Sexual Reproduction in the Citrus Black Spot Pathogen, *Phyllosticta citricarpa*

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ABSTRACT

Citrus black spot (*Phyllosticta citricarpa*) causes fruit blemishes and premature fruit drop, resulting in significant economic losses in citrus growing areas with summer rainfall across the globe. The mating type locus of *P. citricarpa* has recently been characterized, revealing the heterothallic nature of this pathogen. However, insight into the occurrence of mating and the impact of completing the sexual cycle of *P. citricarpa* was lacking. To investigate the occurrence and impact of sexual reproduction, we developed a method to reliably, and for the first time, produce ascospores of *P. citricarpa* on culture media. To demonstrate meiosis during the mating process, we identified recombinant genotypes through

multilocus genotyping of single ascospores. Because the process of fertilization was not well understood, we experimentally determined that fertilization of *P. citricarpa* occurs via spermatization. Our results demonstrate that *P. citricarpa* is heterothallic and requires isolates of different *MAT* idiomorphs to be in direct physical contact, or for spermatia to fulfill their role as male elements to fertilize the receptive organs, in order to initiate the mating process. The impact of mating on the epidemiology of citrus black spot in the field is discussed.

Additional keywords: genetic recombination, *Guignardia*, heterothallism.

Citrus black spot, caused by the fungus *Phyllosticta citricarpa* (McAlpine) van der Aa (synonym: *Guignardia citricarpa* Kiely), is an economically important fungal disease in citrus-producing areas around the world that experience hot and humid summers (Fourie et al. 2013; Kotzé 1981; Yonow et al. 2013). The disease was first officially reported on citrus in 1895 from Australia (Benson 1895), and has been subsequently detected in other parts of the world, including parts of Asia, Africa, South America, and North America (Brentu et al. 2012; Kotzé 1981; Schubert et al. 2012; Yonow et al. 2013). It is assumed that the fungus coevolved with citrus hosts originating in South and Southeast Asia, particularly Northeast India, China, and Australia (Hynniewta et al. 2014; Scora 1975). Disease symptoms are expressed as fruit blemishes, resulting in fruit unacceptable for fresh markets (Baayen et al. 2002; Kotzé 1981), whereas heavy infection near the pedicle of the developing fruit can result in premature fruit drop, decreasing yield (Baldassari et al. 2008; Brentu et al. 2012). Management of the disease using fungicides is costly and levels of control needed to render the fruit acceptable to consumers are difficult to achieve (Silva Junior et al. 2016).

P. citricarpa is known to have both asexual (formerly referred to as *P. citricarpa*) and sexual states (formerly referred to as *G. citricarpa*) in its lifecycle (Baayen et al. 2002; Baldassari et al. 2008; Kotzé 1981; McOnie 1964a). The asexual state produces numerous conidia contained in pycnidia, which are readily produced on fruit lesions, dead twigs, and leaf litter in orchards (Kotzé 1981; Spósito et al. 2011). The sexual state, involving the formation of pseudothecia, has thus far only been found in decomposing leaf litter on the orchard floor and requires alternate wetting and drying conditions to produce ascospores within the pseudothecia (Baayen et al. 2002; Baldassari et al. 2008; Kotzé 1981; Reis et al. 2006). Conidia are

easily produced under controlled conditions but production of ascospores in vitro has, until now, eluded researchers.

In the epidemiology of citrus black spot, both conidia and ascospores play a role, with their relative importance depending on environmental conditions and host plant variety. Because conidia are transported by rain splash over short distances, they are mainly responsible for spread of the pathogen within the tree canopy (Kotzé 1981; Spósito et al. 2011), although longer-distance spread is possible, as has been demonstrated by disease movement in Florida (Wang et al. 2016). Conidia are considered particularly important where there is overlap of old diseased fruit in the tree alongside young susceptible fruit (up to 24 weeks old) of the consecutive season (Baldassari et al. 2006; Spósito et al. 2008, 2011). In addition to conidia, ascospores disseminate the pathogen between trees and orchards as they are dispersed by wind over longer distances (Spósito et al. 2007). Ascospores are considered important where sources of conidia are limited in the canopy (i.e., no late hanging diseased fruit or dead twigs) when susceptible fruit are present.

Although the role of ascospores in the epidemiology of citrus black spot is postulated (Baayen et al. 2002; Baldassari et al. 2008; Kotzé 1981; Spósito et al. 2008), firm evidence for their role is lacking. This is largely due to the fact that both *P. citricarpa* and the endophyte *P. capitalensis* Henn., which is homothallic, are often present in the same orchard (Baayen et al. 2002; Wang et al. 2016). Because these two species are morphologically indistinguishable, and both produce ascospores in decaying leaf litter on the orchard floor (Baayen et al. 2002), any statement about the role of ascospores in the disease epidemiology without species identification needs to be considered cautiously. For example, the observations concerning the role of ascospores of *P. citricarpa* by Kiely (1948), McOnie (1964b) and Kotzé (1981) might inadvertently have combined observations of *P. citricarpa* with those of *P. capitalensis*. Furthermore, past studies with *P. citricarpa* ascospores have relied on spores collected from field material (Baldassari et al. 2008; McOnie 1964b), which most likely consisted of mixtures of *P. citricarpa* and *P. capitalensis*. The ability to produce pure *P. citricarpa* ascospores in vitro would be a

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significant aid in elucidating the role of ascospores in infection and disease spread in the epidemiology of citrus black spot. However, infertility in culture and an inability to induce the sexual state of *P. citricarpa* has hindered progress in this area (Amorim et al. 2017; Baayen et al. 2002; Baldassari et al. 2008; McOnie 1964b). Therefore, to improve our understanding of the epidemiology of citrus black spot, it is necessary to be able to produce ascospores of *P. citricarpa* in culture.

Several methods developed in previous studies failed to reliably induce the sexual state of *P. citricarpa* in vitro. Moran Lemir et al. (2000) reported that inoculation of a single mycelium plug or spread of a suspension of mycelia and spores on a medium containing lemon leaf and malt extracts resulted in successful production of ascospores of *P. citricarpa*. However, this process could not be repeated by Baldassari et al. (2008) or Wang et al. (2016). Recently, Amorim et al. (2017) paired isolates of complementary mating types on opposite sides of autoclaved *Citrus* leaf discs placed in Petri dishes of various media and incubated at different conditions. Even so, these authors did not observe any sexual structures from their mating assays. In another citrus pathogen that forms pseudothecia in leaf litter, *Mycosphaerella citri* causing citrus greasy spot, successful production of sexual structures in vitro was obtained through pairing single isolates on autoclaved grapefruit leaf discs subjected to alternate wetting and drying conditions (Mondal et al. 2004). In other ascomycetes such as *P. ampelici* (synonym: *G. bidwellii*), *M. fijiensis*, and *Ascochyta rabiei*, methods used and conditions required for successful in vitro mating have involved several factors from manipulation of environmental (e.g., light and temperature) to nutrient (e.g., the presence of host plant tissues) conditions, which vary between species (Etebu et al. 2003; Jailloux 1992; Trapero-Casas et al. 2012). In the case of *P. citricarpa*, it is possible that the key factors triggering mating of *P. citricarpa* were missing in past studies.

Failure to produce *P. citricarpa* ascospores in vitro is likely due to a lack of information about the mechanisms controlling sexual reproduction of *P. citricarpa*. In all ascomycetes studied to date, sexual reproduction is governed by a single mating type locus (*MAT1*), with two mating types referred to as *MAT1-1*, defined by the presence of the *MAT1-1-1* gene, and *MAT1-2*, containing the *MAT1-2-1* gene (Coppin et al. 1997; Debuchy and Turgeon 2006; Debuchy et al. 2010; Pöggeler 2001; Turgeon and Yoder 2000). The main role of mating types is controlling recognition mechanisms leading to fertilization (Coppin et al. 1997). In homothallic species, both *MAT* genes can be sometimes linked or combined in a single large locus allowing self fertilization (Coppin et al. 1997; Debuchy and Turgeon 2006). In heterothallic ascomycetes, an individual contains either a *MAT1-1* or *MAT1-2* idiomorph, and interactions between partners with complementary *MAT* idiomorphs are required for sexual reproduction to occur (Coppin et al. 1997; Ni et al. 2011). In *P. citricarpa*, single-spored cultures have not been found to reproduce sexually, implying that it is a heterothallic species. This hypothesis was recently supported by full sequence characterization showing that *P. citricarpa* isolates contain single *MAT1-1* or *MAT1-2* idiomorphs (Amorim et al. 2017; Wang et al. 2016). In the Australian *P. citricarpa* population, the two idiomorphs were found in a 1:1 ratio (Carstens et al. 2017; Wang et al. 2016), suggesting the occurrence of sexual reproduction in this population. The next step required to validate the heterothallic nature of *P. citricarpa* is to demonstrate that isolates with different mating types undergo sexual reproduction when they interact.

In heterothallic fungal species, fertilization requires the fusion of a male element to donate a nucleus into a cell of the female organ (receptive element) which can accept this nucleus (Coppin et al. 1997). This fusion can happen via several approaches, with one of those being spermatization (Alexopoulos et al. 1996). Spermatization involves the male gamete (i.e., spermatia, microconidia, or macroconidia) attaching to female receptive cells and donating its nucleus into the receptive cells of the fungus. In *P. citricarpa*, it is not known how fusion occurs, or the nature of the receptive and fertilizing elements. The presence of a spermatial state producing

spermatia contained in spermatogonia on fallen dead leaves and in pure culture has been recorded as a part of the *P. citricarpa* life cycle, although this state has not been formally described (Baayen et al. 2002; Kiely 1948). Spermatia are dumbbell-shaped, 5.0 to 8.0 by 0.5 to 1.0 μm in size, which is smaller than conidia with the size of 8.0 to 10.5 by 5.5 to 7.0 μm (Anonymous 2009). Kiely (1948) observed that spermatogonia always appeared prior to the formation of pseudothecia and hypothesized that spermatia were functioning as male gametes to fertilize a female organ of *P. citricarpa*. However, until the present, no experimentation had been done to test whether or not spermatia play a role in the sexual cycle of *P. citricarpa*.

Lacking a clear understanding of sexual reproduction of *P. citricarpa*, including an inability to produce pure *P. citricarpa* ascospores, has hindered our understanding of several aspects of the citrus black spot disease cycle, including ascospore infection processes and epidemiology. To develop effective disease management strategies based on the disease cycle, the overall objective of this study was to investigate aspects of sexual reproduction of *P. citricarpa* and specifically test the hypotheses that (i) mating-type genes identified in the *P. citricarpa* population are functional and the fungus undergoes sexual reproduction, (ii) the fungus is able to produce ascospores in vitro in the absence of host plant tissue, (iii) there is genetic recombination in the ascospore progeny resulting from in vitro mating, and (iv) spermatia play a role in fertilization of *P. citricarpa*. Testing these hypotheses will significantly enhance our understanding of the disease cycle and may enable the development of more effective disease management strategies.

MATERIALS AND METHODS

Isolates. Twenty-seven isolates of *P. citricarpa*, previously identified by sequence analysis of the internal transcribed spacer region and partial translation elongation factor 1- α gene (Miles et al. 2013) and screened for mating types via polymerase chain reaction (PCR) with specific primers (Wang et al. 2016), were used in this study (Table 1). The isolates were cultured on half-strength potato dextrose agar (1/2 PDA) (BD Difco, BD Australia) at room temperature for 2 weeks prior to commencement of the mating assays.

Vegetative compatibility test. In order to determine whether *P. citricarpa* isolates of different mating types are vegetatively compatible and undergo sexual reproduction through fusion of vegetative mycelia, a vegetative compatibility assay was carried out as follows. Two-week-old isolates of *P. citricarpa* were paired by placing two parallel mycelial strips (30 by 2 mm) on 1/2 PDA plates at a distance of 30 mm from each other. *MAT1-1* isolates (Queensland Plant Pathology Herbarium, Brisbane, Australia [BRIP] 27890, 53711, 53714, 53718, and 54230) and *MAT1-2* isolates of *P. citricarpa* (BRIP 52614, 53719, and 54232) (Table 1) were paired with themselves and with the other isolates in all possible combinations in triplicate. The plates were then incubated at 25°C with a cycle of 12 of light and 12 of darkness for 30 days. After the incubation period, vegetative and sexual compatibility between each isolate combination was evaluated. Isolate combinations were considered vegetatively and sexually compatible via fusion of mycelia if the two colony margins were found to freely grow into each other, and mycelia fused to produce pseudothecia in the intermingling areas.

Sexual compatibility test. In order to determine sexual compatibility between isolates of different mating types of *P. citricarpa*, a “sandwich mating” technique was developed. All isolates (Table 1) were paired in all possible combinations in triplicate. To pair the isolates, 10 mycelium plugs (5 mm in diameter) of one isolate’s 2-week-old mycelium were placed mycelium side down on the surface of the colony of another isolate (Fig. 1). The plates were then kept at ambient conditions on the laboratory bench for 2 weeks. Presence of pseudothecia and ascospores were examined using a Nikon Eclipse Ci-L compound microscope (Nikon Corporation) at $\times 200$ magnification by making wet mounts of mycelium and fruiting bodies obtained from the areas around and under the plugs,

and from examining the inside of the Petri dish lid because it is known that ascospores are vertically ejected up to 12 mm from pseudothecia (Kiely 1948; Kotzé 1963).

Spermatia fertilization. In order to investigate whether spermatia play a role in sexual reproduction of *P. citricarpa*, spermatia suspensions were prepared by flooding 2-week-old *P. citricarpa* cultures with sterile water and incubating for 30 min at room temperature to allow spore release. The resulting suspension containing conidia and spermatia was filtered through two layers of Whatman number 2 filter paper (pore size = 8 µm), then two layers of Whatman number 597 filter paper (pore size = 4 to 7 µm) to separate spermatia from conidia. The resulting spermatia suspension was then adjusted to 10⁵ spermatia/ml. A single droplet of 10 µl of spermatia suspension was aseptically checked for the presence of conidia by placing the droplet in a closed, sterile Petri dish before checking it under a Nikon Eclipse Ci-L compound microscope (Nikon Corporation) at ×100 magnification. Droplets found to only contain spermatia were then applied to the outer perimeter of pycnidia production of actively growing 2-week-old colonies on 1/2 PDA, generally approximately 10 mm inside of the colony margin. Four *P. citricarpa* isolates (BRIP 53711 and 53714 and BRIP 52614 and 54232, representing *MATI-1* and *MATI-2*, respectively) were fertilized with a spermatia suspension of BRIP 53711 and 54232. We used five replicated culture plates (each plate consisted of three colonies × three spermatia droplets applied per colony) per combination. The plates were kept at ambient conditions on the laboratory bench for 2 weeks before being examined for the presence of sexual structures, as described above. To confirm the purity of spermatia suspensions, 24 spermatia droplets of 10 µl each that were not found to have conidia by microscopic examination were plated out on 1/2 PDA to confirm the absence of spermatia germination, as previously observed (Kiely 1948). In addition, a further 15 droplets of 10 µl of spermatia in which conidia were observed after filtering were also plated as positive controls for colony development.

Genotyping. Parental and progeny isolates. To determine genotypes of ascospores produced in vitro, putative single F1 ascospore progeny from three crosses (BRIP 53711 × 54232, 53714 × 53713, and 53714 × 53719), which were previously found to have different alleles of polymorphic simple-sequence repeat (SSR) markers locus 6 and 12 (Wang et al. 2016), were arbitrarily collected from the Petri dish lid of the mated cultures. The Petri dish lids were inverted, sterile water was added to the lids, and a glass

Fig. 1. Sandwich mating technique, in which 2-week-old mycelial plugs (arrow head) grown on half-strength potato dextrose agar (1/2 PDA) of one mating type isolate of *Phyllosticta citricarpa* were placed mycelium side down on the 2-week-old colony surface (arrow) grown on 1/2 PDA of the complementary mating type isolate. Scale bar = 10 mm.

TABLE 1. Details of *Phyllosticta citricarpa* isolates used in this study

BRIP ^a	Host	Location ^b	Collector	Mating type ^c
27888	<i>Citrus reticulata</i> Imperial	Mundubbera	S. L. Willingham	<i>MATI-1</i>
27889	<i>C. reticulata</i> Imperial	Mundubbera	S. L. Willingham	<i>MATI-2</i>
27890	<i>C. reticulata</i> Imperial	Mundubbera	S. L. Willingham	<i>MATI-1</i>
52614 ^d	<i>C. limon</i>	Gayndah	A. K. Miles	<i>MATI-2</i>
53711	<i>C. sinensis</i> Navel	Gayndah	A. K. Miles	<i>MATI-1</i>
53712	<i>C. sinensis</i> Navel	Gayndah	A. K. Miles	<i>MATI-2</i> ^e
53713	<i>C. reticulata</i> Imperial	Gayndah	A. K. Miles	<i>MATI-2</i>
53714	<i>C. reticulata</i> Fremont	Mundubbera	A. K. Miles	<i>MATI-1</i>
53715	<i>C. sinensis</i> Valencia	Mundubbera	P. E. Mayers	<i>MATI-2</i>
53716	<i>C. sinensis</i> Valencia	Mundubbera	P. E. Mayers	<i>MATI-2</i>
53717	<i>C. limon</i>	Tiaro	A. K. Miles	<i>MATI-1</i>
53718	<i>C. limon</i>	Tiaro	A. K. Miles	<i>MATI-1</i>
53719	<i>C. limon</i>	Tiaro	A. K. Miles	<i>MATI-2</i>
53720	<i>C. limon</i>	Emerald	A. K. Miles	<i>MATI-1</i>
54229	<i>C. sinensis</i> Valencia	Beerwah	P. E. Mayers	<i>MATI-2</i>
54230	<i>C. sinensis</i> Valencia	Mundubbera	P. E. Mayers	<i>MATI-1</i> ^f
54231	<i>C. sinensis</i> Valencia	Mundubbera	P. E. Mayers	<i>MATI-1</i> ^f
54232	<i>C. sinensis</i> Valencia	Mundubbera	P. E. Mayers	<i>MATI-2</i>
54233	<i>C. sinensis</i> Valencia	Mundubbera	P. E. Mayers	<i>MATI-1</i>
54234	<i>C. sinensis</i> Valencia	Mundubbera	P. E. Mayers	<i>MATI-1</i>
54235	<i>C. sinensis</i> Valencia	Mundubbera	P. E. Mayers	<i>MATI-2</i>
54236	<i>C. sinensis</i> Navel	Mundubbera	P. E. Mayers	<i>MATI-1</i>
54237	<i>C. sinensis</i> Valencia	Mundubbera	P. E. Mayers	<i>MATI-2</i>
54238	<i>C. limon</i> Meyer	Beerwah	P. E. Mayers	<i>MATI-2</i>
54239	<i>C. sinensis</i> Valencia	Mundubbera	P. E. Mayers	<i>MATI-1</i>
54240	<i>C. sinensis</i> Valencia	Mundubbera	A. K. Miles	<i>MATI-1</i>
54241	<i>C. limon</i>	Mundubbera	A. K. Miles	<i>MATI-1</i> ^f

^a Queensland Plant Pathology Herbarium, Brisbane, Australia (BRIP) accession numbers.

^b Locations in Queensland, Australia.

^c Mating type identified by Wang et al. (2016) and confirmed in the present study by polymerase chain reaction (PCR) and mating assays.

^d Ex-epitype isolate designated by Glienke et al. (2011).

^e Mating type identified in the present study by PCR and mating assays.

^f Identified as heterokaryon in Wang et al. (2016), found to be *MATI-1* in the present study through PCR and mating assays.

spreader was used to dislodge the ascospores. The resulting ascospore suspension was then spread onto 1/2 PDA in order to obtain single germinating ascospores. Daily microscopic examination enabled germinating single ascospores to be transferred to a new 1/2 PDA plate and grown at ambient laboratory conditions to obtain monoascospore cultures.

DNA extraction. In order to determine whether the produced ascospores were the product of outbreeding between isolates of different mating types, the parental and the putative F1 progeny cultures were grown on 1/2 PDA for 2 weeks prior to DNA isolation. After scraping fungal mycelia and spores from culture plates, total genomic DNA (gDNA) was extracted using the Wizard Genomic DNA purification kit (Promega Corp.) following the manufacturer's instructions. DNA concentrations were determined using a DUO spectrophotometer (BioDrop) and diluted using sterile distilled water to a final concentration of 15 to 30 ng/μl prior to multilocus genotyping.

Multilocus genotyping. In order to determine the genotypes and hybrid nature of the ascospore-derived cultures and demonstrate the occurrence of meiotic recombination, parental and single-ascospore isolates were subjected to multilocus genotyping. SSR markers and mating type primers published by Wang et al. (2016) were used. SSR markers 6 and 12 were analyzed for two crosses (BRIP 53711 × 54232 and 53714 × 53713), and marker 6 was analyzed for cross BRIP 53714 × 53719, based on previous SSR analysis showing different multilocus genotypes for the parental isolates (Wang et al. 2016). PCR amplifications of the SSR loci were performed in 30-μl reactions, each containing approximately 20 ng of gDNA (nuclease-free water served as a nontemplate control) (Qiagen), 3.0 μl of 10× PCR buffer, 0.48 μl of 10 mM dNTP, 3.0 μl of 25 mM MgCl₂, 0.3 μl each of 10 mM primer, and 0.15 μl of *Taq* DNA polymerase at 5.5 U/μl, using the following thermocycling conditions: 1 min at 94°C; 30 cycles of 94°C for 20 s, 61°C for 20 s, and 72°C for 20 s each; followed by a final extension of 72°C for 2 min. PCR products were size fractionated on 1.5% agarose gels to examine DNA quality and the presence of a single fragment of the expected size. PCR products were directly sequenced by Macrogen Incorporated using an AB 3730xl DNA Analyzer (Applied Biosystems) to determine the number of repeat motifs of each SSR locus.

In addition to analyzing SSR loci, the mating type of the parental isolates of the three crosses and their respective single-ascospore progenies was determined as follows. DNA of each isolate was subjected to PCR amplification using primer pairs Gcmat1-56f and Gcmat1-686r specific to *MAT1-1*, and Gcmat2-686f and Gcmat2-1185r specific to *MAT1-2* (Wang et al. 2016). PCR was performed in 20-μl reactions, each containing approximately 20 ng of gDNA (nuclease-free water served as a nontemplate control) (Qiagen), 2.0 μl of 10× PCR buffer, 0.4 μl of 10 mM dNTP, 0.6 μl of 25 mM MgCl₂, 0.2 μl each of 10 mM primer, and 0.2 μl of *Taq* DNA polymerase at 5.5 U/μl. Thermocycling conditions consisted of 2 min at 95°C; 30 cycles of 95°C for 15 s, 59°C for 15 s, and 72°C for 30 s each; followed by a final extension of 72°C for 2 min. PCR products were size fractionated on 1.5% agarose gels to examine the length of a single amplified fragment compared with the fragments from the standard mating type tester strains BRIP 53711 and 52614 for *MAT1-1* and *MAT1-2*, respectively (Wang et al. 2016). This PCR was also performed on all other *P. citricarpa* isolates (Table 1) to confirm the mating types previously reported by Wang et al. (2016).

RESULTS

Vegetative compatibility. After a 1-month incubation, all of the combinations of isolates remained as distinct colonies on the plates, including combinations of the same isolate (Fig. 2A to C). No fusion of mycelium of the two colonies or sexual structures was observed.

Sexual compatibility. The sandwich mating technique demonstrated that pseudothecia and ascospores were formed between

reciprocal combinations of different mating types, and never between combinations of the same mating type or the same isolate. Ascospores were found on Petri dish lids, and pseudothecia embedded in the agar, as early as 2 weeks from pairing in all combinations with isolates of different mating type. An exception was isolate BRIP 53712, which never produced sexual structures in any of the pairing

Fig. 2. Mycelium-free zones (arrows) observed between **A**, different and **B** and **C**, the same isolates of *Phyllosticta citricarpa* 1 month after being paired on half-strength potato dextrose agar. Scale bars = 10 mm.

combinations. The morphology of the pseudothecia, asci, and ascospores observed were consistent with previous descriptions of *P. citricarpa* (Baayen et al. 2002; Sutton and Waterston 1966) (Fig. 3).

Fig. 3. Squashed pseudothecia with **A**, asci; **B**, mature asci about to release ascospores; and **C**, ascospores indicated by the arrow produced by *Phyllosticta citricarpa*, 2 weeks after pairing isolates of complementary mating types on half-strength potato dextrose agar using sandwich mating and spermatia fertilization techniques. Scale bars: A = 50 μ m, B = 20 μ m, and C = 10 μ m.

Spermatia fertilization. Pseudothecia and ascospores were found in the spermatia fertilization assays for the combinations between isolates of different mating types but not between pairs of the same mating type or the same isolate. Spermatia suspensions plated onto clean 1/2 PDA plates were not found to grow into colonies, suggesting that they are not vegetatively viable and that the spermatia suspensions used for fertilization did not contain conidia. However, where small numbers of conidia were observed to be present in control spermatia suspensions, *P. citricarpa* colonies developed after 1 week of incubation.

Recombination in ascospores of *P. citricarpa*. Multilocus genotyping using SSR and mating type loci enabled identification of the parental and F1 progeny isolates. Specifically, the results demonstrate recombination in the ascospore progeny produced by *P. citricarpa* in vitro (Table 2). The number of markers used limits the proportion of recombinant progeny to be positively identified, which was 0.40 for BRIP 53714 $\times$ 53713, 0.75 for BRIP 53711 $\times$ 54232, and 0.59 for BIRP 53714 $\times$ 53719, whereas the rest of the progeny could not be distinguished from parental genotypes. As expected, recombinant progeny subjected to genotyping at three loci showed a higher proportion of recombinant offspring than that subjected to only two loci. Amplification using the mating type-specific primers on the parental and progeny isolates showed that both mating types were present among all the isolates, with each isolate being positive for only one of the mating type idiomorphs.

DISCUSSION

We report for the first time the successful mating of *P. citricarpa* on pure culture media through a sandwich mating technique involving direct physical contact between two isolates of complementary mating types. We demonstrated that genetic recombination occurred during mating, resulting in nonparental genotypes in single-ascospore progenies. In addition, we demonstrated that spermatia function as male gametes which fertilize the receptive organs during sexual reproduction of *P. citricarpa*. Hence, we provide evidence that the mating type genes of *P. citricarpa* are functional and that this fungus can undergo sexual reproduction under laboratory conditions, confirming its heterothallic nature.

Since citrus black spot was first reported from Australia over 100 years ago (Benson 1895), the occurrence and role of its sexual cycle in the disease epidemiology has been postulated (Baldassari et al. 2008; Kiely 1948; Reis et al. 2006; Spósito et al. 2011). Although the sexual state of *P. citricarpa* was known, evidence confirming the occurrence of this mode of reproduction was required to improve our knowledge of the disease cycle. In the present study, the sexual state of *P. citricarpa* was successfully induced in vitro through outcrossing of isolates carrying complementary *MAT* idiomorphs, providing the crucial evidence for *P. citricarpa* being heterothallic. In addition, confirmatory mating type identification of the previously characterized Australian *P. citricarpa* isolates (Miles et al. 2013; Wang et al. 2016) showed that single isolates were only either the *MAT1-1* or *MAT1-2* idiomorph. None of these isolates were sexually compatible with themselves or with isolates of the same idiomorph. Instead, they behaved as either *MAT1-1* or *MAT1-2* to form sexual structures when pairing with a complementary partner. The only exception was isolate BRIP 53712, which failed to produce sexual structures when paired with other isolates. This isolate also did not produce conidia or spermatia by itself in the present study (data not shown), indicating a loss of reproductive function for this particular isolate. Additional evidence for heterothallism of *P. citricarpa* was that the monoascospore progeny isolates were found to contain only one or the other *MAT* idiomorph. The conclusion that *P. citricarpa* is heterothallic is consistent with previous reports that single-spored isolates of *P. citricarpa* are self infertile (Baayen et al. 2002; Baldassari et al. 2008), which has recently been shown to be due to the presence of only one gene, either *MAT1-1-1* or *MAT1-2-1*, in a single isolate (Amorim et al. 2017; Wang et al. 2016).

Our newly developed sandwich mating technique was reproducible; all of the crosses were successfully repeated and the pathogen was able to complete a sexual cycle within 2 weeks, from pairing to ascospore release, under laboratory conditions. Previously, the only study reporting successful induction of sexual production in *P. citricarpa* in vitro was that of Moran Lemir et al. (2000). These authors reported successful production of ascospores from an individual isolate, although the successful mating techniques developed in the present study clearly demonstrated that a prerequisite for mating of *P. citricarpa* is close physical contact between isolates carrying complementary *MAT* genes. Because the isolate used by Moran Lemir et al. (2000) was collected from infected lemon fruit with citrus black spot symptoms where both *P. citricarpa* and *P. capitalensis* could have been present, but no species identification was reported, this isolate was possibly a mixture of the two species. Therefore, it is more likely that the ascospores found in the study by Moran Lemir et al. (2000) might have been derived from *P. capitalensis*, which is homothallic (Wang et al. 2016). This would also explain why results of Moran Lemir et al. (2000) could not be replicated by Baldassari et al. (2008) and Wang et al. (2016). Difficulties faced in other studies (Amorim et al. 2017; Baldassari et al. 2008) may be due to lack of vegetative compatibility, as observed in our study, as well as not having access to *MAT* gene information. Recent mating type studies of *P. citricarpa* (Amorim et al. 2017; Wang et al. 2016) and our sandwich mating technique have allowed us to overcome these issues.

Genotyping of the ascospores produced in vitro clearly demonstrated that new recombinant genotypes were formed in the *P. citricarpa* F1 progeny, confirming that genetic recombination had occurred. Generally, recombination in fungi can be the result of sexual or sometimes parasexual reproduction (Alexopoulos et al. 1996; Pontecorvo 1956; Varga et al. 2014). However, based on two key observations, recombinations found in this study are unlikely to be a result of any events other than sexual reproduction. First, sexual structures, including pseudothecia, asci, and ascospores, were identified in our sandwich mating experiments and spermatia fertilization assays. Second, no evidence for *P. citricarpa* being vegetatively compatible was found, which precludes formation of heterokaryons required for somatic hybridization and parasexuality (Alexopoulos et al. 1996; Leslie 1993; Meijer et al. 1994). The progeny populations show a range of different recombinant genotypes which, in nature, may lead to potentially advantageous combinations of genes, therefore increasing the genetic diversity and adaptability of the pathogen (Glass and Kuldau 1992; McDonald and Linde 2002). Adaptations may include resistance to fungicides or the ability to overcome host plant resistance (McDonald and Linde 2002). These advantages have been documented with examples, such as the fact that frequent sexual recombination in *M. graminicola* (synonym: *Septoria tritici*) plays an important role in overcoming resistance to newly bred wheat cultivars and rapidly developing resistance to fungicides (Cowger et al. 2000; Zhan et al. 2007). Therefore, these issues need to be taken into account in fungicide development and resistance breeding studies with citrus host plants. In addition to genetic recombination, the ability of *P. citricarpa* to form airborne ascospores has further major epidemiological consequences for the dissemination of the pathogen and its ability to cause disease. In particular, airborne ascospores are, in general, suited for longer-distance dispersal and have a greater ability than conidia to survive for longer periods of time in the environment outside of the host (Aanen and Hoekstra 2007). Hence, in citrus black spot endemic areas where *P. citricarpa* simultaneously produces both sexual and asexual spores, each spore type can contribute to epidemic development.

This study demonstrates the sexual function of spermatia as a male gamete in the life cycle of *P. citricarpa*, and shows that fertilization in this fungus is via spermatization. In addition, germination tests showed that spermatia were not capable of germinating and forming colonies on culture media, supporting

their role in sexual reproduction rather than vegetative growth. This finding supports the hypothesis of Kiely (1948) that spermatia function as male gametes in *P. citricarpa*, because this author observed that spermatia were always present prior to development of pseudothecia. Spermatization has also been reported as a mode of fertilization in other ascomycete fungi such as *Elsinoë thurmalacharii* (Chiplonkar 1968) and *Botrytis cinerea* (Fukumori et al. 2004). Under field conditions, spermatia of *P. citricarpa* are found abundantly on both fruit lesions and leaf litter (Kiely 1948). However, sexual reproduction of *P. citricarpa* has only been observed on decaying leaf litter and never on diseased lesions on fruit or any other parts of the tree (Baayen et al. 2002; Baldassari et al. 2008). Therefore, we hypothesize that this is a result of only one mating type being present in a single diseased lesion on aboveground parts of the tree. Because citrus black spot lesions are often confined to discrete spots on the outer surface of the fruit, leaves, and twigs, opportunities for spermatia to move between lesions and fertilize a complementary partner on the other lesion are probably limited. On the other hand, leaf litter on the orchard floor more readily allows the two mating types to come in close contact for spermatia to fertilize the receptive organs. This might occur via rain splash washing spermatia horizontally within the leaf litter or in a downward direction from the canopy to the leaf litter. Future studies on distribution of mating types of *P. citricarpa* at the lesion, fruit, and tree level, and movement of spermatia and their mode of attachment to the female receptive elements, are required to test this hypothesis in order to explain the occurrence of sexual reproduction of *P. citricarpa* on leaf litter under orchard conditions. Moreover, investigating the ability of spermatia to germinate on living tissues or infect citrus fruit would provide a better understanding of this spore type in the disease cycle of citrus black spot on *Citrus* spp.

The vegetative compatibility test found no evidence for *P. citricarpa* isolates being vegetatively compatible so that the sexual state could not

TABLE 2. Genotyping of *Phyllosticta citricarpa* parental isolates and their ascospore progenies produced from in vitro sexual compatibility test using two simple-sequence repeat (SSR) loci and the mating type locus

Cross, genotypes ^a	N ^b	Loci examined		
		SSR locus 6 (No. CAA)	SSR locus 12 (No. ACC) ^c	Mating type
1				
Parent 1 (BRIP 53714) ...		15	14	MATI-1
Parent 2 (BRIP 53713) ...		8	11	MATI-2
Parental genotype 1	2	15	14	MATI-1
Parental genotype 2	4	8	11	MATI-2
Progeny hybrid 1	2	15	14	MATI-2
Progeny hybrid 2	1	8	14	MATI-1
Progeny hybrid 3	1	8	14	MATI-2
Total progeny	10	...	...	...
2				
Parent 1 (BRIP 53711) ...		8	14	MATI-1
Parent 2 (BRIP 54232) ...		12	11	MATI-2
Parental genotype 2	2	12	11	MATI-2
Progeny hybrid 1	2	12	14	MATI-2
Progeny hybrid 2	1	12	11	MATI-1
Progeny hybrid 3	2	8	11	MATI-1
Progeny hybrid 4	1	12	14	MATI-1
Total progeny	8	...	...	...
3				
Parent 1 (BRIP 53714) ...		15	...	MATI-1
Parent 2 (BRIP 53719) ...		8	...	MATI-2
Parental genotype 1	1	15	...	MATI-1
Parental genotype 2	6	8	...	MATI-2
Progeny hybrid 1	5	15	...	MATI-2
Progeny hybrid 2	5	8	...	MATI-1
Total progeny	17	...	...	...

^a Isolates in bold (in parentheses) are parental isolates retrieved from Queensland Plant Pathology Herbarium, Brisbane, Australia (BRIP).

^b Number of progeny.

^c SSR locus 12 was not applicable to the third cross.

be induced after attempting vegetative mycelium interactions. On 1/2 PDA medium, colonies of *P. citricarpa* simply stopped growing toward each other once in close proximity, preventing direct interaction between mycelia. In addition, use of a modified in vitro procedure developed by Mondal et al. (2004) to pair *M. citri* that involves two mycelial plugs placed opposite one another at the edge of an autoclaved lemon leaf disc was not successful for *P. citricarpa* because the mycelia did not interact to produce sexual structures (data not shown). Similarly, Amorim et al. (2017) were not successful in their efforts to induce the sexual state of *P. citricarpa* through pairing isolates of complementary mating types on citrus leaf discs. Therefore, it is likely that sexuality in *P. citricarpa* does not occur via mycelia interactions due to the lack of vegetative compatibility among isolates. Whether or not the observation that mycelia of *P. citricarpa* avoid growing into each other is indicative of vegetative incompatibility needs further investigation because, to the best of our knowledge, this feature has never been reported in *P. citricarpa*.

Through confirming heterothallism of *P. citricarpa*, this study reveals that, in order for an epidemic in a new area to include aerial dispersal via ascospores, it will be necessary for individuals of both mating types to be present. Epidemics without aerial ascospore dispersal have only been reported in Florida and Zimbabwe, although a small number of ascospores were found in the latter case (Wang et al. 2016; Whiteside 1967). Moreover, by reliably producing ascospores of *P. citricarpa* in culture media, the present study will facilitate future studies related to the occurrence and impact of the sexual cycle to epidemiology of citrus black spot in the field. It will also facilitate studies of inheritance, gene mapping, and genetic exchange between and among populations. Studies on the pathogenicity of ascospores to citrus fruit are underway, aiming to test the hypothesis that *P. citricarpa* ascospores are able to cause direct infection. Furthermore, the ability to produce ascospores of *P. citricarpa* in vitro will enable the study of genes involved in sexual reproduction of *P. citricarpa*, which will provide potential new target genes for fungicide development or alternative means to manage the disease through RNA interference approaches (Duan et al. 2012; Nowara et al. 2010). Thus, knowledge gained from the present study may aid in the development of better management approaches to control citrus black spot and, ultimately, reduce the impact of the disease.

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