compared with sequences from the draft genomes of three tebuconazole-sensitive *S. caricae* isolates and two additional tebuconazole-sensitive isolates. There were five different haplotypes among the *S. caricae* isolates (Fig. 1). The upstream promoter region, the first exon, and both introns were identical among the haplotypes. Tebuconazole-sensitive isolates were represented haplotypes 1 and 2. Haplotype 1 (GenBank Accession KU847917), which had two synonymous substitutions in the third exon, was shared by two tebuconazole-sensitive isolates (C166 and ATCC5257). Haplotype 2 (GenBank Accession KU847918), shared by nine highly tebuconazole-resistant isolates (GA6057, GA6081, GA8007, GA8016, GA8036, GA8051, GA8061, GA8091, and GA8098) and three sensitive isolates (RG3, CBS102399, and CSB248.90), was the most common. Highly resistant isolate GA8002 had haplotype 3 (GenBank Accession KU847919), which contained a non-synonymous substitution in the second exon (isoleucine (I) to threonine (T) at codon position 113). Haplotype 4 (GenBank Accession KU847920) was shared by highly resistant isolate 2245 and moderately resistant isolate GA8025, and contained a non-synonymous substitution in the third exon (glycine (G) to serine (S) at codon position 463). The other two moderately resistant isolates (GA6011 and GA6075) shared haplotype 5 (KU827921), which had one non-synonymous substitution (alanine (A) to glycine (G) at codon position 464) in the third exon. None of the substitutions was associated with tebuconazole resistance.

No difference in expression levels of *Cyp51* and *ScAtrG* between resistant and sensitive isolates

Using the expression level of *Actin* as an internal control, the induced expression levels of *Cyp51* and *ScAtrG* for isolates were calculated by averaging the two experimental replicates since there was no significant difference between experiments (P = 0.305 for *Cyp51*, and P = 0.519 for *ScAtrG*) or isolate by experiment interaction (P = 0.539 for *Cyp51*, and P = 0.263 for *ScAtrG*). For *Cyp51*, the relative expression levels among tebuconazole-resistant *S. caricae* ($\bar{x}$ = 1.077), tebuconazole-sensitive *S. caricae* ($\bar{x}$ = 1.003), and tebuconazole-sensitive *S. citrulli* ($\bar{x}$ = 1.057) and *S. cucurbitacearum* ($\bar{x}$ = 0.993) were not significantly different. There were no significant differences in expression levels between the three resistant *S. caricae* isolates and the moderately resistant *S. caricae* isolate. In addition, the average expression levels of the ABC transporter, *ScAtrG,* for the four isolates were also similar with no significant differences detected among tebuconazole-resistant *S. caricae* ($\bar{x}$ = 1.307), tebuconazole-sensitive *S. caricae* ($\bar{x}$ = 1.354), and tebuconazole-sensitive *S. citrulli* ($\bar{x}$ = 1.080) and *S. cucurbitacearum* ($\bar{x}$ = 1.287) isolates.

DISCUSSION

Tebuconazole, a widely used triazole fungicide, is considered very effective in the management of gummy stem blight (GSB) of cucurbits in the southeastern U.S. (Keinath and Hansen 2013; Thomas et al. 2012b). However, our identification of resistant isolates collected from watermelon fields in Georgia and observations of resistance in a field trial in Georgia (unpublished data) indicate that the efficacy of triazole fungicides in the southeastern U.S. could decline in the next few years. In a tebuconazole fungicide

sensitivity assay, we identified 16 highly resistant (two from Tift County in 2013 and 14 from Cook County in 2014) and 3 moderately resistant (two from Tift County in 2013 and one from Cook County in 2014) GSB isolates. One isolate from Brazil was also identified as highly resistant. All of the tebuconazole-resistant GSB isolates identified from the southeastern U.S. and Brazil were *S. caricae*, and all *S. caricae* isolates from the southeastern U.S. were highly resistant or moderately resistant to tebuconazole. However, isolates of *S. caricae* sampled from watermelon in California, cucumber in Michigan, a cucurbit in Brazil, and papaya in Hawaii, Brazil, and Chile were sensitive to tebuconazole (Table 1), which indicates that *S. caricae* is not simply a more resistant species overall. Although the date when the sensitive *S. caricae* isolates were sampled is unknown, they were all obtained prior to 2013 when tebuconazole resistance was first detected in isolates from Georgia. *Stagonosporopsis citrulli,* the dominant species causing GSB in the southeastern U.S. (Stewart et al. 2015), remains sensitive to tebuconazole. The three isolates of *S. cucurbitacearum* collected from regions outside the southeastern U.S. were also sensitive to tebuconazole.

Although *S. citrulli* and *S. caricae* are morphologically indistinguishable, distinct differences in tebuconazole sensitivity are evident between these two species causing GSB in the southeastern U.S. The decline in efficacy of MBC (Keinath and Zitter 1998), QoI (Keinath 2009; Stevenson et al. 2004), and SDHI (Keinath 2012; Thomas et al. 2012a) fungicides against GSB fungi resulted from increased frequency of resistant isolates due to strong selection pressure from each fungicide. It is not clear how or from where the tebuconazole-resistant *S. caricae* populations arose in the southeastern U.S (Li and Brewer, 2016), but as tebuconazole use for GSB continues, it is possible that selection for resistant

isolates of *S. caricae* with greater fitness and against sensitive isolates of *S. citrulli* may result in changes in the species composition of GSB fungi in the southeastern U.S. Alternatively, *S. caricae* could be introduced from the papaya-growing area in Central America by way of infested seed. With that, this species could accumulate within the watermelon field in the southeastern U.S.

We investigated the underlying genetic basis of tebuconazole resistance within *S. caricae* by comparing the nucleotide sequence of *Cyp51* in resistant and sensitive isolates. Triazole molecules bind to lanosterol 14α-demethylase (Kelly and Kelly 2013), and, therefore, a single-site mutation in *Cyp51* causing an amino acid change in the enzyme is responsible for triazole resistance in several plant-pathogenic fungi, including *Erysiphe necator* (Frenkel et al. 2015), *Phakopsora pachyrhizi* (Schmitz et al. 2014), and *Zymoseptoria tritici* (Cools et al. 2011). We sequenced and compared the coding region (1,676 bp) and promoter region (1,314 bp) of *Cyp51* from 11 highly resistant, three moderately resistant, and five sensitive *S. caricae* isolates. The promoter regions were compared because insertions were found in the promoter regions of *Cyp51* in triazole-resistant *Venturia inaequalis* (Schnabel and Jones 2001) and *Z. tritici* (Cools et al. 2012), which were responsible for increasing transcription. However, the promoter region, both introns, and the first exon were identical among sequences for all 19 isolates of *S. caricae* that were compared. Although there were polymorphic sites in the second and third exons, none of the haplotypes was clearly associated with the tebuconazole-resistant phenotype. Moreover, the most common haplotype was shared among resistant and sensitive isolates. Frequently reported mutational sites and amino acid substitutions identified in several other plant-pathogenic fungi, e.g. Y136F (Cools et al. 2013; Price et al. 2015), were not

found in any of the tebuconazole-resistant *S. caricae* isolates. Thus, triazole resistance in *S. caricae* in the southeastern U.S. is not likely due to mutations in the *Cyp51* gene or upstream promoter region.

Increased expression of *Cyp51* is another mechanism for triazole resistance in fungi that we investigated in *S. caricae*. In addition to insertions in the promoter region of *Cyp51* leading to increased expression (Cools et al. 2012; Schnabel and Jones 2001), other unknown transcriptional enhancing factors in triazole-resistant isolates may act on the promoter region of *Cyp51,* as is seen in resistant *S. homoeocarpa* (Ma and Tredway 2013). After growing on tebuconazole-amended media for 7 days, the average expression levels of *Cyp51* in tebuconazole-resistant *S. caricae*, tebuconazole-sensitive *S. caricae*, and other tebuconazole-sensitive GSB fungi were not significantly different, therefore, gene duplication of *Cyp51* and other unknown transporters may be responsible for the tebuconazole resistance of *S. caricae.*

Differences in expression of some transporters, which are integral proteins that move molecules, including nutrients, and secrete substrates, such as toxic compounds, across membranes (Perlin et al. 2014), have been identified as a mechanism of triazole fungicide resistance. Transporters in the ABC and MFS superfamilies are responsible for decreasing fungicide accumulation within cells of many pathogenic fungi (Price et al. 2015). The ABC transporter homolog *ScAtrG,* identified and investigated in *S. caricae* in this study, did not show significant increases in expression in tebuconazole-resistant isolates. Homologs of other transporters that could be involved in fungicide efflux from the cell were not readily identified in the draft genome sequence of *S. caricae*, but it is possible that an unidentified transporter or transporters may contribute to triazole resistance.

Another mechanism for triazole resistance identified in fungi is multiple copies of *Cyp51* genes. Paralogs of *Cyp51,* designated as *Cyp51A* and *Cyp51B,* were first identified in the human pathogen *Aspergillus fumigatus* (Mellado et al. 2001). Both genes belonged to the lanosterol 14α-demethylase family. In a study of plant-pathogenic fungi, Yin et al. (2009) found that tebuconazole induced the expression of both *Cyp51A* and *Cyp51B* in *Fusarium asiaticum* and *F. graminearum*, and determined that the sequences of *Cyp51A* were different between the two species. In another study of *F. graminearum* populations, polymorphism was found among *Cyp51A* sequences, but not among *Cyp51B* sequences (Talas and McDonald, 2015). *Cyp51* homologs identified in *D. exigua* and the genomes of the three *Stagonosporopsis* species were orthologous to the more conserved *Cyp51B* of *A. fumigataus* and *F. graminearum*. Paralogs of *Cyp51B,* including *Cyp51A,* were not identified within the genomes of *D. exigua* (Mellado et al. 2001; Yin et al. 2009) or the three *Stagonosporopsis* species causing GSB. Interestingly, in recent studies of *Rhynchosporium commune*, three paralogs were identified, including *Cyp51A, Cyp51B*, and a pseudogene of *Cyp51A*, designated as *Cyp51A-p*, and the presence of *Cyp51A* was associated with triazole resistance (Brunner et al. 2016). Paralogs of *Cyp51*, including *Cyp51A*, could be present in resistant isolates of *S. caricae* and serve as a possible mechanism for tebuconazole resistance. The three draft genomes of *S. caricae* searched for *Cyp51* homologs were all generated from triazole-sensitive isolates, so it would be worthwhile to sequence the genome of a resistant isolate to scan for paralogs.

Although the molecular mechanism(s) for resistance to triazoles in *S. caricae* is not clear, we can rule out some of the known resistance mechanisms identified in other fungi, and now know that the underlying basis of resistance is more complex than originally

hypothesized. Studies investigating additional mechanisms are warranted, because knowledge of the molecular basis of resistance will inform us of the evolutionary processes leading to triazole resistance in GSB fungi, and possibly allow us to develop a molecular marker to quickly and accurately monitor triazole resistance in field populations. Future approaches could include RNA-seq to identify genes that are expressed at higher levels in resistant isolates or if paralogs of *Cyp51* are expressed. Recent work on *C. beticola* identified 110 genes that were expressed at different levels in triazole-resistant isolates (Bolton et al. 2015). Until the molecular basis of resistance to triazoles is identified, the PCR-based marker for distinguishing *Stagonosporopsis* species causing GSB (Brewer et al. 2015) can be used to screen for *S. caricae*, which is more likely to be triazole-resistant and resistance can then be tested by fungicide sensitivity assays.

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Table 4.1. Resistance or sensitivity to tebuconazole for isolates of *Stagonosporopsis* spp. causing gummy stem blight of cucurbits or fruit rot of papaya

Species (isolate)	Sampling location (year[a])	Original host	No. of isolates	Tebuconazole phenotype[b]
Field isolates				
S. citrulli	Cook Co., GA (2013)	Watermelon (*Citrullus lanatus*)	53	S
S. citrulli	Tift Co., GA (2013)	Watermelon	95	S
S. citrulli	Suwannee Co., FL (2013)	Watermelon	72	S
S. citrulli	Colleton Co., SC (2013)	Watermelon	22	S
S. citrulli	Cook Co., GA (2014)	Watermelon	61	S
S. caricae	Tift Co., GA (2013)	Watermelon	2	HR
S. caricae	Tift Co., GA (2013)	Watermelon	2	MR
S. caricae	Cook Co., GA (2014)	Watermelon	14	HR
S. caricae	Cook Co., GA (2014)	Watermelon	1	MR
Isolates with sequenced genomes				
S. citrulli (C5-5)	Tift Co., GA (2010)	Watermelon	1	S
S. citrulli (DIDYNY)	Onondaga Co., NY (N.A.)	Muskmelon (*Cucumis melo*)	1	S
S. citrulli (AcSq5)	Johnson Co., NC (2000)	Acorn squash (*Cucurbita pepo*)	1	S
S. cucurbitacearum (RT2)	MI (1997)	Butternut squash (*Cucurbita moschata*)	1	S
S. cucurbitacearum (GSB26)	Tompkins Co., NY (1992)	Muskmelon	1	S
S. cucurbitacearum (GSB29)	NY (1992)	Watermelon	1	S
S. caricae (RG3)	CA (N.A.)	Watermelon	1	S
S. caricae (ATCC5257)	HI (N.A.)	Papaya (*Carica papaya*)	1	S
S. caricae (C166)	MI (N.A.)	Cucumber (*Cucumis sativus*)	1	S

Other *S. caricae* isolates				
S. caricae (2245)	Brazil (N.A.)	Unknown cucurbit	1	HR
S. caricae (CBS102399)	Brazil (N.A.)	Papaya	1	S
S. caricae (CBS248.90)	Chile (N.A.)	Papaya	1	S

[a]N.A. indicates that the sampling year was unknown

[b]S indicates sensitive (relative growth (RG) < 25%), MR indicates moderately resistant (25% < RG < 75%), and HR indicates highly resistant (RG > 75%) based on *in vitro* mycelial growth assay on tebuconazole-amended media.

Table 4.2. Nucleotide sequence and purpose of primers used in this study

Primer	Sequence	Purpose(s)
ScCyp51_F-175	5'-TTTATCGCCCGATCTGCTCC-3'	Amplification of *Cyp51* coding region Sequencing of *Cyp51* coding region
ScCyp51_R1774	5'-TAACACACCAGGTAGTGCGG-3'	Amplification of *Cyp51* coding region Sequencing of *Cyp51* coding region
ScCyp51u_F-1314	5'-TAACCACCGTCGCTACACAC-3'	Amplification of *Cyp51* promoter region Sequencing of *Cyp51* promoter region
ScCyp51u_R215	5'-GGGTCCATGCCATAGGTGAC-3'	Amplification of *Cyp51* promoter region Sequencing of *Cyp51* promoter region
ScCyp51_F289	5'-GAGGCAACGCGCTGACTC-3'	Sequencing of *Cyp51* coding region
ScCyp51_F590	5'-GCACTCCGCTCCTACGTTAC-3'	Sequencing of *Cyp51* coding region
ScCyp51_R1055	5'-CCAGGCGAAGGAGAATCCAA-3'	Sequencing of *Cyp51* coding region
ScCyp51_R1390	5'-AGCTTCTGGTTCTTCCTCTTTGT-3'	Sequencing of *Cyp51* coding region
ScCyp51u_F-839	5'-GGTGCCACGAAGATGCTGAA-3'	Sequencing of *Cyp51* promoter region
ScCyp51u_R-188	5'-GCGATAAACTGACGACACGC-3'	Sequencing of *Cyp51* promoter region
ScCyp51_F1212	5'-CACGCACCCATTCACTCCATT-3'	Determining expression level of *Cyp51*
ScCyp51_R1321	5'-GAGAAGCCAGGCGAAGACATC-3'	Determining expression level of *Cyp51*
ScAct_F	5'-CTCCCATCAACCCCAAGTCCA-3'	Determining expression level of *Actin*
ScAct_R	5'-CAGCCTGGATGGAGACGTAGA-3'	Determining expression level of *Actin*
ScAtrG_F	5'-ACTGCTACTCCTAGCCAAGGGT-3'	Determining expression level of *AtrG*
ScAtrG_R	5'-GTCTGACCGTTGTCACCGATG-3'	Determining expression level of *AtrG*

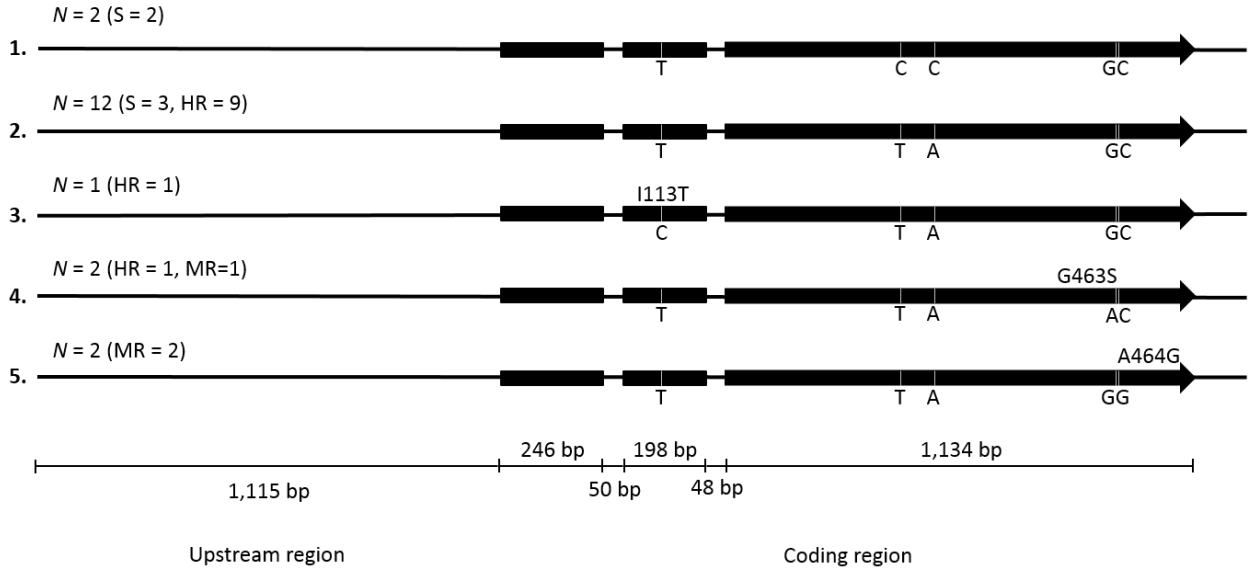

Fig. 4.1. Schematic of the *Cyp51* gene and upstream region of five genotypes from five tebuconazole-sensitive, 11 highly tebuconazole-resistant, and three moderately tebuconazole-resistant *Stagonosporopsis caricae* isolates. Total number of isolates (*N*), and number of sensitive (S), highly resistant (HR), and moderately resistant (MR) isolates with each genotype is shown. Black rectangles represent exons and arrows indicate the direction of transcription. Letters below the bars indicate polymorphic sites among the five genotypes. The amino acid locations of the putative protein for non-synonymous substitutions are indicated above the bar.

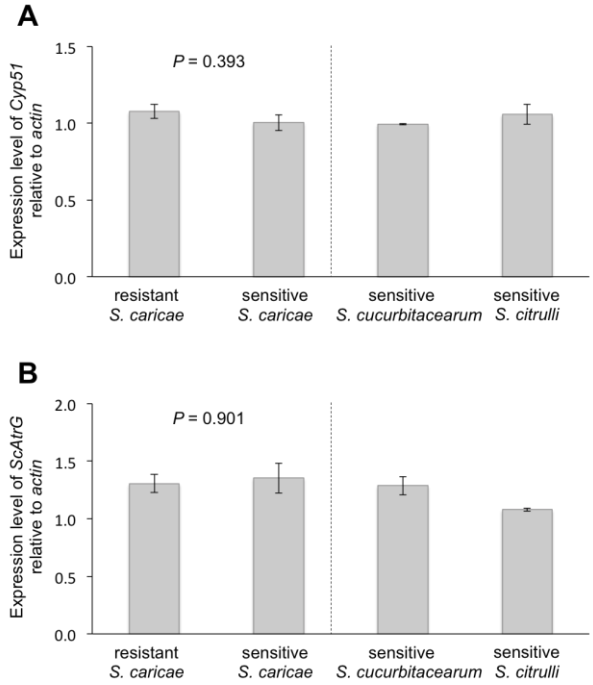

Fig. 4.2. Expression levels of **A**, *Cyp51* and **B**, *ScAtrG* relative to *Actin* among gummy stem blight fungi grown on media amended with tebuconazole (0.3 µg/ml) for 7 days. Error bars indicate the standard error of the four biological replicates of four tebuconazole-resistant and four tebuconazole-sensitive *S. caricae* isolates each averaged over two experiments, as well as two biological replicates of tebuconazole-sensitive *S. citrulli* and *S. cucurbitacearum* isolates averaged over two experiments. ANOVA was conducted among four highly tebuconazole-resistant and four tebuconazole-sensitive isolates of *S. caricae.* The *P*-value is shown to the left of the dashed reference line.

CHAPTER 5

IDENTIFICATION AND EVOLUTION OF MATING-TYPE GENES IN THREE *STAGONOSPOROPSIS* SPECIES[1]

[1] Li, H., T. M. Gottilla, and M. T. Brewer. To be submitted to *Current Genetics*.

ABSTRACT

Speciation and the mechanisms by which evolutionary change leads to the origin of new species serve as the foundation of all biodiversity. The divergence of closely related clades can result from reproductive differences that lead to genetic isolation and ultimately speciation. Numerous fungal diseases are caused by morphologically identical species that are genetically divergent and represent cryptic species. We were interested in identifying if differences in the mating system or reproductive genes among three *Stagonosporopsis* species (*S. citrulli*, *S. cucurbitacearum*, and *S. caricae*) causing gummy stem blight (GSB) of cucurbits underlie species divergence. The mating-type loci (*MAT1*) of three isolates from each of the three species were identified in assembled draft genome sequences. For the three species, *MAT1* was structurally identical and contained both mating-type genes necessary for sexual reproduction, which suggests that all three species are all homothallic. However, both *MAT1-1-1* and *MAT1-2-1* showed rapid evolution with a much greater number of amino acid-changing substitutions detected for both of the reproductive genes compared with genes flanking *MAT1*. Positive selection was detected in *MAT1-2-1*, especially in the highly conserved high mobility group (HMG) domain. Thus, positive selection on mating-type genes may have lead to reproductive isolation and driven speciation in GSB fungi and prevent the exchange of genes underlying recently evolved traits, such as fungicide resistance.

INTRODUCTION

The origin of new species represents the separating of lineages that purportedly evolve along independent trajectories due to the cessation of gene flow with other lineages.

The divergence of closely related clades on the evolutionary tree of life can result from differences in reproduction that lead to genetic isolation among clades. In fact, reproductive traits are often the most variable, both genetically and phenotypically (Billard et al. 2012). Eukaryotic organisms vary in their reproductive mode and may propagate by asexual reproduction to rapidly produce genetically identical clones (Anderson and Kohn 1995), sexual reproduction between two parents resulting in recombination and genetically diverse offspring (Heitman et al. 2013), or by both modes of reproduction. Sexual reproduction impacts the evolution of eukaryotes by driving genetic recombination and purging deleterious mutations (Ni et al. 2011), but there are costs to sexual reproduction, including having to find a mate, exposing oneself to disease, and contributing only half the number of genes as would be contributed in asexual reproduction (Otto 2009). In the process of speciation, divergence in reproductive mode or mating system or rapidly diverging reproductive genes can produce barriers between species, preventing gene flow and leading to reproductive isolation (Restrepo et al. 2014). Often there is a fitness cost to mating between closely related species, so it is advantageous for rapid evolution to occur on reproductive genes in order to prevent mating. Genes that have been subjected to selection can be detected by many methods (Aguileta et al. 2009), but one of the most widely used is measuring the ratio of nonsynonymous (d_N) to synonymous nucleotide (d_S) substitutions, d_N/d_S or ω (Nielseon 2005). If genes are diverging among species, or rapidly evolving under positive selection, then nonsynonymous mutations that change the amino acid sequence occur more frequently than synonymous mutations leading to $\omega > 1$ (Aguileta et al. 2009). Conversely, under purifying selection, nonsynonymous mutations are often deleterious, so they occur less frequently leading to ω

< 1. If a gene is not under selection, as in the case with pseudogenes, then nonsynonymous mutations and synonymous mutations are equally as likely to occur ($\omega \approx 1$). Knowledge of the mechanisms leading to reproductive isolation can help explain the formation of new species.

Selection on reproductive genes has been identified as a driver of speciation in many fungi (Stewart et al. 2011), including those in the phylum Ascomycota. Fungi in the phylum Ascomycota exist as haploids for most of their life cycle. Sexual reproduction occurs when two sexually compatible haploid individuals undergo plasmogamy producing dikaryotic hyphae that develop into the sexual fruiting body, or ascocarp. Within the ascocarp, karyogamy occurs and is quickly followed by meiosis and a mitotic division, producing eight ascospores in each ascus. Mating in Ascomycota, from recognition of compatible individuals to production of sexual structures, is regulated by a single mating-type locus, *MAT1*. Individuals have one of two possible forms at this locus, named *MAT1-1* and *MAT1-2*, respectively (Yoder et al. 1986). They are defined as idiomorphs rather than alleles, because of their dissimilar sequences and having descended from unrelated ancestors (Metzenberg and Glass 1990). The proteins encoded by mating-type genes are transcription factors (TFs) responsible for regulating cell identity and sexual development that control the mating ability of the fungus (Ni et al. 2011). The major gene of the *MAT1-1* idiomorph is *MAT1-1-1*, which encodes a TF with an α-box motif (Nelson 1996). This motif regulates the expression of pheromone and receptor genes responsible for mating recognition (Butler 2006). Some species contain other genes within the *MAT1-1* idiomorph, such as *MAT1-1-2* and *MAT1-1-3*, which have high-mobility-group (HMG) domains and are involved in the mating process. Within the *MAT1-2* idiomorph, *MAT1-2-1* is the major

mating-type gene. The product of this gene is a TF with an HMG domain that bends target DNA to activate transcription (Coppin et al. 1997). Heterothallic, or self-incompatible species in this phylum can mate if they are each different idiomorphs. Conversely, homothallic, or self-compatible, species have the major mating-type genes of both idiomorphs within their genomes (Roach et al. 2014). Therefore, they can complete sexual reproduction with themselves or outcross with different individuals. In homothallic species, the organization of mating-type genes within the genome is diverse. The genes can be adjacent or distantly located from one another. The direction of open reading frames (ORFs) varies among species (Debuchy and Paret 2006). Information on molecular structure and selection pattern of genes that are active in sexual reproduction is essential to predict the speciation of fungi in phylum Ascomycota.

An example of a recently diverged group of fungi that may have different reproductive modes or fast evolving mating-type genes are the gummy stem blight fungi. These fungi cause the plant disease gummy stem blight (GSB) that results in severe yield loss of crops in the family Cucurbitaceae around the world (Keinath 2011; van Steekelenburg 1983; Wiant 1945). Recently, Stewart et al. (2015) determined GSB is caused by three closely related *Stagonosporopsis* species with similar morphology: *S. cucurbitacearum* (syn*. Didymella bryoniae*), *S. citrulli*, and *S. caricae*. Additionally, *S. caricae* causes a fruit rot of papaya. Sexual reproduction of GSB fungi is known to occur in cucurbit fields, with the ascocarps, or pseudothecia, occasionally observed at the end of season. The mature pseudothecia are dark, globular and embedded within the host plant tissue or artificial media (Punithalingam and Holliday, 1972). The ascospores are forcibly discharged from each ascus and disseminated out of the pseudothecium through the ostiole (Chiu and

Walker, 1949). When conditions are suitable, the ascospores germinate to produce hyphae. Asexual spores, or conidia, are produced by GSB fungi in structures called pycnidia. The conidia also germinate to produce hyphae. Abundant pycnidia produced on infected plant tissue contribute to GSB epidemics (Schenck, 1968). Studies on the reproductive mode of GSB fungi have suggested that they are homothallic (Chiu and Walkers, 1949; Keinath, 2012); however, it is not clear which of the three species were investigated in these studies, since it has only recently been discovered that this group is composed of three different species (Stewart et al., 2015). Therefore, it is not clear if all three species have a homothallic mating system. Repeated attempts under numerous conditions to induce selfing or crossing within and among these three GSB species in our lab have been unsuccessful. The structure of the mating-type locus controlling and regulating mating in GSB fungi remains unknown. Insight into the genetic structure and evolutionary pressure on the *MAT1* locus could provide information on divergence and speciation of GSB fungi. Knowledge of the mating system and reproductive modes of all three species would be informative for disease epidemiology and management since sexual reproduction plays an important role in the disease epidemics of plant pathogenic fungi (McDonald and Linde 2002).

Within the same genus, another complex of three closely related, morphologically indistinguishable species causing a plant disease was identified. *Stagonosporopsis chrysanthemi*, *S. inoxydabilis* and *S. tanaceti* cause ray blight of pyrethrum (*Tanacetum cinerariifolium*), a plant in the family Asteraceae (Vaghefi et al. 2012). Among them, *S. tanaceti*, the species reported only in Australia and lacking a known sexual reproductive stage, was verified as a heterothallic species with only a single mating type, *MAT1-1* with

the major gene *MAT1-1-1,* at the *MAT1* locus. The globally distributed species *S. chrysanthemi* and *S. inoxydabilis* were both identified as homothallic, and contain both *MAT1-1-1* and *MAT1-2-1* at adjacent positions within the same genomes (Vaghefi et al. 2015). Genomes have recently been sequenced in our lab for three individuals of each of the three species of GSB fungi making investigation into the mating-type locus fairly straightforward. In our study of the *MAT1* locus of the *Stagonosporopsis* species causing GSB, the objectives were to: (i) identify the *MAT1* locus and mating-type genes of the three *Stagonospropsis* species; (ii) compare the structure and variation of the *MAT1* loci among the three species to provide insight into their mating systems; and (iii) determine if the mating-type genes in these species are rapidly evolving, which may have contributed to species divergence and continued reproductive isolation.

MATERIALS AND METHODS

DNA extraction and genome sequencing

To investigate the *MAT1* locus, three isolates of each *Stagonosporopsis* species causing GSB (or papaya fruit rot) were selected for whole genome sequencing. Isolates of *S. citrulli* included: C5-5, from watermelon (*Citrullus lanatus*) in Georgia; DIDYNY, from muskmelon (*Cucumis melo*) in New York; and AcSq5, from acorn squash (*Cucurbita pepo*) in North Carolina. The three isolates of *S. cucurbitacearum* were: GSB26, from muskmelon in New York; GSB29, from watermelon in New York; and RT2, from butternut squash (*Cucurbita moschata*) in Michigan. The *S. caricae* isolates were: ATCC5257, from papaya in Hawaii; C166, from cucumber (*Cucumis sativus*) in Michigan; and RG3, from watermelon in California.

A protocol developed by Fulton et al. (1995) was used to extract high quality DNA from all nine isolates. After growing at 25°C in the dark for 7 days on quarter-strength potato dextrose agar (qPDA) covered with sterile cellophane, approximately 500 mg mycelium of was removed and added to 17.5 ml cetyl trimethylammonium bromide (CTAB) lysis buffer. The lysis buffer of each reaction was pre-mixed with the following components: 6.5 ml of Buffer A (0.35 M sorbitol; 0.1 M Tris-HCl, pH 9; and 5 mM EDTA, pH 8), 6.5 ml of Buffer B (0.2 M Tris-HCl, pH 9; 50 mM EDTA, pH 8; 2 M NaCl; 2% CTAB), 2.6 ml of Buffer C (5% Sarkosyl), 1.75 ml PVP (0.1%), and 1.25 µl Proteinase K. Using 2010 Geno/Grinder (SPEX SamplePrep, Metuchen, NJ, United States), the mixture was agitated with two 5-mm glass beads (VWR Soda Lime, Radnor, PA, United States) at 1,750 RPM for 2 min. After adding 5.75 ml potassium acetate (5 M), tubes were inverted ten times and incubated on ice for 30 min. They were then centrifuged for 20 min at 14,000 *g*, then the supernatant was added to one volume of chloroform-isoamylalcohol (v/v 24:1). Another centrifugation for 10 min at 14,000 *g* followed, and then the supernatant was added to 100 µl RNase A (10 mg/ml) and incubated at 37°C for 120 min. Next, isopropanol at equal volume and sodium acetate at 1/10 volume were added. The mixture was then incubated at 25°C for 5 min. Centrifugation at 14,000 *g* for 30 min followed, and the supernatant was discarded. After rinsing twice with 70% ethanol and air-drying overnight, the DNA pellet was dissolved in 100 µl deionized H_2O.

Sequence annotation and comparison of *MAT1* locus

Genomic DNA for the nine isolates was submitted to the Georgia Genomics Facility (Athens, GA, United States) for next-generation sequencing (NGS) library preparation. Illumina sequencing reactions followed the NextSeq platform, based on a paired end 150-

bp (PE150) protocol (Illumina Inc. San Diego, CA, United States). All files of forward and reverse reads for each isolate were trimmed and concatenated. Using ABySS v.1.3.6 (Simpson et al. 2009) on the Georgia Advanced Computing Resource Center Linux cluster, each concatenated file with optimal 64-mer values, i.e., substrings of length 64 bp in DNA sequence data, were assembled *de novo* into draft genomes.

Sequences of each of the nine draft genomes were searched for homologous genes of the *MAT1* locus using megaBLAST (Altschul et al. 1990). The searches were conducted with nucleotide sequences from the *MAT1* locus of the homothallic species *S. chrysanthemi* (GenBank accession number KJ139676; Vaghefi et al. 2015), including the entire open reading frame (ORF) of *MAT1-1-1* and *MAT1-2-1*, as well as the *MAT1* locus of *Leptographium proganum* (GenBank accession number KC883458; Duong et al. 2013), containing the entire open reading frames (ORF) of *MAT1-1-1*, *MAT1-1-2*, and *MAT1-1-3*. Single contigs from draft genomes of the *Stagonosporopsis* species where the *MAT1* locus was located were further annotated. The entire ORF of *MAT1* flanking genes were predicted using the nucleotide sequence of the *MAT1* locus from *Leptosphaeria maculans* (GenBank accession number AY174048; Cozijnsen and Howlett 2003). Nucleotide sequences of identified *MAT1-1-1* and *MAT1-2-1* coding regions were used to search the GenBank protein database by BLASTx (Altschul et al. 1990), in order to locate conserved protein domains.

Measuring genetic diversity and selection

Once the *MAT1* locus, mating-type genes, and *MAT1*-flanking genes, *pyridoxamine 5´-phosphate oxidase* (*PPO*) and *DNA lyase* (*APN2*) of the three *Stagonosporopsis* species had

been identified, estimates of genetic diversity and selection were measured. Nucleotide sequences in each of the eight isolates where the *MAT1* locus was identified were aligned and compared using Geneious R9 (Biomatters Ltd. Auckland, New Zealand). The conserved regions of *MAT1-1-1* (α-box motif) and *MAT1-2-1* (HMG domain) were also compared. DnaSP v5.10.01 (Librado and Rozas 2009) was used to estimate measures of sequence diversity including the number of unique haplotypes (h); the number of segregating sites (S); the number of synonymous (d_S) and nonsynonymous (d_N) substitutions in coding regions; Nei's (1987) pairwise nucleotide diversity (π); and Watterson's (1975) unbiased estimation of nucleotide diversity (θ). Analyses were conducted within and among the three *Stagonosporopsis* species.

Selection on genes within and flanking the *MAT1* locus of the three *Stagonosporopsis* species was estimated based on d_N/d_S, or ω. Consensus sequences were generated for each of the three species for the following genes that were identified: *MAT1-1-1*, *MAT1-2-1, PPO*, and *APN2*, as well as the α-box motif of *MAT1-1-1* and HMG domain of *MAT1-2-*. Sequences of the conserved gene *Actin* from a previous study (Li et al. 2016; GenBank Accession Number: KX246907-KX246909) were also included for comparison to a region unlinked to *MAT1*. Alignment of each gene was conducted by Clustal Omega (EMBL-EBI, Hinxton, UK). A Neighbor-Joining tree was built for each alignment with the Jukes-Cantor model of evolution. Using sequence alignment and neighbor-joining trees, ω values were calculated with the maximum likelihood method by CODEML in PAML v4.8 package (Yang 2007). In order to detect the statistical power of positive selection, likelihood ratio tests were applied on three pairs of models in the program: positive selection (M2a) *vs*. nearly neutral (M1a); β (M7) *vs.* β & ω (M8); and one ratio (M0) *vs*. discrete (M3). Amino acid sites under

positive selection were estimated by Bayes empirical Bayes (BEB) analysis (Yang et al. 2005).

RESULTS

Identification of *MAT1*

We identified the *MAT1* locus in the draft genomes of the three *Stagonosporopsis* species that causes GSB (Fig. 5.1). The locus is also identified in an isolate of *S. caricae* that causes papaya fruit rot. The *MAT1* locus is not identified in one of the nine sequenced isolates, C5-5 of *S. citrulli*, most likely because it had lower coverage of assembled contigs. However, nucleotide sequences of the *MAT1* locus and flanking regions are identical between the other two isolates (AcSq5 and DIDYNY) of *S. citrulli*, as well as among all the three isolates (GSB26, GSB29, and RT2) of *S. cucurbitacearum*. The three sequenced isolates of *S. caricae* have identical sequence, as well, with one exception: in the second exon of *MAT1-1-1*, there is a synonymous substitution at site 1,027 in RG3. Among all the three species, the *MAT1-1-1* and *MAT1-2-1* genes are identified, but no other mating-type genes were found. The sequences of *MAT1* flanking genes in all three GSB species identical to those of the *Stagonosporopsis* species causing ray blight of pyrethrum. *PPO* is upstream of the *MAT1* locus and a ρ-GTPase activator (data not shown) is located upstream of *PPO*.

Structure and sequence diversity of *MAT1* loci

Among the three *Stagonosporopsis* species, the structure of *MAT1* is nearly identical (Fig. 5.1). *MAT1-1-1* contains two exons that are 232 bp and 983 bp. The intron of *MAT1-1-1* for *S. caricae* is 52 bp, compared to 50 bp for *S. citrulli* and *S. cucurbitacearum*. The

conserved α-box motif of *MAT1-1-1* was identified. It is 570 bp and spans the intron. *MAT1-2-1* is located 394 bp upstream of *MAT1-1-1*. The coding region of *MAT1-2-1* also consists of two exons, which are 439 and 107 bp. The conserved HMG domain is 135 bp and spans the 55-bp intron. *PPO* is located upstream of *MAT1-2-1*. The non-coding region between *PPO* and *MAT1-2-1* varies in length among the species: it is 1,115 bp in *S. cucurbitacearum*, and 1,123 bp in the other two species. *PPO* contains two exons that are 96 bp and 657 bp, with a 183 bp intron. *APN2* is located downstream of *MAT1-1-1*, with the opposite reading direction of the three genes upstream. The distance between the ORFs of *MAT1-1-1* and *APN2* is 1,247, 1,228, and 1,252 bp for *S. citrulli*, *S. cucurbitacearum*, and *S. caricae*, respectively. *APN2* contains three exons that are 217, 1,630 and 91 bp. The first intron is 55 bp in all three species and the second intron is 123 bp in *S. cucurbitacearum*, and 132 bp in the other two species. All of the sequence length differences among the three *Stagonosporopsis* species are in non-coding regions.

There is diversity of nucleotide sequences among the three *Stagonosporopsis* species (Table 5.1). The *MAT1* flanking gene *APN2* (π = 0.022, θ = 0.017) has higher nucleotide diversity than *MAT1-1-1* (π = 0.017, θ = 0.014). Excluding insertions and deletions, there were 95 segregating sites out of 2,125 bp *APN2* ORF compared to 46 segregating sites out of 1,265 bp ORF of *MAT1-1-1*. *MAT1-2-1* has lower nucleotide diversity (π = 0.007, θ = 0.005) than the upstream flanking gene *PPO* (π = 0.008, θ = 0.006). However, within the coding region, *MAT1-1-1* has 27 nonsynonymous substitutions and 16 synonymous substitutions, whereas *MAT1-2-1* has 6 nonsynonymous substitutions and one synonymous substitution, compared to 28 nonsynonymous substitutions and 60 synonymous substitutions of *APN2,* and zero nonsynonymous substitutions and 8 synonymous

substitutions for *PPO*. The nucleotide diversity of the conserved α-box motif is similar to that of the entire gene of *MAT1-1-1* (π = 0.013, θ = 0.010). However, there is a high ratio of nonsynonymous substitutions (d_N = 10) to synonymous substitutions and (d_S = 5) in this region. The nucleotide diversity of the conserved HMG domain of *MAT1-2-1* is relatively low (π = 0.007, θ = 0.006). This region has two segregating sites that are nonsynonymous.

Positive selection on *MAT1* locus

Analyses of evolutionary pressures acting on genes within the *MAT1* locus, the conserved regions of the genes, and the *MAT1*-flanking genes were conducted (Table 5.2). The two flanking genes and *Actin* show low ω values, i.e. the ratio of nonsynonymous (d_N) and synonymous substitutions (d_S), with ω = 0 for both *PPO* and *Actin*, and ω = 0.190 for *APN2. MAT1-1-1* has a relatively high ω value (0.894) and an even higher value for the conserved α-box motif (0.940). Amino acid site 383S is under positive selection based on BEB analysis; this site was not within the α-box motif. *MAT1-2-1* has a high ω value of 2.203, along with five amino acid sites under positive selection. The ω value fro the conserved HMG domain of this region is extremely high. Based on BEB analysis of the HMG domain, all 45 amino acid sites are under positive selection. However, the *p*-values of the likelihood ratio tests from three pairs of model comparisons are not all less than 0.05.

DISCUSSION

Based on the structure of *MAT1* identified in the genome sequences generated from this study, we conclude that the three closely related *Stagonosporopsis* species causing GSB or papaya fruit rot (*S. citrulli*, *S. cucurbitacearum*, and *S. caricae*) are all homothallic,

because both major mating-type genes, *MAT1-1-1* and *MAT1-2-1*, exist within each of the genomes. Despite of minor variations in the size of non-coding introns and intergenic regions among the three species, they all contained *MAT1-2-1* adjacent to *MAT1-1-1*. The structure of the *MAT1* locus in these three species is the same as two homothallic *Stagonosporopsis* species causing ray blight of pyrethrum: *S. chrysanthemi* and *S. inoxydabilis* (Vaghefi et al. 2015). Flanking the *MAT1* locus were *GTPase* and *PPO* in the upstream positions and *APN2* in the downstream positions. This architecture is similar to *Leptosphaeria maculans* causing blackleg of canola (*Brassica napus*) (Cozijnsen and Howlett 2003), which is in the same order Pleosporales of class Dothideomycetes as *Stagonosporopsis*. Other mating-type genes reported in some filamentous Ascomycota (Duong et al. 2013; Wey et al. 2016), but not required for sexual reproduction were not found. This is the first direct evidence, to our knowledge, since early reports on the disease (Chiu and Walkers, 1949; Keinath, 2012) that all three *Stagonosporopsis* species causing GSB are homothallic. Our results also provide evidence that *S. caricae* causing fruit rot of papaya has the genetic architecture of a homothallic fungus.

Homothallism impacts the population biology of *Stagonosporopsis* species, since selfing has the same clonal consequence as asexual reproduction by generating genetically identical offspring (Billiard et al. 2012). Populations of homothallic fungal species nonrandomly mate and are often in linkage disequilibrium since they contain large amounts of sexual clones. On the other hand, homothallic populations are able to maintain diverse genotypes from occasional outcrossing that can generate genotypic diversity. This type of population genetic pattern was reported in many homothallic fungi that are plant pathogens, such as *Leptosphaeria maculans* (Travadon et al. 2011), *Monilinia vaccinii-*

corymbosi (Burchhardt and Cubeta 2015) and *Sclerotinia sclerotiorum* (Atallah et al. 2004; Attanayake et al. 2012). A previous population genetic study of *S. citrulli* causing GSB in the southeastern U.S. at both a regional and fine-scale level within field revealed a similar pattern to other homothallic fungi showing numerous clones, linkage disequilibrium in total and clone-corrected populations, and evidence of outcrossing (Li and Brewer 2016). Although homothallic, there was high genetic diversity within each population that could facilitate adaptation to a changing environment, host, and disease management strategies, but currently adapted clones with higher fitness in the short term could rapidly accumulate and become widespread across different populations (Heitman et al. 2013).

Results from this study suggest that the *MAT1* locus may have played a role in the divergence of three *Stagonosporopsis* species. Previous work from Stewart et al. (2015) revealed nucleotide diversity ($0.002 < \pi < 0.011$) in four housekeeping genes: internal transcribed spacer region (*ITS*), b-tubulin (*BTUB*), chitin synthase I (*CHS*), and calmodulin (*CAL*). Diversity in nucleotide sequences was also observed from mating-type genes: *MAT1-1-1* ($\pi = 0.017$) and *MAT1-2-1* ($\pi = 0.007$), as well as the flanking regions ($\pi = 0.022$ for *APN2*, and $\pi = 0.088$ for *PPO*). The conserved α-box motif of *MAT1-1-1* ($\pi = 0.013$) and HMG domain of *MAT1-2-1* ($\pi = 0.007$), which play key roles in fungal mating, were diverse. In addition to the diverse nucleotide sequences among three species, the majority of segregating sites in the mating-type genes (27 of 46 for *MAT1-1-1* and 6 of 8 for *MAT1-2-1*) were nonsynonymous. Changes to the protein products of mating-type genes can be advantageous to the evolution of closely related species (Aguileta et al. 2009). The ratio of nonsynonymous to synonymous substitutions (ω) showed patterns of positive selection acting on the *MAT1* locus. The ω values were much lower for the flanking genes and an

unlinked housekeeping gene. Within *Stagonosporopsis* species, *MAT1-1-1* had a high ω value (0.849) and the value for its conserved α-box motif was even higher (0.940). The other mating-type gene, *MAT1-2-1* had an even greater ω value (2.203 overall), especially for the conserved HMG domain, where $\omega >> 1$, because there were no synonymous substitutions. BEB analysis showed that all 45 amino acid sites were under positive selection. Although the likelihood ratio tests comparing evolutionary models were not significant, we could still conclude that the *MAT1* locus, especially on the conserved HMG domain of *MAT1-2-1*, is under positive selection based on the other comparative analyses. As a faster rate of evolution is often associated with genes related to reproductive isolation (Wu and Ting, 2004), the *MAT1* locus is a hot spot of divergence in the evolutionary history of the three *Stagonosporopsis* species causing GSB. The divergence at the *MAT1* locus could be aprezygotic isolation barrier among three *Stagonosporopsis* species (Heitman et al. 2013).

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Table 5.1 Nucleotide diversity of mating-type locus (*MAT1*), with conserved region, and flanking genes of three gummy stem blight species

Gene	Species[a]	No. of Total sites	No. of haplotypes (h)	No. of segregating sites (S)[b]	No. of synonymous sites (dS)	No. of nonsynonymous sites (dN)	Nucleotide Diversity (π)	Watterson's θ
MAT1-1-1								
	S. caricae	1267	2	1	0	1	0.001	0.001
	S. citrulli	1265	1	0	0	0	0	0
	S. cucurbitacearum	1265	1	0	0	0	0	0
	Overall	1267	4	46	16	27	0.017	0.014
α-box motif								
	S. caricae	570	1	0	0	0	0	0
	S. citrulli	570	1	0	0	0	0	0
	S. cucurbitacearum	570	1	0	0	0	0	0
	Overall	570	3	15	5	10	0.013	0.010
MAT1-2-1								
	S. caricae	601	1	0	0	0	0	0
	S. citrulli	601	1	0	0	0	0	0
	S. cucurbitacearum	601	1	0	0	0	0	0
	Overall	601	3	8	1	6	0.007	0.005
HMG domain								
	S. caricae	135	1	0	0	0	0	0
	S. citrulli	135	1	0	0	0	0	0
	S. cucurbitacearum	135	1	0	0	0	0	0
	Overall	135	3	2	0	2	0.007	0.006
APN2								
	S. caricae	2125	2	3	2	0	0.001	0.001
	S. citrulli	2125	1	0	0	0	0	0
	S. cucurbitacearum	2116	1	0	0	0	0	0
	Overall	2125	4	95	60	28	0.022	0.017
PPO								
	S. caricae	936	1	0	0	0	0	0
	S. citrulli	936	2	1	1	0	0.001	0.001
	S. cucurbitacearum	936	1	0	0	0	0	0
	Overall	936	4	15	8	0	0.008	0.006

[a] *S. citrulli* contains two isolates; *S. caricae* and *S. cucurbitacearum* contain three isolates. Overall tests were based on all eight isolates.
[b] Number of segregating sites excludes insertions and deletions.

Table 5.2 Selection analyses on mating-type locus (*MAT1*), with conserved region, flanking genes, and *Actin* gene of three gummy stem blight species

Coding region	No. of nucleotides	No. of amino acids	ω [a]	No. of positively selected sites [b]
MAT1-1-1	1,212	404	0.849	1
α-box motif	570	190	0.940	0
MAT1-2-1	543	181	2.203	5
HMG domain	135	45	>> 1	45
APN2	1,935	645	0.190	0
PPO	750	250	0.000	0
Actin	1,125	375	0.000	0

[a] mean ratio of the nonsynonymous substitution rate (*dN*) and the synonymous substitution rate (*dS*)

[b] number of amino acid sites under positive selection based on Bayes empirical Bayes (BEB) analysis (Yang et al. 2005)

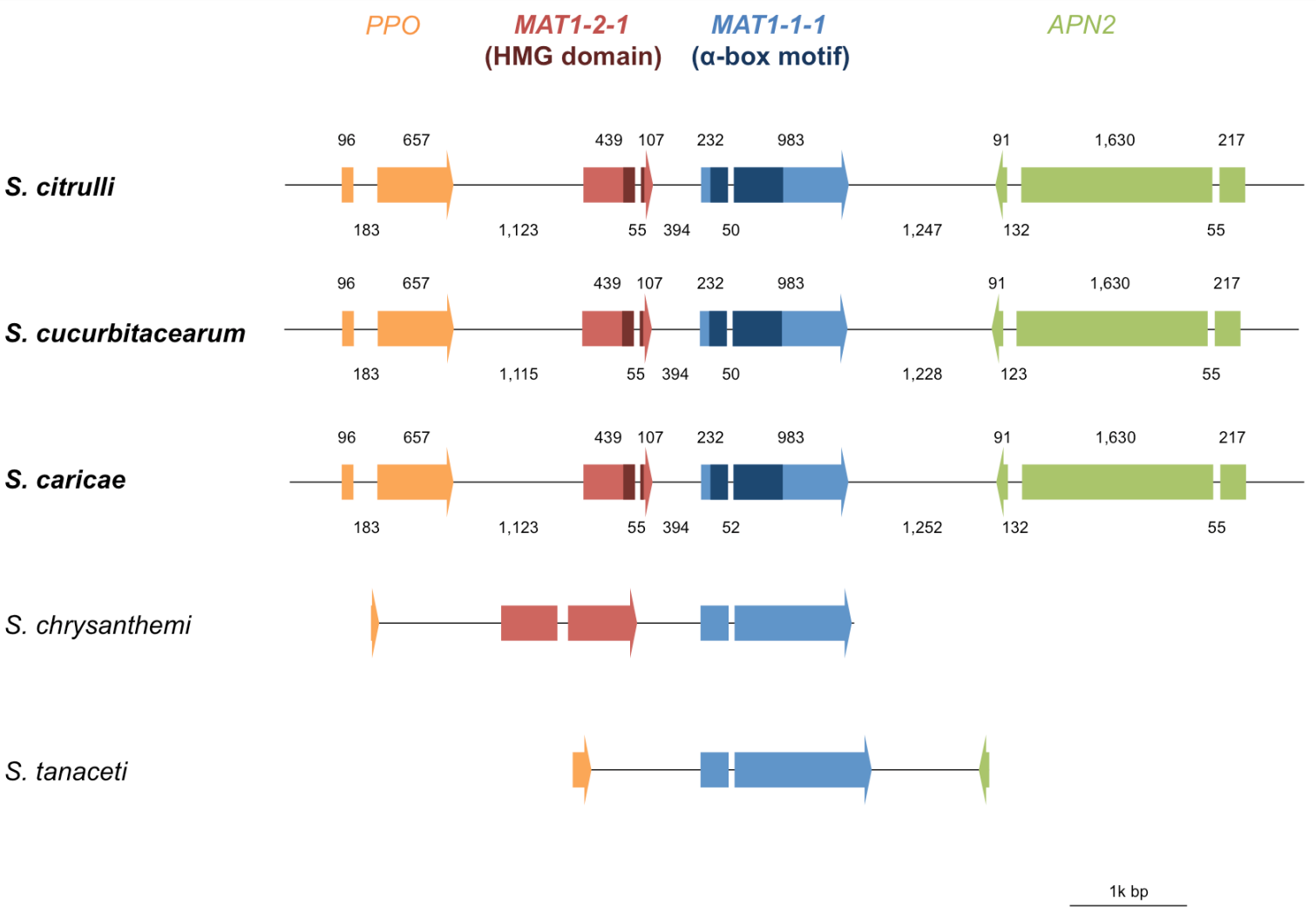
PPO
MAT1-2-1
(HMG domain)
MAT1-1-1
(α-box motif)
APN2
S. citrulli
96 657 439 107 232 983 91 1,630 217
183 1,123 55 394 50 1,247 132 55
S. cucurbitacearum
96 657 439 107 232 983 91 1,630 217
183 1,115 55 394 50 1,228 123 55
S. caricae
96 657 439 107 232 983 91 1,630 217
183 1,123 55 394 52 1,252 132 55
S. chrysanthemi
S. tanaceti
1k bp

Fig. 5.1 Schematic structure of mating-type locus (*MAT1*) and flanking region among three *Stagonosporopsis* species causing gummy stem blight on cucurbits and fruit rot on papaya, with homothallic species *S. chrysanthemi* (Genebank accession: KJ139673; Vaghefi et al. 2015) and heterothallic species *S. tanaceti* (Genebank accession: KJ139672; Vaghefi et al. 2015) as references. Coding regions of ortholog genes were illustrated in the same color. Conserved regions of two mating-type genes were shown in darker color. Arrows indicated the direction of reading. The number, where above numbers were coding region and below numbers were non-coding regions, represented size of the regions.