Mating Type and Simple Sequence Repeat Markers Indicate a Clonal Population of *Phyllosticta citricarpa* in Florida

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ABSTRACT

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Phyllosticta citricarpa, the citrus black spot pathogen, was first identified in Florida in March 2010. Subsequently, this pathogen has become established in Florida but can be easily confused with the endemic nonpathogenic citrus endophyte *P. capitalensis*. In this study, the mating-type (*MAT*) loci of *P. citricarpa* and *P. capitalensis* were identified via draft genome sequencing and were characterized at the structural and sequence levels. *P. citricarpa* was determined to have an

idiomorphic, heterothallic *MAT* locus structure, whereas *P. capitalensis* was found to have a single *MAT* locus consistent with a homothallic mating system. A survey of *P. citricarpa* isolates from Florida revealed that only the *MAT1-2* idiomorph existed in the Floridian population. In contrast, isolates collected from Australia exhibited a 1:1 ratio of *MAT1-1* and *MAT1-2* isolates. Development and analysis of simple sequence repeat markers revealed a single multilocus genotype (MLG) in the Floridian population ($n = 70$) and 11 MLG within the Australian population ($n = 24$). These results indicate that isolates of *P. citricarpa* from Florida are likely descendent from a single clonal lineage and are reproducing asexually. The disease management focus in Florida will need to be concentrated on the production and dispersal of pycnidiospores.

Citrus black spot caused by *Phyllosticta citricarpa* (McAlpine) van der Aa (teleomorph: *Guignardia citricarpa* Kiely) has been reported from many citrus-growing areas with humid subtropical climates, including regions in Southeast Asia, Oceania, Africa, South America, and most recently, North America (Kotzé 1981; Paul et al. 2005; Schubert et al. 2012; Yonow et al. 2013). The disease affects all commercially grown citrus cultivars, including sweet orange, mandarin, grapefruit, and lemon, among which lemon is the most susceptible (Baldassari et al. 2008; Kotzé 1981). Although sour orange (*C. aurantium*) and Tahiti lime (*C. latifolia*) do not produce citrus black spot symptoms, *P. citricarpa* can none-the-less be isolated from asymptomatic host tissue (Baldassari et al. 2008; Kotzé 1981). Citrus black spot can cause multiple, rind-limited fruit symptoms including hard spot, false melanose, freckle spot, cracked spot, and virulent spot (de Goes et al. 2000; Kiely 1948, 1949; Kotzé 1981). While blemished fruit is unappealing and unsuitable in the fresh market, the internal juice quality is not affected (Kotzé 1981). However, when citrus groves are severely infected by citrus black spot, fruit yield declines due to premature fruit drop (Kiely 1949; Kotzé 1981; Spósito et al. 2011), which is a particular concern for juice processors. Furthermore, *P. citricarpa* is currently listed as an A1 quarantine pest by the European and Mediterranean Plant Protection Organization. The European Union (EU) quarantine measures for citrus black spot have considerably slowed or prevented citrus export to the EU from countries with the disease (European Food Safety Authority (EFSA) 2014).

While citrus black spot inoculum can consist of either ascospores or pycnidiospores (conidia originating from pycnidia), epidemiological

studies outside of North America indicate the most important inoculum for the spread of citrus black spot is ascospores, which are solely produced in decomposing leaf litter under alternating wetting and drying cycles (Kotzé 1981; Reis et al. 2006; Spósito et al. 2008). During leaf-wetting events, ascospores are forcibly ejected up to 1.2 cm from mature pseudothecia and carried over some distance (<24.7 m) by wind currents to susceptible plant tissue (Spósito et al. 2007). Conidia produced by pycnidia on symptomatic fruit, dead twigs, or decomposing leaves are considered a source of secondary inoculum. This secondary inoculum plays an important role in spread within plant canopies in areas such as Brazil and, presumably, Florida, where an overlap of fruit maturities and frequent summer rains occur (Spósito et al. 2011). However, in South Africa and Australia, conidia are less important to the disease epidemiology, because the seasonal rainfall is limited in the summer, the bloom is restricted by cooler weather and irrigation practices, and fruit is usually harvested from trees before new fruit set begins (Kotzé 1981; Spósito et al. 2008).

Despite the well-documented role of ascospores in the epidemiology of citrus black spot (Kotzé 1981; McOnie 1964; Reis et al. 2006; Spósito et al. 2008), the factors regulating *P. citricarpa* ascospore production are unclear. Sexual reproduction in the Ascomycota is controlled by a single *MAT* locus with two idiomorphs, known as *MAT1-1* and *MAT1-2* (Debuchy and Turgeon 2006; Lee et al. 2010; Ni et al. 2011). The *MAT1-1* idiomorph contains the *MAT1-1-1* gene encoding an α -box protein, and the *MAT1-2* idiomorph carries the *MAT1-2-1* gene encoding a high mobility group (HMG)-domain protein (Lee et al. 2010; Ni et al. 2011). In heterothallic species, sexual reproduction occurs when opposite mating type partners interact (Lee et al. 2010; Ni et al. 2011). In contrast, homothallic fungi contain both *MAT1-1-1* and *MAT1-2-1* genes either in a fused *MAT* locus or in proximity, which allows the initiation of sexual reproduction without the requirement for an isolate of opposite mating type (Lee et al. 2010; Ni et al. 2011). Although this is the most commonly observed scenario, exceptions to this general *MAT* locus structure and regulation of mating have been reported

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(Butler et al. 2009; O'Connell et al. 2012; Vaillancourt et al. 2000). Because *P. citricarpa* is not fertile in single spore-derived cultures, this species is presumed heterothallic but the *MAT* locus structure and underlying regulation has not been characterized.

Given that *P. citricarpa* is an emerging pathogen in Florida, establishing the genetic structure of its one or more populations and determining whether the populations are undergoing random mating can provide valuable information for epidemiological modeling and disease management. A previous study evaluated the genetic diversity of *P. citricarpa* isolates from Florida, using multilocus sequence analysis with four conserved loci (ITS, TEF1, ACT, and GPDH) and a small number of *P. citricarpa* isolates from Florida, Brazil, South Africa, Zimbabwe, and Australia (Zavala et al. 2014). This study found that isolates, regardless of origin, were genotypically indistinguishable. This finding is likely attributed to the conserved nature of the four chosen markers. Greater genotypic resolution using markers such as simple sequence repeat (SSR) loci are needed to determine the genetic structure of *P. citricarpa* populations in Florida and compare this to populations from other citrus production areas.

In addition to *P. citricarpa*, *P. capitalensis* is commonly isolated from both diseased and healthy citrus fruit (Baayen et al. 2002; Glienke et al. 2011). *P. capitalensis* is a ubiquitous endophyte asymptotically colonizing citrus and many other hosts (Glienke et al. 2011). These two *Phyllosticta* species are morphologically very similar, but a single culture of *P. capitalensis* can produce fertile pseudothecia on artificial media (Baayen et al. 2002; Baldassari et al. 2008) whereas *P. citricarpa* cannot (Baayen et al. 2002; Baldassari et al. 2008; McOnie 1964). From these observations, *P. capitalensis* has been presumed to have a homothallic mating system and *P. citricarpa* a heterothallic system. A single report (Moran Lemir et al. 2000) concerning *P. citricarpa* ascospore production from a single isolate on an artificial medium has not been repeated by other groups (Baldassari et al. 2008), including our own. Whether the conditions for ascospore production by *P. citricarpa* have not been appropriate or whether *P. citricarpa* requires an opposite *MAT* type isolate to achieve sexual reproduction remains unknown. The overall objectives of this study were to identify and characterize *MAT* loci of *P. citricarpa* and *P. capitalensis* and to assess the genetic structure of the Floridian population of *P. citricarpa*. We utilized genomic sequence data and SSR markers generated in this study to test the hypothesis that *P. citricarpa* has a heterothallic mating structure and is randomly mating in Florida. The knowledge gained in this study will greatly improve our understanding of the epidemiology of citrus black spot in Florida and facilitate the development of disease control strategies aimed at limiting the spread of the disease.

MATERIALS AND METHODS

Fungal isolation and maintenance. The 113 *P. citricarpa* isolates and one *P. capitalensis* isolate from Florida and DNA samples of 26 *P. citricarpa* isolates from Australia, provided by A. Miles (Miles et al. 2013), used in this study were isolated from freckle spot lesions, hard spot lesions, or pycnidia within lesions on symptomatic citrus fruit (Supplementary Table S1). The identity of all fungal isolates was verified based on the production of yellow pigment on oatmeal agar (Baayen et al. 2002) and using species-specific primers designed by Peres et al. (2007). Afterward, fungal isolates were single-spored, were kept on colonized sterile filter paper in sterile coin envelopes, and were stored in plastic containers with desiccants in a -20°C freezer, as described by Hincapie et al. (2014). When needed, small pieces of filter paper were transferred onto half-strength potato dextrose agar ($1/2\text{PDA}$) (Difco, BD, Sparks, MD) and were maintained at 24°C under a 12-h photoperiod until use.

DNA isolation and genome sequencing. To isolate genomic DNA (gDNA), the method described by Hu et al. (2014) was

adopted. Briefly, fungal mycelia were grown on $1/2\text{PDA}$ with a layer of cellophane at 24°C under a 12-h photoperiod for 6 days. Following this growth period, 100 mg of fungal mycelia was scraped from the cellophane, was transferred into 2.0-ml screw cap tubes with sterile 5-mm stainless steel beads, was flash frozen with liquid nitrogen, and was homogenized twice for 30 s at 30 Hz, using a Qiagen TissueLyser II (Qiagen, Valencia, CA). gDNA was then isolated using the Qiagen plant mini kit (Qiagen), based on the manufacturer's protocol. The quality and quantity of gDNA was determined by measuring the absorbance at 260 to 280 nm and 260 to 230 nm, using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). All DNA samples were stored at -20°C until use.

To obtain gDNA for genome sequencing, gDNA samples from arbitrarily selected Floridian *P. citricarpa* isolate Gc12 and Floridian *P. capitalensis* isolate Gm33 were further purified by phenol-chloroform extraction and ethanol precipitation. The quality and quantity of gDNA was determined as above and by electrophoresis on a 0.6% agarose gel. More than 5 μg of gDNA, each, of *P. citricarpa* Gc12 and *P. capitalensis* Gm33 were submitted to the Interdisciplinary Center for Biotechnology Research (ICBR) Genomics Core at the University of Florida. Single-end sequencing was performed twice independently for each species on the Ion Torrent Personal Genome Machine (PGM) platform using 200- or 400-bp chemistry on an Ion 318 chip (Life Technologies, Carlsbad, CA).

Identification of *MAT* loci. Raw sequencing data from Ion Torrent PGM were de novo assembled using the de Bruijn algorithm implemented in the CLC Genomics Workbench version 5.0.1 (CLC Bio, Aarhus, Denmark) and the SPAdes genome assembler (version 3.5) (Bankevich et al. 2012). Multiple criteria including N50 value, total contig number, and the average contig length were used to decide the best assembly. Final assembly parameters utilized a window size of 20 and 30 nucleotides, a bubble of 175 and 400 nucleotides, and a minimum contig length of 200 nucleotides for sequencing data of *P. citricarpa* Gc12 and *P. capitalensis* Gm33, respectively. Protein-encoding genes were predicted from the assembly using the *ab initio* algorithm GeneMark-ES (Ter-Hovhannisyan et al. 2008). Homology searches were performed using BLASTn and BLASTp algorithms against the National Center for Biotechnology Information (NCBI) database and were displayed using EPoS 0.9.1 (Griebel et al. 2008) to search for *MAT*-associated genes, including *MAT1-1-1*, *MAT1-2-1*, *apn2* (DNA lyase), and *sla2* (cytoskeleton assembly control protein) genes.

During the analysis of genomic data from *P. citricarpa* Gc12, a *P. citricarpa* isolate from China, CGMCC3.14348, became available in the NCBI database (accession number AOTE000000000). This Chinese *P. citricarpa* genome was annotated and analyzed as above to identify and comparatively analyze its *MAT* locus relative to the *MAT* locus from Gc12.

Amplification of the flanking region and the coding sequence of *MAT1-2-1* of *P. citricarpa*. To determine the flanking sequence of *MAT1-2-1* of *P. citricarpa*, conventional polymerase chain reaction (PCR) using GcMAT-HMG-7 and GcMAT-PH-4 primers (Table 1) was performed in a 25- μl reaction volume containing 1 to 10 ng of gDNA, 0.2 μM of each primer, 0.2 mM of each dNTP, 1 $\times$ PCR reaction buffer, and 1 U of HotStar *Taq* polymerase (Qiagen), using an MJ Research PTC-200 Peltier thermal cycler (Bio-Rad Laboratories, Hercules, CA), according to the following conditions: 95°C for 5 min, 40 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, followed by a final extension of 5 min at 72°C . PCR amplicons were cloned into the pGEM-T Easy vectors (Promega Corp., Madison, WI) and were sequenced by Sanger chemistry at the ICBR, followed by BLASTn analysis against the NCBI database.

To determine the coding sequence of *MAT1-2-1* of *P. citricarpa*, total RNA was extracted using the Qiagen RNeasy plant mini kit (Qiagen), following the instructions of the manufacturer, was

quantified using a NanoDrop ND-1000, and was reverse transcribed into complementary DNA using SuperScript III reverse transcription (Invitrogen, Grand Island, NY) and oligo-dT₂₀, based on the instructions of the manufacturer. PCR reactions using GcitMAT-HMG-1 (5'-TTTCCTCTACTCAAGACCCTTCCC-3') and oligo-dT₂₀ were performed according to the conditions above, except the annealing temperature was 50°C. Subsequently, a nested PCR was performed as above, using primer GcitMAT-HMG-3 (5'-GCTCTCCACACGTTCCAGTCCC-3') and oligo-dT₂₀ with 100-fold diluted PCR products, and the resulting amplicons were cloned and sequenced as above.

Sequence alignment and phylogenetic analysis. Dot plot analysis of *MAT1-1* and *MAT1-2* of *P. citricarpa* was performed using the nucleic acid dot plot program on the Molecular Toolkit website (Maizel and Lenk 1981). Synteny analysis between *MAT1-1* and *MAT1-2* of *P. citricarpa* and between *MAT* idiomorphs of *P. citricarpa* and *P. capitalensis* was performed using the Artemis comparison tool (Carver et al. 2005). Sequence identity was analyzed using the Needleman-Wunsch global alignment algorithm available on the NCBI website. Predicted amino acid sequences of the PH-domain proteins of *P. citricarpa* and *P. capitalensis* were aligned using ClustalW2 along with other PH-domain proteins of Dothideomycetes, obtained from GenBank and the Joint Genome Institute genome databases (Supplementary Table S2). The maximum likelihood phylogenetic tree of amino acid sequences was generated accordingly, using MEGA version 6.0.5 (Tamura et al. 2013) with 1,000 bootstrap replicates under the Le and Gascuel (LG) + Gamma (G) model.

Screening of *MAT* idiomorphs of *P. citricarpa*. To determine the mating type of Floridian *P. citricarpa* isolates, specific primers targeting the coding sequence of *MAT1-1-1* or *MAT1-2-1* (Table 1) were designed, using Primer Premier version 5.0 (Premier Biosoft International, Palo Alto, CA) with the default settings. To validate the primer specificity, PCR reactions were performed under the following cycling conditions: 95°C for 5 min, 40 cycles of 95°C for 30 s, 55°C or 60°C for 45 s, and 72°C for 45 s, with a final extension of 10 min at 72°C. The resulting PCR products were analyzed by electrophoresis on a 1% agarose gel with SYBR Safe DNA stain (Invitrogen) in 1× Tris-acetate-EDTA (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.4) buffer at 110 V for 30 min and were visualized under ultraviolet light using a UVP-MultiDoc-It digital imaging system (UVP, Upland, CA). gDNA of *P. citricarpa* isolates BRIP 27888 from Australia and Gc12 from Florida were used as positive controls for *MAT1-1* and *MAT1-2* idiomorphs, respectively. gDNA from *P. capitalensis* isolate Gm33 was used as a negative control. Nuclease-free water (Qiagen) was used as a nontemplate control. The 113 Floridian isolates collected from 2010 through 2013 and 26 gDNA samples from the Australian population were evaluated for mating type loci using primers. χ^2 tests using PROC FREQ in SAS version 9.4 (SAS Institute, Cary, NC) were performed at $P = 0.05$, to compare the *MAT1-1*:*MAT1-2* ratio in each population to a 1:1 ratio.

SSR marker screening and primer design. Genome sequences of the Floridian isolate Gc12 and the Chinese isolate CGMCC3.14348 were analyzed with the default settings of the online tool BatchPrimer3 (You et al. 2008) to identify potential SSR markers and corresponding

primer pairs. Afterward, primer pairs were manually compared with select polymorphic SSR markers between the genomes of the two isolates. In total, 13 primer pairs were selected for further PCR assays. A summary of SSR markers and primers is shown in Table 2. To adopt a method for economically labeling PCR fragments with a fluorescent dye (Schuelke 2000), an M13 tail (5'-TGTAACACGACGGCCAGT-3') was linked to the 5' end of the forward primer of each primer pair, and the universal M13 primer (5'-TGTAACACGACGGCCAGT-3') labeled with 6-FAM was then used for incorporation of the fluorescent dye into PCR products. To fit the 96-well format of downstream processes, gDNA of the 72 Floridian *P. citricarpa* isolates, representative of the geographical and temporal diversity of all isolates, and 26 arbitrarily selected Australian isolates were chosen for genotyping analysis. DNA was exhausted in preliminary trials from two of the original 26 Australian isolates and DNA from two of the 72 gDNAs from the Floridian isolates failed to amplify. These isolates were eliminated from further analysis such that, in total, 70 Floridian isolates and 24 Australian isolates were analyzed. Touchdown PCR was performed in 15- μ l reaction volumes (0.5 μ l of gDNA [5 to 10 ng], 0.05 μ M of M13-tailed forward primer, 0.2 μ M of reverse primer, 0.2 μ M of 6-FAM-labeled M13 universal primer, 0.2 mM of each dNTP, 1× PCR reaction buffer, 2 mM of MgCl₂, and 0.025 U of *Taq* DNA polymerase per microliter [Qiagen]), under the following cycling conditions: 95°C for 5 min, 20 cycles of 95°C for 30 s, 60°C for 30 s, with a decrease of annealing temperature of 0.5°C in each cycle, and 72°C for 30 s, followed by 25 cycles of 95°C for 30 s, 50°C for 45 s, and 72°C for 45 s, with a final extension of 45 min at 72°C.

Fragment length analysis and population genetic statistics. Fragment analysis of PCR products was performed on an Applied BioSystems 3730xl DNA Analyzer at the ICBR with the GeneScan 600 LIZ size standard (Applied Biosystems by Life Technologies). Allele size was determined using GeneMarker Software version 2.6.3 (SoftGenetics, State college, PA) and analyzed using GenAlEx version 6.5 (Peakall and Smouse 2012). Population genetic statistics including Nei's unbiased genotypic diversity (Hexp), Simpson's index of diversity (1-D), evenness (E.5), number of multilocus genotypes (MLG), number of expected MLG at the smallest sample size (eMLG), Shannon-Wiener index of MLG diversity (H), Stoddart and Taylor's index of MLG diversity (G), index of association (I_A), and standardized index of association ($\bar{r}_d$) were determined with the *poppr* package in R (version 2.0.2) (Kamvar et al. 2014). Hexp is the probability of two randomly chosen genotypes being different and ranges from zero to one. 1-D is also the probability that two randomly selected individuals belong to different genotypes. E.5 is the distribution of genotype abundances and ranges from zero, where a single genotype is dominant within a population, to one, where genotypes are equally abundant within a population. Under the random mating scenario, eMLG would be equal to MLG, and a larger H or G would be revealed due to the high genetic diversity of a population. For I_A and $\bar{r}_d$, the null hypothesis was that the loci are independently associated and the population is expected to undergo random mating. If the observed I_A and $\bar{r}_d$ were not significantly different from those obtained from the randomized

TABLE 1. Primers used for amplification of the mating-type (*MAT*) locus of *Phyllosticta citricarpa*

Name	Primer sequence (5' to 3') ^a	Tm ^b (°C)	Amplicon size (bp)	Target
Gcmat1-56f	CTCAAGCCCAATCAATCAG	60	630	<i>MAT1-1-1</i>
Gcmat1-686r	AGGATGTCAATGTGAGGGCA	60	630	<i>MAT1-1-1</i>
Gcmat2-686f	CTTCAGCCAGCTTCTTCTTG	55	500	<i>MAT1-2-1</i>
Gcmat2-1185r	AGAGCAGAAGGTCTTGCGTA	55	500	<i>MAT1-2-1</i>
GcMAT-HMG-7	CATCGCCCAACACATCAAAGCGACCTA	56	448	Intergenic region
GcMAT-PH-4	CGGTGCTCTCCTTTTTCCTGGA	56	448	Intergenic region

^a Primers specifically targeting either *MAT1-1-1* or *MAT1-2-1* of *P. citricarpa* were designed using Primer Premier software version 5.0.

^b Tm = melting temperature.

data, the null hypothesis would not be rejected, indicating the population is consistent with random mating.

RESULTS

Identification of *MAT* loci. Initial attempts to isolate sequences of the *P. citricarpa* *MAT* locus followed a PCR-based strategy, using degenerate primers designed from *MAT* genes from *Cochliobolus heterostrophus*, *Alternaria alternata*, and *Stagonospora nodorum* (Arie et al. 1997, 2000; Bennett et al. 1999). None of the chosen primer combinations amplified a specific *MAT* locus sequence from *P. citricarpa* (data not shown). Subsequently, a draft genome-sequencing strategy was pursued for *P. citricarpa* Gc12 and *P. capitalensis* Gm33, using two runs of semiconductor-based next generation sequencing technology for each species. For *P. citricarpa* (Gc12), 1.0×10^7 reads totaling 2,360 Mbp (1,889 Mbp of Q20 nucleotides) with a mean read length of 226 bp were obtained. For *P. capitalensis* (Gm33), 9.3×10^6 reads totaling 2,281 Mbp (1,745 Mbp of Q20 nucleotides) with a mean read length of 245 bp were obtained. This whole-genome shotgun project has been deposited in the DDBJ/ENA/GenBank database under accession LOEN000000000 for *Phyllosticta citricarpa* Gc12 and under accession LOEO000000000 for *Phyllosticta capitalensis* Gm33. The versions described in this paper are versions LOEN01000000 and LOEO01000000, respectively.

From the assembled genome and predicted proteins of *P. citricarpa* isolate Gc12, BLAST queries using *C. heterostrophus* MAT1-2-1 identified a 1,463-bp fragment on contig 3787 (contig length = 16,464 bp) with an E-value of $9e-19$. This fragment encoded a protein with high similarity to MAT1-2-1 from *Sphaeropsis sapinea* (E-value = $5e-31$). Contig 3787 also contained a putatively conserved

flanking gene of the *MAT* locus, *apn2*. Sequences of genes, including *sla2*, which have been reported to flank *MAT* loci of other ascomycete fungi were not predicted on this contig. Using BLASTn and BLASTp searches with *C. heterostrophus* MAT1-1-1 gene and protein as query sequences, no gene with homology to MAT1-1-1 was identified in the *P. citricarpa* Gc12 genome. Contig 513 (contig length = 29,282 bp) in the assembled genome of *P. capitalensis* isolate Gm33 was identified by BLAST queries to encode proteins homologous to MAT1-1-1 from *Neofusicoccum parvum* with an E-value of $8e-47$ and MAT1-2-1 of *Diplodia seriata* with an E-value of $8e-30$. Similar to *P. citricarpa*, *apn2* was the only conserved flanking gene identified in this contig. Following BLAST queries of the *P. citricarpa* isolate CGMCC3.14348 genome using *C. heterostrophus* *MAT* genes as above, a gene of 1,941 bp on contig 4071 (9,524 bp) was identified with an E-value of $3e-29$. This gene was further verified to encode a homolog of MAT1-1-1 from *N. parvum* (E-value = $5e-51$), based on the subsequent BLASTp search of NCBI. No sequences sharing homology with MAT1-2-1 sequences were identified on contig 4071 or elsewhere in the *P. citricarpa* CGMCC3.14348 genome.

The structural organization of the *P. citricarpa* Gc12 MAT1-2 locus was predicted by comparative sequence analysis with *P. capitalensis* Gm33 contig 338. Subsequently, *P. citricarpa* PCR primers GcMAT-HMG-7 and GcMAT-PH-4 were designed and used to amplify sequences that joined contigs 3787 and 2477 to provide a complete MAT1-2 locus sequence. The AUGUSTUS gene prediction tool was used to generate gene models for this joined contig, as well as for contig 513 from Gm33 and contig 4071 from *P. citricarpa* CGMCC3.14348. Multiple sequence alignments with related sequences from GenBank were used to confirm the coding sequence predictions. Reverse transcription-PCR was used to confirm the coding sequence of *P. citricarpa* MAT1-2-1. The

TABLE 2. Simple sequence repeat markers and primers developed for *Phyllosticta citricarpa*

Marker	Repeat motif in ^a		Primer sequence (5' to 3')	Population							
				Australian				Floridian			
	Gc12	CGMCC3.14348		Na ^b	Hexp ^c	1-D ^d	E.5 ^e	Na ^b	Hexp ^c	1-D ^d	E.5 ^e
6	(CAA) ₈	(CAA) ₁₁	F: GGCCTGCAGTACGATTTTA R: ATATCCACGTCCATCAACTC	3	0.81	0.54	0.89	1	–	–	–
7	(GTG) ₅	(GTG) ₈	F: AAGGTGGTCGTGGTCATC R: CAAGTTCCTGGGAGTACATCA	2	0.16	0.08	0.46	1	–	–	–
12	(ACC) ₁₁	(ACC) ₇	F: TAAAGTAATGACGCTCGACTC R: GAGAGAAAGGAGACGTGACA	2	0.83	0.41	0.85	1	–	–	–
19	(ACC) ₉	(ACC) ₈	F: GCAGGCACTACCTTAGACC R: GTCGAGGATGACAGTACCC	4	0.54	0.42	0.58	1	–	–	–
20	(CTG) ₁₃	(CTG) ₁₄	F: GTTTCGGCATCTTTGTTTT R: GATTCCCTAAACCTGCTGTTG	3	0.34	0.23	0.50	1	–	–	–
22	(AAG) ₆	(AAG) ₉	F: GAGTCCAAGCCTTCAAAAG R: ACTGAACCATCAGCTCAAGTA	1	–	–	–	1	–	–	–
28	(CTC) ₇	(CTC) ₈	F: ATGCCGTCAATGTATAACAAC R: ACTATCACGGTGAATGGAAC	1	–	–	–	1	–	–	–
29	(AGC) ₇	(AGC) ₆	F: GTCCTCCCTCGTACTCATTA R: TCTTAGAATGCTTTTCACGAC	1	–	–	–	1	–	–	–
32	(GGCT) ₅	(GGCT) ₇	F: TGTCTGAGGCTAAGAGTTCTG R: AGAAGGGAGAAGAGAGTTGAA	2	0.16	0.08	0.46	1	–	–	–
34	(AGGT) ₆	(AGGT) ₁₃	F: GTAGCATTGAGGACGAG R: ACCACTCAATTAACCACCATT	1	–	–	–	1	–	–	–
36	(CAA) ₉	(CAA) ₁₀	F: AATATGTCGAGCTACACAAGC R: GTTCCATAGAGAGCAAAGGTC	1	–	–	–	1	–	–	–
37	(CTC) ₈	(CTC) ₇	F: GCATCTCTTCTCCTTCTTCTT R: AAATCGAGACTGTGCTATTTG	2	0.16	0.08	0.46	1	–	–	–
41	(GGT) ₈	(GGT) ₉	F: TTGAACATTTTGAAGACCAAC R: GTGTCTGCCTCGAATCTATAA	1	–	–	–	1	–	–	–

^a Gc12 = *P. citricarpa* isolate with the MAT1-2 idiomorph; CGMCC3.14348 = *P. citricarpa* isolate with the MAT1-1 idiomorph.

^b Na = allele number.

^c Hexp = Nei's unbiased genotypic diversity, the probability of two randomly chosen genotypes being different, ranging from zero to one; the greater the value, the greater the diversity.

^d 1-D = Simpson's index of diversity, the probability that two randomly selected individuals belong to different genotypes, ranging from zero to one; the greater the value, the greater the diversity.

^e E.5 = evenness, the distribution of genotype abundances, ranging from zero, where a single genotype is dominant within a population, to one, where genotypes are equally abundant within a population.

resulting structural prediction of the *P. citricarpa* Gc12 and *P. capitalensis* Gm33 *MAT* loci are shown in Figure 1 and sequences were deposited in GenBank (accession numbers KT708823 and KT708824, respectively).

Defining boundaries of the *MAT* loci. Synteny mapping between the *P. citricarpa* *MAT* idiomorphs determined the sequence boundaries of the *MAT1-1* and *MAT1-2* idiomorphs. The conserved flanking sequences of the *MAT* locus begin upstream at the start of the *oml1* (overlapping *MAT* locus 1) 5' coding sequence and end downstream in the intergenic region of the *40S S9* gene (Figs. 1 and 2A and B). While the BLASTp search revealed that OML1 of *P. citricarpa* is homologous to MAT1-1-4 of *Diplodia pinea* (E-value = 2e-06), the *oml1* gene is primarily located in the conserved flanking sequences of both *MAT* idiomorphs of *P. citricarpa* and, therefore, as a whole is not considered part of the *MAT* locus (Figs. 1 and 2A and B). From this analysis, the sequences that comprise the *MAT1-1* and *MAT1-2* idiomorphs are 6,005 and 6,623 bp, respectively.

Structurally, the *MAT1-1* idiomorph contained one additional predicted gene downstream of *MAT1-1-1*; this gene encodes a protein with a putative integral membrane domain (the pleckstrin homology [PH] domain) (Fig. 1). The *MAT1-2* idiomorph also encoded a PH-domain protein downstream of the conserved *MAT1-2-1* gene, but it appears to be only distantly related to the PH-domain gene of the *MAT1-1* idiomorph, as the two sequences share only 56% identity at the nucleotide level. The *MAT1-2* idiomorph also encoded a unique hypothetical protein located downstream of *MAT1-2-1* and upstream of the PH-domain gene (Fig. 1). A BLASTp search of the NCBI database revealed that this hypothetical protein shared low identity to MAT1-2-5 of *D. pinea* (E-value = 1.9) (Bihon et al. 2014). However, since the designation of *MAT1-2-5* has been assigned to the cytochrome *c* oxidase subunit VIa gene (*cox13*) in *Coccidioides* species (Mandel et al. 2007), *MAT1-2-9* was, instead, used to designate this gene in the *MAT1-2* idiomorph according to the nomenclature proposed by Turgeon and Yoder (2000).

Gene prediction and BLAST analysis of the *P. capitalensis* contig 338 revealed that the *MAT* locus of *P. capitalensis* contained both *MAT1-1-1* and *MAT1-2-1* genes in close proximity (Fig. 1). This locus also contained the *MAT1-2-9* gene, a PH domain-encoding gene, and the conserved flanking genes found associated with the *P. citricarpa* *MAT* idiomorphs (Fig. 1). Synteny mapping between the *MAT* idiomorphs of *P. citricarpa* and the *MAT* locus of

P. capitalensis revealed a conservation of gene composition between the species with a fused *MAT1-1* and *MAT1-2* idiomorph structure in *P. capitalensis* (Fig. 2). Pairwise comparison between the *MAT1-1-1* and *MAT1-2-1* genes of *P. citricarpa* with the *P. capitalensis* orthologs revealed 68 to 70% nucleotide sequence identity (59 to 65% amino acid identity) (Table 3). The *oml1* gene of *P. capitalensis* shared similar nucleotide sequence identity (55 to 57%) with the allelic copies of the *P. citricarpa* orthologs, which were 92% identical to each other (Table 3). The PH-domain gene from *P. capitalensis* shared greater nucleotide identity (72%) with the *P. citricarpa* *MAT1-2* PH-domain gene than with the *P. citricarpa* *MAT1-1* PH-domain gene (54%) (Table 3). Maximum-likelihood phylogenetic analysis using PH-domain amino acid sequences from *P. citricarpa*, *P. capitalensis*, and other Dothideomycetes also showed well-supported clades for Pleosporales, Botryosphaerales, and Capnariales (Fig. 3). However, while the two PH-domain proteins of *P. citricarpa* clustered most closely with the PH-domain protein from *P. capitalensis*, the separate branching of the *MAT1-1* copy of the *P. citricarpa* PH-domain protein reflected its significant divergence (Fig. 3).

Mating type screen of the *P. citricarpa* population in Florida.

In preliminary trials with the Australian BRIP 27888 and the Floridian Gc12 isolates, the newly designed *MAT1-1*-specific primer pair (Gcmat1-1) amplified a 630-bp band from isolate BRIP 27888 only and the *MAT1-2*-specific primer pair (Gcmat1-2) produced a 500-bp amplicon from Gc12 only (Fig. 4). These amplicons were easily differentiated by agarose gel electrophoresis, and DNA sequencing of the amplicons verified their specific *MAT* locus identities (data not shown). Neither primer pair generated an amplicon when *P. capitalensis* gDNA was used as template, thus demonstrating the species-specificity of the primers (Fig. 4A). Among the entire collection ($n = 113$) of Floridian *P. citricarpa* isolates, a single 500-bp amplicon was generated from the gDNA for all isolates with the *MAT1-2*-specific primers (Table 4; Fig. 4B). No amplicon was produced with the *MAT1-1*-specific primers. Of the 26 gDNA samples of *P. citricarpa* from Australia, a nearly equal portion of amplicons from the *MAT1-1*-specific and the *MAT1-2*-specific primer pairs were generated. These were of the expected 630- and 500-bp size, respectively (Table 4; Fig. 4B). A χ^2 analysis showed that the ratio of *MAT1-1* and *MAT1-2* *MAT* amplicons in the Australian population was not significantly different from 1:1 ($\chi^2 = 0.0435$, $P = 0.8348$). In addition, three gDNA samples from Australian isolates amplified both a 500- and

Fig. 1. Structural organization of the mating-type (*MAT*) loci of *Phyllosticta citricarpa* and *P. capitalensis*. Arrowed boxes with gene names above indicate the position and orientation of open reading frames. Similar gene sequences are marked by the same shade. Black lines within the arrowed boxes indicate introns. The light gray boxes represent the idiomorphic region of the *MAT* locus of *P. citricarpa*. Black lines underneath *MAT* genes of *P. citricarpa* indicate amplified regions using *MAT*-specific primers. *apn2*, DNA lyase; *40S S9*, ribosomal protein 40S S9; *MAT1-1-1*, α box-containing protein; *MAT1-2-1*, high mobility group domain-containing protein; *PH-domain*, putative pleckstrin homology domain-containing protein (integral membrane protein); *oml1*, hypothetical protein (overlapping *MAT* locus 1); *MAT1-2-9*, hypothetical protein. The figure is to scale.

a 630-bp product, indicative of potential heterokaryosis among field isolates (Table 4).

Screening and amplification of SSR markers. Based on the outputs of BachPrimer3 for the two *P. citricarpa* genomes (isolates Gc12 and CGMCC3.14348), 13 primer pairs identical in both genomes but predicted to amplify polymorphic SSR loci were selected for touchdown PCR amplification (Table 2). Each primer pair, except marker 28, consistently amplified a single fragment from Floridian and Australian isolates. All markers were monomorphic across all isolates from the Floridian population tested (Table 2), except two isolates in which the primers failed to amplify.

Seven of the tested markers showed polymorphism for the Australian population (Table 2). The allele number of each marker varied from 2 to 4, and the Hexp (unbiased genotypic diversity index) values ranged from 0.16 to 0.81 (Table 2).

Diversity and population structure of *P. citricarpa* isolates. All 70 isolates tested from Florida belonged to a single multilocus genotype (MLG); however, the Australian population consisted of 11 unique MLGs resolved from seven SSR markers (Table 5; Supplementary Table S1; Fig. 5). Of the Australian population, four isolates belonged to the same MLG, MLG5, as the Floridian population. No genotype diversity was present in the Floridian

Fig. 2. Synteny between *MAT1-1* and *MAT1-2* idiormorphs of *Phyllosticta citricarpa* and the fused idiormorph of *P. capitalensis*. **A**, A dot plot straight black diagonal line indicates highly similar regions between idiormorphs. **B and C**, Lines generated by the Artemis Comparison Tool analysis indicate highly similar regions in the forward and reverse directions between idiormorphs.

population ($G = 1$) and significant genotype diversity was present in the Australian population ($G = 5.88$) (Table 5). The unbiased genotypic diversity was also greater in the Australian population ($H_{exp} = 0.87$) than in the Floridian population ($H_{exp} = 0$). The observed I_A and $\bar{r}_d$ values for the Australian population were 0.17 and 0.03, respectively, which did not significantly differ from those obtained from randomized data ($P = 0.131$ for I_A and $P = 0$ for $\bar{r}_d$).

DISCUSSION

In areas of the world where citrus black spot has become established, pseudothecia are produced in the leaf litter, and ascospores are thought to be responsible for the majority of new fruit infections and spread within the grove (Kiely 1948; Kotzé

1981; McOnie 1964; Reis et al. 2006; Spósito et al. 2008). Given this epidemiology, regulation of sexual reproduction in *P. citricarpa* and critical factors involved for this process are of both practical and fundamental value. Deciphering the makeup and structure of the *MAT* locus as well as the distribution of idiomorphs within the populations, as reported here, is an important step in gaining this understanding. We first discovered that the Floridian isolate of *P. citricarpa* (Gc12), characterized in this study, only contained the *MAT1-2* idiomorph. The presence of only one idiomorph is consistent with the lack of self-fertility in culture (Baayen et al. 2002; Baldassari et al. 2008) and is supported by multiple other lines of evidence. First, from the draft genome sequence established here, the assembled 28.9-kb contig containing the *MAT1-2-1* coding sequence contained an additional 13.1 kb of upstream and 14.3 kb of downstream sequence, with no sequence homology to known *MAT1-1* genes. Further, given the significant sequencing coverage (approximately 75×), if *MAT1-1-1* genes existed in close proximity with the *MAT1-2-1* gene, as found in homothallic *P. capitalensis* Gm33 in this study, it would be expected to be found within this region. Third, *MAT1-1-1* sequences, but no evidence of *MAT1-2-1* sequences, were identified in the *P. citricarpa* CGMCC3.14348 genome. Fourth, mating type screening using *MAT*-specific primers clearly showed that *P. citricarpa* isolates in the Floridian and Australian populations were either *MAT1-1* or *MAT1-2*, with the exception of a few presumed heterokaryotic isolates identified in the Australian population. It is possible that these isolates were impure, as DNA from single-spored isolates was not available at the time of the study. Thus, the evidence presented here indicates that *P. citricarpa* is heterothallic.

The boundaries of fungal *MAT* loci can generally be determined based on the conserved flanking genes and genetic content. However, these criteria were not completely applicable to *P. citricarpa*. Studies have reported conserved genes that commonly flank the *MAT* loci of ascomycetes and zygomycetes (Debuchy and Turgeon 2006;

TABLE 3. Percent nucleotide and amino acid identity of the mating-type (*MAT*) loci between *Phyllosticta citricarpa* and *P. capitalensis*^a

<i>P. capitalensis</i>	<i>P. citricarpa</i>		Sequence identity (%) ^b
	Idiomorph	Gene	
<i>oml1</i>	<i>MAT1-1</i>	<i>oml1</i>	55 (40)
	<i>MAT1-2</i>	<i>oml1</i>	57 (24)
<i>MAT1-1-1</i>	<i>MAT1-1</i>	<i>MAT1-1-1</i>	68 (59)
<i>MAT1-2-1</i>	<i>MAT1-2</i>	<i>MAT1-2-1</i>	70 (65)
<i>MAT1-2-9</i>	<i>MAT1-2</i>	<i>MAT1-2-9</i>	69 (48)
PH domain-encoding gene	<i>MAT1-1</i>	PH domain-encoding gene	54 (41)
	<i>MAT1-2</i>	PH domain-encoding gene	72 (67)

^a Nucleotide and amino acid sequences were predicted using the AUGUSTUS web interface with *Botrytis cinerea* as the model organism.

^b Sequence identity was analyzed using the Needleman-Wunsch global alignment algorithm available on the website of the National Center for Biotechnology Information. Nucleotide identities are presented with amino acid identities in parentheses.

Fig. 3. Maximum likelihood tree constructed using the amino acid sequences of pleckstrin homology (PH)-domain proteins from *Phyllosticta citricarpa* idiomorphs, *P. capitalensis*, and other Dothideomycetes. The tree was constructed using MEGA version 6.0 and was rooted with *Saccharomyces cerevisiae*. Levels of branch support obtained with 1,000 bootstrap replicates are indicated at nodes when they exceeded 70%. Fungal families are indicated with vertical bars. The scale bar represents 0.5 substitutions per site.

Gryganskyi et al. 2010; Lee et al. 2010). Of them, *apn2* and *sla2* are most commonly found adjacent to *MAT* loci in ascomycetes (Debuchy and Turgeon 2006; Lee et al. 2010). But in Dothideomycetes, only *apn2* is commonly found in the flanking region of the *MAT* locus (Debuchy and Turgeon 2006) and other genes, such as the *40S S9* gene that we observed in *P. citricarpa* and the *gap1* gene (GTPase activating protein) in *Leptosphaeria maculans* (Cozijnsen and Howlett 2003), rather than the *sla2* gene, commonly define the other flank. This limited conservation of the downstream flank explains our failure to amplify the *MAT* locus in initial cloning attempts. Syntenic analysis between the *MAT* idiomorphs of *P. citricarpa* revealed that the conserved flanking sequences of the *MAT* locus begin upstream in the 5' end of the *oml1* gene rather than in an intergenic region. This structure is rare but found in the *MAT* loci of *Magnaporthe grisea*, *Cordyceps takaomontana*, *Aspergillus fumigatus*, and *Pyrenopeziza brassicae*, in which one

of their *MAT* genes or lineage-specific genes extends from the idiomorphic region into the flanking region (Debuchy and Turgeon 2006; Paoletti et al. 2005; Singh and Ashby 1998; Yokoyama et al. 2003). The encoded OML1 protein of *P. citricarpa* shared homology with MAT1-1-4 of *D. pinea*, which is located in its *MAT1-1* idiomorph (Bihon et al. 2014), implying a possibility that the incorporation of the *oml1* gene into the *MAT* locus of *P. citricarpa* is ongoing. The expansion of *MAT* loci has been observed in *Coccidioides* species, *Mycosphaerella* species, and, the most striking case, *Cryptococcus neoformans*, in which extensive rearrangements of genes were found between the two idiomorphs (over 100 kb) (Arzanlou et al. 2010; Lengeler et al. 2002; Mandel et al. 2007). These findings are consistent with the evolution of *MAT* loci toward sex chromosomes (Lengeler et al. 2002).

In contrast to *P. citricarpa*, the *MAT* locus of *P. capitalensis* harbors both *MAT1-1-1* and *MAT1-2-1* genes as well as the *MAT1-2-9* ortholog and a single PH domain-encoding gene. This organization was also present in the *MAT* locus of another presumably homothallic Botryosphaeriales fungus, *B. dothidea*. The structurally homothallic *MAT* locus in *P. capitalensis* is consistent with the previous observation that *P. capitalensis* can produce pseudothecia and ascospores in single spore-derived cultures (Baayen et al. 2002; Baldassari et al. 2008). The presence of a single PH domain-encoding gene in the homothallic locus as well as in each of the heterothallic idiomorphs presents an additional instance in which the distinction between conserved flanking sequence and idiomorphic sequences becomes blurred. Surprisingly, in the *P. citricarpa* idiomorphs the two PH-domain genes shared only 56% nucleotide identity, whereas the *P. citricarpa* *MAT1-2* PH-domain gene shared 72% nucleotide identity with the *P. capitalensis* PH-domain gene. Based on this intraspecies sequence divergence, the PH-domain genes are considered part of the *P. citricarpa* idiomorph. Our analysis of other available genome sequences (Supplementary Table S3) determined that, with the exception of *P. citricarpa*, the PH-domain gene is often found adjacent to the *MAT* idiomorphs in Dothideomycetes rather than a member of the idiomorph. This evidence combined with our findings of the divergent 5' sequences of *oml1* indicates that *P. citricarpa* has expanded its idiomorphic regions by the incorporation of flanking sequences, including the PH-domain gene that is frequently found adjacent to *MAT* idiomorphs.

Analysis of the *MAT* locus also gave us our first indication that genetic diversity in the Floridian population of *P. citricarpa* was limited. Isolate screening revealed that only the *MAT1-2* idiomorph was present in the *P. citricarpa* isolates collected in Florida. This result implies that *P. citricarpa* only reproduces asexually in Florida and there is a possibility of the existence of a single clonal population. Previously, Zavala et al. (2014) investigated the genetic diversity of *Phyllosticta* spp. collected in Florida, using multilocus

Fig. 4. Specificity verification of the mating-type (*MAT*) primers for *Phyllosticta citricarpa* by conventional polymerase chain reaction (PCR). **A**, M, 100-bp DNA marker; lanes 1 and 5, *P. citricarpa* BRIP 27888 from Australia served as a positive control of the *MAT1-1* idiomorph; lanes 2 and 6, *P. citricarpa* Gc12 served as a positive control of the *MAT1-2* idiomorph; lanes 3 and 7, *P. capitalensis* Gm33 served as a negative control; lanes 4 and 8, nuclease-free water served as a nontemplate control. **B**, Representative samples from Australia (BRIP 54234-54241) and Florida (GCCN1s-8s).

TABLE 4. Evaluation of mating-type (*MAT*) idiomorphs among *Phyllosticta citricarpa* isolates collected in Florida from 2010 to 2014 and genomic DNA samples from Queensland, Australia, using *MAT*-specific primers designed in the study

Population	Total number of isolates	Number of isolates		
		<i>MAT1-1</i>	<i>MAT1-2</i>	<i>Heterokaryon</i>
Florida, U.S.A.	113	0	113	0
Queensland, Australia	26	12	11	3

TABLE 5. Genotypic diversity of the Floridian and Australian populations of *Phyllosticta citricarpa*

Population	N ^a	MLG ^b	eMLG ± SE ^b	H ^c	G ^c	Hexp ^d	E.5 ^e
Australia	24	11	11.0 ± 0.0	2.04	5.88	0.87	0.73
Florida	70	1	1.0 ± 0.0	0.00	1.00	0.00	NaN ^f

^a N, number of individuals.
^b MLG = number of multilocus genotypes observed; eMLG ± SE = number of expected MLG at the smallest sample size ≥10 based on rarefaction ± standard error. Under random mating, eMLG would be equal to MLG.
^c H = Shannon-Wiener index of MLG diversity, G = Stoddart and Taylor's index of MLG diversity; the greater H or G, the more diverse the population.
^d Hexp = Nei's unbiased genotypic diversity, the probability of two randomly chosen genotypes being different, ranging from zero to one; the greater the value, the greater the diversity.
^e E.5 = evenness, the distribution of genotype abundances, ranging from zero, where a single genotype is dominant within a population, to one, where genotypes are equally abundant within a population.
^f NaN = not a number.

phylogenetic analysis, with sequences from ITS, ACT, TEF1, and GPDH loci. Their results revealed no sequence polymorphisms within loci among *P. citricarpa* isolates from Florida, Brazil, Australia, and Africa. In an independent study with these same loci, *P. citricarpa* isolates from Australia clustered in the same clade and exhibited limited phylogenetic diversity (Glienke et al. 2011). These results suggested that markers based on other than highly conserved genes were needed to resolve phylogenetic relationships

and population structure within the species. Our test of this hypothesis with newly developed SSR markers established that MLG5 is the only genotype in the Floridian population of *P. citricarpa*. In contrast, 11 MLG were revealed among the 24 Australian isolates, with high genetic diversity relative to the lack of diversity in Florida.

The clonality of the *P. citricarpa* population in Florida is likely derived from a founder effect (Provine 2004) similar to what has

Fig. 5. Distribution of multilocus genotypes (MLG) of **A**, Australian and **B**, Floridian populations of *Phyllosticta citricarpa*. This result shows that 11 MLG were found in Australia but only one MLG was found in the Floridian population.

been observed in *Cryptococcus gattii* (Carriconde et al. 2011) and the global population of *Phytophthora infestans* before 1976 (Fry et al. 1993). Expanded genotypic diversity in the Australian population (11 MLGs among 24 isolates) may be attributed to the length of time *P. citricarpa* has been in Australia (Benson 1895), the possibility of multiple and larger past introductions and regular sexual recombination due to the presence of both mating types. Although four Australian isolates shared their MLG with the Floridian population, this is not evidence for the origin of the Floridian population in Australia. A broader, global survey of populations with an expanded set of genotypic markers will be required to generate hypotheses on the center of *P. citricarpa* diversity and the source of the Floridian population. Screening of new *P. citricarpa* isolates is ongoing to monitor for new introductions that could establish the opposite mating type and, potentially, the sexual cycle in Florida.

In other parts of the world, the disease cycle of citrus black spot is thought to depend on ascospores to initiate infections and spread the disease through and between groves (Kotzé 1981; McOnie 1964; Reis et al. 2006; Spósito et al. 2008). However, accounts from early stages of disease establishment in Rhodesia (Zimbabwe) determined that most fruit infections occurred from conidial inoculum and only small numbers of ascospores were found in leaf litter (Whiteside 1967). Because this study was conducted prior to the advent of molecular identification, it is uncertain whether the detected ascospores were from *P. citricarpa* or *P. capitalensis*. Even in the presence of ascospores, conidia produced under favorable environmental conditions, such as frequent summer rains, can become dominant in the spread of the disease within tree canopies, as shown in Brazil (Spósito et al. 2011). With the presence of only a single mating type in Florida, ascospore inoculum is unlikely to exist. This places citrus black spot epidemiology in Florida in a unique situation in which inoculum production and spread would solely depend on conidia produced on dead twigs, fruit lesions, leaves, and in leaf litter. These forms of conidial inoculum have been demonstrated to be important in Brazil (Spósito et al. 2011), where climate and production patterns are similar to Florida. It will be interesting to determine the presence and pattern of mating type idiomorphs and the population structure among Brazilian isolates of *P. citricarpa* to better understand the relative contributions of the sexual and asexual cycles to disease development.

The findings of this study should be applicable to reducing the severity and the spread of citrus black spot. While the majority of citrus produced in Florida is grown for juice processing, certain citrus types, such as grapefruit and specialty fresh fruit, are destined for the fresh fruit market and export (Florida Agricultural Statistics Service 2015). As such, it is important to continue to reduce disease spread to facilitate market access. Current measures to restrict the movement of infected plant debris including the removal of debris from and disinfection of grove equipment are still highly advisable, as conidia can be produced in large numbers on infected twigs and leaf litter. For disease management, further emphasis should be placed on the removal of dead wood from trees. Disease management in the more susceptible cultivars, such as Valencia, is more complicated because the mature fruit overlap with young fruit, leading to greater inoculum potential for the younger fruit. Fungicide applications during this period (May to September) should be considered important for disease management in Florida.

In conclusion, this study identified the *MAT* loci of *P. citricarpa* and *P. capitalensis* and determined the presence of only the *MAT*-2 idiomorph in a clonal Floridian population of *P. citricarpa*. As a consequence, the presumed ascospore production of *P. citricarpa* in leaf litter in Florida is unlikely. This shows that the *P. citricarpa* does have a heterothallic mating type structure, as hypothesized, but the Floridian population is not randomly mating, as only asexual reproduction can currently occur. Therefore, the importance of *P. citricarpa* conidia as the sole inoculum source should not be underestimated. Future research should focus on the impact of the

asexual cycle on the epidemiology of citrus black spot in Florida and the conditions that may reduce conidial inoculum production.

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