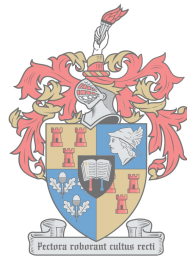# POPULATION STRUCTURE, SEX AND SPATIAL DISTRIBUTION OF *PHYLLOSTICTA CITRICARPA,* THE CITRUS BLACK SPOT PATHOGEN

By

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March 2018

I

DECLARATION

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With regard to the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
My contribution – experimental design, field work, experimental work and wrote the manuscript.	55

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1.   the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors

2.   no other authors contributed to besides those specified above, and

3.   potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in this dissertation.

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SUMMARY

Citrus Black Spot (CBS), caused by *Phyllosticta citricarpa,* is a fungal disease that influences citrus industries worldwide. All commercial *Citrus* spp. are susceptible to the disease. The pathogen was first described 117 years ago from Australia; subsequently, from summer rainfall citrus production regions in China, Africa, and South America; and, recently, the United States. Limited information is available on the pathogen's population structure, mode of reproduction, and introduction pathways at a global scale and at a regional (provincial) scale in South Africa. This is also true for the effect of distance (spatial), season (temporal) and *Citrus* spp. on population structure at the orchard scale. The aforementioned aspects were investigated in the current study. Since limited co-dominant markers are available for *P. citricarpa* population genetic analyses, one of the first aims of the study was to develop new simple-sequence repeat (SSR) markers.

The population structure of *P. citricarpa* was investigated at a global scale in 12 populations from South Africa, the United States, Australia, China, and Brazil. Seven published and eight newly developed polymorphic SSR markers were used for genotyping populations. The Chinese and Australian populations had the highest genetic diversities, whereas populations from Brazil, the United States, and South Africa exhibited characteristics of founder populations. Based on population differentiation and clustering analyses, the Chinese populations were distinct from the other populations. High connectivity was found, and possibly linked introduction pathways, between South Africa, Australia and Brazil. With the exception of the clonal United States populations that only contained one mating type, the other populations contained both mating types in a ratio that did not deviate significantly from 1:1. Although most populations exhibited sexual reproduction, linkage disequilibrium analyses indicated that asexual reproduction is also important.

The effects of distance (spatial) and season (temporal) on the population structure of *P. citricarpa* were investigated over two seasons, in two lemon orchards in South Africa; one in the Mpumalanga province and the other in the North West province. Spatial analyses indicated that subpopulations separated by a short distance (within 200 m) were typically not significantly genetically differentiated, but that those separated by longer distances were sometimes significantly differentiated. Temporal analyses in the North West orchard showed that seasonal populations were not significantly genetically differentiated. In contrast, seasonal populations from the Mpumalanga orchard were significantly differentiated, most likely due to higher rainfall and disease pressure, and the spatial scale of sampling. Based on linkage disequilibrium analyses, sexual and asexual reproduction occurred in both orchards. In each orchard, two dominant

multilocus genotypes (MLGs) were identified in most of the subpopulations, as well as in the seasonal populations. Pycnidiospores are therefore important in the development of CBS at the temporal and spatial scales in South African lemon orchards.

Population genetic studies on a regional (provincial) scale in South Africa showed that ten *P. citricarpa* populations, representing five provinces (North West, Mpumalanga, Limpopo, KwaZulu-Natal and Eastern Cape), were not significantly genetically differentiated. Based on gene and genotypic diversities and private allele richness, the KwaZulu-Natal or the Limpopo provinces are likely the provinces where the pathogen was first introduced. There might have been at least two separate introductions of the pathogen into the country. The Eastern Cape province was confirmed as being the province where the latest introduction occurred in South Africa. Despite lemon trees having overlapping fruit crops, potentially providing increased opportunities for clonal reproduction, *Citrus* spp. (lemons vs. oranges) did not have an effect on population structure; not all lemon populations were significantly genetically differentiated from all orange populations.

The current study has revealed novel information on the population structure of *P. citricarpa* at global and regional (South Africa) scales, which have implications for the epidemiology and management of the disease. The finding that pycnidiospores, in addition to ascospores, are also important in the epidemiology of the disease in South Africa, contradicts previous reports that pycnidiospores are of minor significance. Future studies should re-investigate the role of these spore types in the epidemiology of CBS in South Africa using conventional orchard inoculation and leaf removal studies, combined with a population genetic data analyses. The role that distance and season have on the population structure should also be considered in orchard trial designs. Ascospore spore trap data should be generated that involve the differentiation of *P. citricarpa* from *P. capitalensis*.

OPSOMMING

Sitrus Swartvlek (SSV) is 'n swamsiekte wat deur *Phyllosticta citricarpa* veroorsaak word, en wat sitrusbedrywe wêreldwyd beïnvloed. Alle kommersiële *Sitrus* spp. is vatbaar vir die siekte. Die patogeen is 117 jaar gelede vir die eerste maal in Australië beskryf en daarna van sitrus produserende streke in somerreënval gebiede in Sjina, Afrika en Suid-Amerika en mees onlangs van die Verenigde State. Beperkte inligting oor die patogeen se populasie-struktuur, wyse van voortplanting en introduksie roetes is op 'n globale vlak beskikbaar, sowel as op 'n provinsiale vlak in Suid-Afrika. Op 'n boordvlak, is inligting ook beperk oor die effek wat afstand ("spatial"), seisoen (temporaal) en *Sitrus* spp. op die populasie-struktuur het. Voorafgenoemde aspekte is in die studie ondersoek. Aangesien beperkte dominante merkers vir *P. citricarpa* populasie genetiese analises beskikbaar is, was een van die eerste doelstellings van die studie om nuwe mikrosatelliet merkers te ontwikkel.

Die populasie-struktuur van *P. citricarpa* is op 'n globale vlak in 12 populasies van Suid-Afrika, die Verenigde State, Australië, Sjina en Brasilië ondersoek. Sewe gepubliseerde en agt nuut ontwikkelde polimorfiese mikrosatelliet merkers is gebruik om die populasies te genotipeer. Die Sjinese en Australiese populasies het die hoogste genetiese diversiteit getoon, terwyl populasies van Brasilië, die Verenigde State en Suid-Afrika eienskappe van stigterspopulasies toon. Gebaseer op populasie-differensiasie en groeperings-analises verskil die Sjinese populasies van die ander populasies. Hoë konnektiwiteit en moontlik gedeelde introduksie roetes is tussen Suid-Afrika, Australië en Brasilië gevind. Met die uitsondering van die klonale populasies van die Verenigde State, met net een paringstipe, het die ander populasies beide paringstipes gehad in 'n verhouding wat nie beduidend van 1:1 afwyk nie. Alhoewel die meeste populasies geslagtelike voortplanting getoon het, het "linkage disequilibrium" analises getoon dat ongeslagtelike voortplanting ook belangrik is.

Die effek van afstand (ruimtelik) en seisoen (temporaal) op die populasie-struktuur van *P. citricarpa* is oor twee seisoene in twee suurlemoenboorde in Suid-Afrika ondersoek; een boord in die Mpumalanga-provinsie en die ander in die Noordwes-provinsie. Ruimtelike analises het getoon dat subpopulasies wat deur 'n kort afstand (binne 200 m) geskei word, tipies nie betekenisvol geneties gedifferensieerd was nie, maar dat die wat deur langer afstande geskei is, soms betekenisvol gedifferensieerd was. Temporale analises in die Noordwes boord het getoon dat seisoenale populasies nie betekenisvol geneties gedifferensieerd was nie. In teenstelling hiermee, was seisoenale populasies van die Mpumalanga-boord betekenisvol gedifferensieerd, waarskynlik weens hoër reënval en siektedruk en die ruimtelike skaal van monsterneming.

Gebaseer op "linkage disequilibrium" analises, het geslagtelike en ongeslagtelike voortplanting in beide boorde plaasgevind. In elke boord het twee dominante multi-lokus genotipes (MLG's) in die meeste van die subpopulasies, sowel as in die seisoenale populasies, voorgekom. Piknidiospore is dus belangrik in die ontwikkeling van SSV op temporale en ruimtelike vlakke in Suid-Afrikaanse suurlemoenboorde.

Populasie genetika studies op 'n streeks- (provinsiale) vlak in Suid-Afrika het getoon dat tien *P. citricarpa*-populasies wat vyf provinsies verteenwoordig (Noordwes, Mpumalanga, Limpopo, KwaZulu-Natal en Oos-Kaap), nie betekenisvol geneties gedifferensieerd was nie. Gebaseer op geen- en genotipiese diversiteit en die aantal privaat allele, is die KwaZulu-Natal provinsie of die Limpopo provinsie waarskynlik die provinsies waar die patogeen eerste gevestig het. Daar is moontlik ten minste twee afsonderlike introduksies van die patogeen. Daar is bewys dat die Oos-Kaap die provinsie is waar die laaste introduksie in Suid-Afrika plaasgevind het. Ten spyte daarvan dat suurlemoenbome wat oorvleuende oeste het, moontlik verhoogde geleenthede vir klonale voortplanting bied, het *Citrus* spp. (suurlemoene vs. lemoene) nie 'n effek op die populasie-struktuur gehad nie, omdat nie al die suurlemoenpopulasies betekenisvol geneties gedifferensieerd van al die lemoenpopulasies was nie.

Die studie het nuwe inligting oor die populasie-struktuur van *P. citricarpa* op 'n globale en streeks- (Suid-Afrika) vlak gebring, wat implikasies vir die epidemiologie en bestuur van die siekte inhou. Die bevinding dat piknidiospore, bykomend tot askospore, ook belangrik in die epidemiologie van die siekte in Suid-Afrika is, weerspreek vorige verslae dat piknidiospore van geringe belang is. Verdere studies moet die rol van hierdie spoortipes in die epidemiologie van SSV in Suid-Afrika deur middel van konvensionele boord-inokulasies en blaarverwyderingstudies ondersoek. Dit moet met 'n populasie genetika studie gekombineer word. Die rol wat afstand en seisoen op die populasie-struktuur het, moet ook vir die ontwerp van boordproewe oorweeg word. Askospoor lokval-data moet gegenereer word wat tussen *P. citricarpa* en *P. capitalensis* kan onderskei.

ACKNOWLEDGEMENTS

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CHAPTER 1

A review of *Phyllosticta citricarpa*, the citrus black spot pathogen

INTRODUCTION

The citrus industry in South Africa was founded in 1654 when the seafarer Commander Jan van Riebeeck planted the first orange trees in the Cape Colony on his farm and in the Company's Garden (Chapot, 1975). The first trees were brought from the Island of St Helena, which was a stopover for ships on their way from Asia to Europe. The tradesmen on these ships planted the fruit trees on the Island of St Helena (Powell, 1930; Allwright, 1957). Other records of imported citrus material into South Africa, before the first trained horticulturists arrived in the Cape to develop the fruit industries, included orange trees imported from India in 1656 and grafted trees in 1850 from Brazil (Allwright, 1957). These plantings were seen as the ancestors of the citrus trees that subsequently moved inland from the Cape with the pioneer settlers (Oberholzer, 1969). The first citrus exports took place in 1902 when fruit was shipped from South Africa to England. In 1906, the South African citrus industry won a gold medal at a Trade Show in England. Exports reached the one million box mark in 1925 (CGA, 2017).

Today, the citrus industry in South Africa is an export-driven industry that produces a variety of citrus types. The South African citrus industry is one of South Africa's major agricultural industries with regard to exports. South Africa is the second largest international exporter of fresh citrus fruit and considering the current 70 055 planted hectares, South Africa is the tenth largest international producer of fresh citrus fruit (CGA, 2017). Citrus for fresh fruit production is produced in the Limpopo, Eastern Cape, Western Cape, Mpumalanga, KwaZulu-Natal, Northern Cape and North West provinces. The main citrus producing areas are situated in the Limpopo (42%), Eastern Cape (26%) and Western Cape (17%) provinces. The smallest production area is situated in the North West province with only 161 hectares planted (CGA, 2017). About 60% of the crop is sweet oranges (Valencias 38% and navels 22%), 16% soft citrus, 13% lemons and limes and 11% grapefruit. Currently 76% of the total crop is exported, of which 45% is exported to the European countries and 21% to the Middle Eastern countries (CGA, 2017). Valencias and navels are the major export products with 39% and 24% being exported respectively. The remaining 47% consist of lemons (14%), grapefruit (12%) and soft citrus (11%) (CGA, 2017).

Citrus production in South Africa is hampered by the presence of many pests and diseases. The international fresh fruit trade has always been influenced by plant health (McRae

et al., 2002). Some of South Africa's citrus trade partners have identified pests and diseases that are present in South Africa, but absent from other citrus producing regions in the world. These pests and diseases are thus of quarantine importance (Paul et al., 2005; Carstens et al., 2012). *Phyllosticta citricarpa* (McAlpine) Aa, which causes citrus black spot (CBS), is present in South Africa (Kiely, 1948; Kotzé, 1981; Carstens et al., 2012) and has been identified as being of quarantine importance by these trade partners. This includes the European Union, since CBS is not known to occur in any of the European regions. Specific requirements, such as area freedom, consignments inspections and pre- and post-harvest treatments, to ensure that consignments of citrus fruit are free from CBS has been specified in bilateral export protocols and import requirements (Carstens et al., 2012; E Phoku, Department: Agriculture, Forestry and Fisheries, Republic of South Africa, personal communication). Aside from restricting market access, CBS is also of economic importance in local markets, since it causes blemishes on fruit that affect fruit quality. CBS fruit blemishes are only cosmetic since the pathogen does not cause fruit rot. Fruit symptoms can develop after harvest while the fruit is in storage (Kiely, 1848; Kotzé, 1981; Agostini, et al., 2006).

CBS occurs worldwide where climatic conditions are suitable for disease establishment and spread. Climatic conditions play an important role in the occurrence and severity of the disease and it is only present in citrus producing countries that have a warm, humid, summer rainfall climate (Kotzé, 1981; Paul et al., 2005; Carstens et al., 2012; Yonow et al., 2013). The pathogen most likely spread on a global scale to new areas through infected budwood, trees and leaves. No insect vectors are known to disperse the pathogen. After the first CBS symptoms are observed in a region, the spread of the disease is very slowly. In South Africa, it took about 10 years for it to become a serious disease, and in Brazil it took 12 years to move from the first detection site to São Paulo (Kiely, 1948; Wager, 1952; McOnie, 1965; Kotzé, 1981, 2000; USDA APHIS, 2010). Valencia oranges and lemons are regarded as the most susceptible citrus types. The pathogen can infect all commercial citrus types and all plant parts above the ground with infection mostly remaining latent and asymptomatic. The most obvious symptoms are found on the fruit (Kiely, 1948; Kotzé, 1981).

Phyllosticta citricarpa produces two types of spores, i.e. asexual pycnidiospores and sexual ascospores. The sexual reproductive system of *P. citricarpa* was only recently elucidated as being that of a heterothallic fungus requiring both mating types. In Australia and Brazil both mating types of the pathogen are known to occur, enabling sexual reproduction (Wang et al*.,* 2016; Amorim et al., 2017; Tran et al., 2017). However, in Florida (USA), and in Portugal, Italy and Malta, only one mating type occurs (Wang et al*.,* 2016; Guarnaccia et al., 2017).

The relative contribution of asexual and sexual reproduction in countries where both mating types occur or are likely to occur, differs. In Australia and South Africa, *P. citricarpa* is believed to mainly reproduce sexually with ascospores playing a prominent role in disease development and pycnidiospores are believed to play a limited role (Kiely, 1948; McOnie, 1965; Kotze, 1981, 2000). However, in Brazil, pycnidiospores have been shown to play a more prominent role in disease development (Spósito et al., 2007, 2008, 2011).

Due to the importance of CBS in citrus production in South Africa and worldwide, a lot of research has been conducted to better understand the epidemiology of the disease and how to manage the disease. However, none of the studies used a population genetic approach to better understand the biology of the pathogen. This literature review will review CBS with regards to known symptoms, the disease cycle and the epidemiology, focussing on investigating the role of pycnidiospores and ascospores in the epidemiology of the disease. The review will end with a brief summary of population genetics tools that can be used to better understand the biology and epidemiology of plant pathogens. Information on molecular markers that have been evaluated for *P. citricarpa,* and that can be used in population genetic studies will be provided.

HISTORY AND GEOGRAPHICAL DISTRIBUTION OF CITRUS BLACK SPOT

A. H. Benson was the first to officially recognize and describe CBS. Although he did not study the disease, he made drawings in 1895 of the symptoms he found on sweet oranges in New South Wales, Australia (Benson, 1895). The first measurements of one of the spore types of CBS from the fruit was made by Cobb in 1897 (Cobb, 1897). At that stage, a *Colletotrichum* sp*.* was regarded as the causal organism. In 1899, McAlpine provided the first detailed description of the causal organism. He based his description on the structure of the asexual (pycnidial) form found in lesions on citrus fruit and described the causal organism as a new species*, Phoma citricarpa* McAlpine (McAlpine, 1899). Experiments conducted in 1906 confirmed the suspected latent nature of the pathogen on fruit (Kiely, 1948). The first results on possible control methods with chemicals was published in 1916 (Darnell-Smith, 1916). Kiely, who described the pseudothecial stage of the fungus, *Guignardia citricarpa* Kiely, was the first to discover the importance of ascospores in the life cycle of CBS and that leaves can be latently infected (Kiely, 1948). Later in 1973, the asexual form was renamed *Phyllosticta citricarpa* (McAlpine) Aa (Van der Aa, 1973).

The sexual form of the pathogen was known for many years as *Guignardia citricarpa* and the asexual form as *Phyllosticta citricarpa*. The recent changes in fungal nomenclature abolished the separate names for the two forms of a fungus, and a single name for each fungus based on

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the earliest description was adopted. Accordingly, the CBS pathogen is now known as *Phyllosticta citricarpa* (Rossman and Samuels, 2005; Wikee et al., 2011).

The first official record of the disease outside Australia was in 1920 from China which is hypothesised to be the centre of origin of *Citrus* (Lee, 1920; Scora, 1975). The second notification was from Argentina in 1928 (Marchionatto, 1928). The third international notification was from South Africa in 1929 (Doidge, 1929). Although Brazil and the United States of America are the second and third largest producers of citrus, CBS was only reported to be present in 1980 (Paul et al., 2005) and in 2010 (Schubert et. al., 2012) in these countries, respectively.

In South Africa, CBS was first described in 1929 from orange orchards, nearby Pietermaritzburg in the Natal province (currently known as the KwaZulu-Natal province) (Doidge, 1929). Today, KwaZulu-Natal is the fifth largest citrus producing region in South Africa and the third largest production region for grapefruit (CGA, 2017). In 1952, Wager reported that the disease only became notable in 1940 in the Pietermaritzburg area (Wager, 1952). In 1945, it was found in other areas in Natal and was also reported for the first time from another province in South Africa, namely the Northern Transvaal (currently known as the Limpopo province). In 1946, the disease was reported from other provinces including the Western Transvaal (currently known as the North West province) and Eastern Transvaal (currently known as the Mpumalanga province). By 1950 the disease was wide spread in these citrus producing areas. In 1953, Wager reported that CBS was not reported from the western side of the Western Transvaal (North West), the Western Cape and the Eastern Cape. The absence of CBS from the Western Cape was supported by McOnie (1964a). The disease was noticed in the Eastern Cape in the early 1970s (C. Kellerman, Citrus Consulting Association (SASSCON), personal communication). Until today, no CBS has been reported from the Western Cape and two of the magisterial districts in the western part of the North West province (Carstens et al., 2012).

Although CBS has an almost global distribution, it has to date only been recorded from citrus producing countries and regions having a warm, humid, summer rainfall climate. The disease has been reported from Africa (Ghana, Nigeria, Kenya, Uganda, Zambia, Zimbabwe, Mozambique, Swaziland and parts of South Africa), Asia and Oceania (Hong Kong, Bhutan, parts of China, Indonesia, Philippines, Taiwan and parts of Australia), South America (Argentina, Brazil and Uruguay) and North America (Florida - USA) (CABI, 2017). The CBS disease has never been reported from countries or areas within countries where citrus is produced under Mediterranean climates and climates with winter rains and hot, dry summers (Broadbent 1995; Carstens et al., 2012; Paul et al., 2005; Yonow et al., 2013). *Phyllosticta citricarpa* was recently reported to be present in citrus leaf litter from the Mediterranean countries, Portugal, Malta and Italy, where citrus

is produced. However the disease was apparently absent as no symptoms were present in the orchards where the pathogen was found (Guarnaccia et al., 2017). In Australia, the disease is absent from the Murray Valley in Riverina and Riverland (Miles et al*.,* 2008). In South Africa, the disease is absent from the Western and Northern Cape citrus producing provinces (Carstens et al., 2012). Also in China, CBS is restricted to production regions with warm, humid, summer rainfall conditions (Wang et al., 2012). CBS is absent for Europe, Central America, the Caribbean Region and New Zealand (Everett and Rees-George, 2006; CABI, 2017).

PHYLLOSTICTA SPECIES ASSOCIATED WITH *CITRUS* SPECIES

Prior to the 1970s, it was believed that *G. citricarpa* had both a pathogenic and a non-pathogenic variant since a *Guignardia* sp. was isolated from citrus trees showing no CBS symptoms and other host plant species (Kiely, 1948; McOnie, 1964b). The pathogenic variant causing CBS symptoms was restricted to *Citrus* spp., while the symptomless non-pathogenic variant had a broader host range and wider distribution. Morphologically it was not possible to distinguish between the two variants. However, molecular studies based on analyses of the sequence of the internal transcribed spacer (ITS) region, and amplified fragment length polymorphic fingerprint (AFLP) patterns revealed two distinct species, *G. citricarpa* and *G. mangiferae* A.J. Roy (anamorph *P. capitalensis* Henn) (Baayen et al., 2002). The study of Baayen et al., (2002) confirmed that *G. citricarpa* is the CBS pathogen and that it is of phytosanitary importance to the international citrus trade. *Guignardia mangiferae* was renamed to *Phyllosticta capitalensis* following phylogenetic studies conducted in 2011 (Glienke et al., 2011). *Phyllosticta capitalensis* is an endophyte with a wide host range and is not known to cause a plant disease. Subsequently, molecular and phylogenetic analyses of the ITS region and additional gene regions of more *Phyllosticta* isolates have identified other *Phyllosticta* species that are associated with Citrus. Some of the species cause symptoms on fruit and leaves while others are endophytic. Noteworthy is that the economic or phytosanitary importance of these species have not yet been fully elucidated. The plant pathogenic species associated with different citrus types apart from *P. citricarpa* are *P. citriasiana* on pumeloes in Thailand and China (Wulandari et al*.,* 2009*,* Wang et al., 2012), *P. citrichinaensis* on grapefruit, mandarins and oranges in China (Wang et al., 2012), *P. citrimaxima* on pumeloes in Thailand (Wikee, et al., 2013) and *P. paracitricarpa* on leaf litter of lemon orchards in Greece (Guarnaccia et al., 2017). *Phyllosticta paracitricarpa* has been shown to produce atypical necrotic lesions on artificially inoculated fruit only, but no symptoms have been observed in the field (Guarnaccia et al., 2017). The endophytic species associated with *Citrus* spp. other than *P. capitalensis* included *P. spinarum* and *P. citribraziliensis* on lemons in Brazil

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(Stingari et al., 2009; Glienke et al., 2011) and *P. paracapitalensis* on *Citrus* spp. in Italy and Spain (Guarnaccia et al., 2017).

Symptoms caused by the different plant pathogenic *Phyllosticta* species (*P. citricarpa, P. citriasiana, P. citrichinaensis* and *P. paracitricarpa*) are very similar and are also morphologically similar. Symptoms are variable in appearance and can easily be confused with symptoms caused by pathogens other than *Phyllosticta* species. To ensure the correct identification of symptoms and *Phyllosticta* species, specific diagnostic procedures have been developed that include isolation onto specialised agar media and molecular detection using species-specific primers (FAO, 2014). One of the cultural characteristics of *P. citricarpa* is that it forms a yellow halo around colonies after 7-days' growth on oatmeal agar when plates are incubated at 25°C (FAO 2014). Species-specific primers, or sequence data must be used to genotypically differentiate *P. citricarpa* from *P. capitalensis* (Meyer et al., 2006; Peres et al., 2007).

SYMPTOMS CAUSED BY *P. CITRICARPA*

CBS symptoms develop on leaves and fruit. On fruit, the symptoms can develop while still on the tree or after harvest (Kiely, 1948; McOnie, 1967; Kotze, 1981, 2000). However, symptoms on the fruit mostly become visible on mature fruit after colour break (Kotzé, 2000). Symptom expression after harvest and the viability of the fungus in fruit lesions can be influenced and controlled by low storage temperatures and standard packhouse treatments (Korf et al., 2001; Agostini et al., 2006; Schreuder, 2017).

For symptoms to develop on the fruit, the mycelium must grow into the rind (Kotzé, 2000). The pathogen is not known to cause fruit rot, only necrotic lesions. Six symptoms are known to be associated with CBS. The most common of these six symptoms include hard spot, false melanose, freckle spot and virulent spot, whereas lacy spot and cracked spot are less common. Pycnidia producing pycnidiospores are not produced in all of the symptom types (Kiely, 1948; Kotzé, 1981, 2000; De Goes et al., 2000; De Goes, 2001; Aguilar-Vildoso et al., 2002).

Hard spot is the most typical fruit symptom and consists of more or less circular, depressed, brick red lesions that turn brown to black over time, with black margins and grey necrotic tissue in the centres. Sometimes a yellow or green halo may be found around the lesions, depending on the colour of the fruit. Pycnidia often, but not always, develop in the lesions. False melanose or speckled blotch are devoid of pycnidia and can appear on green fruit as dark brown to black lesions. Freckle spots are grey, tan, reddish or colourless, with no halo around them and are mostly devoid of pycnidia. The spots may develop into virulent spots later in the season or during storage. Virulent spots, irregular in shape, are the most damaging and numerous pycnidia

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can develop in these lesions (Kiely, 1948; Kotzé, 1981, 2000). The other two symptoms, lacy spot and cracked spot are less common and are devoid of pycnidia (De Goes et al., 2000; De Goes, 2001; Aguilar-Vildoso et al., 2002).

Leaf symptoms are rare and have only been reported on leaves of lemons and Valencia oranges (Kiely, 1948; Kotzé, 2000; De Oliveira Silva et al., 2017). The disease mainly occurs on leaves as latent infections without visible symptoms. If symptoms on leaves are present, they consist of small round sunken necrotic spots surrounded by a dark brown ring. Sometimes a yellow halo may be present around leaf lesions. Twig symptoms are rare and are characterised by small, round, sunken necrotic spots with grey centres, surrounded by a dark brown ring (Kiely, 1948; Kotzé, 2000; FAO, 2014).

HOSTS OF CITRUS BLACK SPOT

All commercially grown *Citrus* species that include sweet oranges, lemons, limes, pumeloes, grapefruit, mandarins, limes and their hybrids, are susceptible (Kiely, 1948, Kotzé, 1981). Of all the citrus types, lemons and Valencia oranges are regarded as the most susceptible (Kiely, 1948). According to Kiely (1948) and Kotzé (1981), CBS symptoms will first be noticed on lemons in a new area. To date, there is no documentation indicating symptom development on Tahiti limes and sour oranges and their hybrids, although the pathogen has been isolated from Tahiti limes (Kotzé, 1981; Baldassari et al., 2008).

LIFE CYCLE OF *P. CITRICARPA*

The pathogen has a primary life cycle involving sexual ascospores, and secondary life cycle involving asexual pycnidiospores. Pycnidia containing pycnidiospores can be found in lesions on fruit, dead twigs, leaves and living branches, while the ascopores can only be found on leaf litter (Kotzé 1981, 2000; De Oliveira Silva et al., 2017). The availability of the two spore types and infection by the spores requires different climatic conditions and have different ways of dispersal. These aspects will be discussed under the epidemiology section below.

Phyllosticta citricarpa is a heterothallic fungus that requires two mating types for sexual reproduction and the formation of ascospores (Wang et al., 2016; Amorim et al., 2017). Although the importance of ascospores in the life cycle has been known since 1948 (Kiely, 1948), the mating types genes and the mechanism responsible for sexual reproduction of the pathogen were only recently resolved (Wang et al., 2016; Amorim et al., 2017; Tran et al., 2017). A factor that hampered the unravelling of the heterothallic nature of the pathogen was that it has been impossible to produce ascospores in culture until very recently. Tran et al. (2017) was the first to

report a method to produce ascospores in culture. The difficulty in accomplishing this in the past, is due to the fact that *P. citricarpa* is vegetatively incompatible, coupled with the fact that it was not possible to know the mating type of isolates used in matings (Tran et al., 2017). The latter has recently been resolved when the mating type locus was identified for the first time in *P. citricarpa.* This enabled the development of mating type specific primers for the MAT1-1-1 and the MAT1-2-1 genes (Wang et al., 2016; Amorim et al., 2017). The study by Tran et al. (2017) revealed that successful mating requires opposite mating types in direct physical contact. Recent studies determined that both mating types were present in *P. citricarpa* populations from Australia and Brazil. Furthermore, the frequency of the mating types did not deviate significantly from a 1:1 ratio based on Chi-square analyses (Wang et al., 2016, Amorim et al., 2017). Populations obtained from the USA (Florida) and Europe (Portugal, Italy and Malta) have, however, been reported to only contain one of the mating types (Wang et al., 2016; Guarnaccia et al., 2017).

Ascospores are regarded as the primary source of infection of susceptible plant parts. These spores are only produced in fruiting bodies (pseudothecia) on infected leaf litter when certain conditions prevail (Kiely, 1948; Kotzé, 1981; Truter, 2010; Fourie et al., 2013). Under suitable environmental conditions ascospores are ejected from pseudothecia and infected susceptible plant parts. Plant parts are only susceptible while on the tree and only green leaves up to the age of ten months are susceptible (Truter, 2010). Fruit are susceptible from fruit set until four to five months after fruit set (Kiely, 1948; Kotzé, 2000).

Primary infections caused by ascospores, depending on the plant part infected, will develop into lesions in which pycnidia can develop. The most obvious presence of pycnidia are found within fruit lesions. However, as discussed under the symptom section above, not all fruit lesions produce pycnidia. Pycnidia are found in lesions on infected twigs and leaves (dead and green), living branches and sometimes on fruit stalks. Pycnidia produce pycnidiospores that are reported to be short-lived (Kiely, 1948). The spores are produced in gelatinous masses and need water to be release from the mucilaginous mass to infect the susceptible plant parts. Pycnidiospores can cause infections of susceptible fruit and also leaves (Kiely, 1948; Whiteside, 1967; Kotzé, 1981; De Oliveira Silva et al., 2017). Fallen leaves on the ground cannot be infected by pycnidiospores (Truter, 2010).

EPIDEMIOLOGY

Studies on the epidemiology of the disease focused mainly on weather parameters that influence disease development and the dispersal of the two spore types (ascospores and pycnidiospores). The production of pycnidiospores and ascospores occur under different conditions, which

influences the epidemiology differentially (Magarey et al., 2015). Pseudothecia will only develop and produce spores for infection after recurrent wetting and drying periods after 40 to 180 days at temperatures between 15°C to 35°C (Kiely, 1948; McOnie, 1964c; Lee and Huang, 1973; Kotzé, 1981; Truter, 2010; Fourie et al., 2013). Fourie et al. (2013) found, using a modelling approach, that temperature had a major influence on the maturation of pseudothecia. The pseudothecia were only mature and ascospores ready for release after 907.1 degree days >10˚C (Fourie et al., 2013). In unsuitable climates such as too wet or too hot or too dry, the leaf litter decomposes or is colonised by saprophytes or dries out and the pathogen is killed inside the leaf litter before any ascospores can be produced (Kiely, 1948, Lee and Huang, 1973, Truter, 2010). Precipitation (rainfall) is needed for the ascospores to be forcibly discharged from asci within pseudothecia (Kiely, 1948; Kotzé, 1963, 1981; McOnie, 1964c; Reis et al., 2006; Dummel et al., 2015). For ascospores to germinate and infect susceptible plant parts, at least 15 hours of continuous wetness of the plants parts at an optimal temperature of 27°C is required. Optimal conditions for the germination of pycnidiospores and the infection of suseptible plant parts differ from ascospores. Pycnidiospores require a wet period of at least 12 hours at 25°C for infection (Noronha, 2002).

Several modelling approaches, using weather parameters along with other factors, have been used to develop models for determining if the climate in Europe is suitable for CBS to establish and develop. This information is required to determine the risk of introduction of CBS on imported fruit from infected locations (Magarey et al., 2015).

CLIMEX, a mechanistic model which uses literature, weather data and distribution records was used to determine if CBS can establish in Europe. The first study using CLIMEX concluded that it was unlikely that CBS could establish in European regions (Paul et al., 2005). A follow-up study by Yonow et al. (2013), similar to Paul et al. (2005), came to the same conclusion that there is not a risk for CBS establishing in Europe.

A few studies have also used infection models to predict whether CBS will be able to establish in Europe. Magarey et al. (2011) developed an infection model using daily weather data, but did not include an ascospore dispersal model. EFSA (2008, 2014) used advanced infection models to predict the number of CBS infection periods in Europe, South Africa and Australia. Whilst infection events were predicted in most localities, the number of infections were always significantly higher in warm, summer rainfall climates where CBS is known or expected to occur. Fourie et al. (2013) published models for the effects of temperature and wetness on *Phyllosticta* ascospore dispersal using ascospore trapping data and weather data. Magarey et al. (2015) used the ascospore dispersal model (T-model) described in Fourie et al. (2013), ascospore and

pycnidiospore infection models (Magarey et al., 2011) and hourly weather data in order to develop a model to define the thresholds for the number of infection periods required for a site or year to be classified as favourable for CBS development.

The dispersal distance of *P. citricarpa* ascospores and pycnidiospores differ. Ascospores are windborne and responsible for dispersal of the pathogen over distances typically up to 25 meters (Kiely, 1948; Wager, 1953; McOnie, 1964d, 1965; Kotzé, 1981, 2000; Spósito et al., 2007). In contrast to ascospores, pycnidiospores are waterborne spores that mostly disperse at short distances (Spósito et al., 2011; Hendricks et al., 2017). Due to this short-distance dispersal, pycnidiospores are not regarded as an important contributor to disease development within orchards or dispersal of the pathogen to new areas in South Africa and Australia (Kiely, 1948; McOnie, 1964d; Kotzé, 1981, 2000). However, in Brazil pycnidiospores are regarded as being important in CBS epidemiology (Spósito et al., 2008, 2011).

STUDIES THAT HAVE BEEN CONDUCTED TO INVESTIGATE THE ROLE OF ASCOSPORES AND PYCNIDIOSPORES IN CBS EPIDEMIOLOGY

Studies conducted in Australia and South Africa in late 1940's and 1960's came to the conclusion that ascopores are more important in disease epidemics than pycnidiospores (Kiely, 1948; McOnie, 1964d; Kotzé, 1981, 2000). However, in Brazil, recent studies have shown that pynidiospores are important. This could be due to differences in management practices and climate in Brazil, in comparison to South Africa and Australia (Spósito et al., 2008, 2011). More recently, investigations into the role of pycnidiospores in the epidemiology of CBS have been facilitated by the recent introduction of *P. citricarpa* into Florida (USA) consisting of a single mating type. This provides the first evidence that the pathogen can spread and persist using only pycnidiospores (Wang et al., 2016). The specific experiments that have been conducted in all of the aforementioned studies will be discussed in more detail in this section.

In South Africa, McOnie (1964c, d) came to the conclusion that pycnidiospores were not important based on experiments using spore trapping, fruit bagging, and staggered spray experiments. These experiments showed that initial fruit infection coincided with the earliest and highest ascospore discharge. However, at that time it was unknown that two *Phyllosticta* species, similar in their ascospore morphology, were present (Meyer et al., 2006). The species identity of the trapped ascospores remains unknown. Pycnidiospores on dead leaves, which are abundant in orchards, were not considered important since they were released prior to the fruit infection period. Interestingly, pycnidia formed on dead leaves along with pseudothecia (McOnie, 1964c). It was also concluded that if dead leaves were an inoculum source of pycnidiospores, low hanging

fruit would have more lesions, which have not been observed in South Africa. Pycnidiospores on fruit were not considered important in South Africa since in all citrus types, except for lemons, the fruit are removed from trees before the onset of the new crop (McOnie, 1964c; Kotzé, 1981).

In Australia, Kiely (1948) showed that pycnidiospores are not important in the epidemiology of the pathogen using spore trapping in an orchard containing severe fruit infections. Microscope slides that were placed between tree rows and around the boundary of an orchard containing severe fruit infections, rarely contained pycnidiospores when slides were placed between rows, and not at all on slides placed at the boundary of the orchard. Ascospores on the other hand, could consistently be trapped on slides between rows and at the boundary of the orchard. Kiely (1948) could only trap pycnidiospores in water sampled from a filter funnel placed at the bottom of trees. This suggested that the source of pycnidiospores is only relevant within trees, but rarely between trees. The spores trapped from within trees were thought to originate from fruit, since spores were only present once virulent type lesions started forming on fruits (Kiely, 1948).

Studies conducted in the 2000s in Brazil showed that pycnidiospores are important in the disease cycle. The difference in the role of pycnidiospores in Brazil, as opposed to South Africa and Australia, is thought to be due to several cultural and environmental conditions differing between the regions. In Australia and South Africa, in contrast to Brazil, there are few off-season fruit, the period of fruit infection is restricted to four to five months, and no overlapping of old and new fruit crops within trees occurs in most citrus types. Furthermore, differences exist in the frequency, type of pruning and the management of pruning in orchards that influence the amount of dead twigs. In Brazil, three studies were conducted on the spatial behaviour of *P. citricarpa* to deduce the role of pyncidiospores in CBS epidemiology. The focus of the first study was to determine the dispersal of *P. citricarpa* in citrus orchards by counting the trees with symptomatic fruit and by plotting the position of the trees on maps of the orchards over a 3-year period (Spósito et al., 2007). In the second study, the incidence of symptomatic fruit and their aggregation patterns within the tree were measured over a 2-year period, to determine the role of asexual and sexual spores in disease epidemics (Spósito et al., 2008). The studies concluded that the pathogen was only dispersed over a short distance, and that pycnidiospores play an important role in the dispersal of the pathogen within trees and within orchards. In 2011, Spósito et al. (2011) showed that the placement of *P. citricarpa* inoculum, consisting of infected fruits or dead twigs in trees in a CBS-free orchard, was able to cause new fruit infections, but at short distances (<0. 8 m). Pycnidiospores produced in fruit lesions were an important inoculum source since fruit infections were more severe in the second fruit crop, when there was an overlap between young and old

fruit on trees. Lastly, the removal of all leaf litter and therefore ascospore inoculum, from the orchard floor in orchards where there was no overlap of fruit crops within trees, did not prevent disease development (Spósito et al., 2011).

In Florida, where only pycnidiospores are known to be available (Wang et al., 2016), a study has been conducted where the position of trees within orchards with CBS fruit were plotted using the position of the trees on maps of the orchards over a 3-year period. The study suggested that based on expansion of the disease foci, pycnidiospores can be dispersed over longer distances (>0.8 m) than previously reported in Brazil. It was concluded that pycnidiospores can contribute to dispersal within orchards and disease expansion for a distance of at least 6.7 m (Hendricks et al., 2017). However, the methodology employed could not exclude the possibility of spread of the pycnidium-containing leaves or twigs by other means, most notably the frequent tropical storms under south-Florida conditions.

MANAGEMENT OF CITRUS BLACK SPOT

Orchard sanitation

A key management strategy of CBS is to establish new orchards using CBS free trees. It is best to obtain these trees from nurseries in areas that are CBS free (Kotzé, 2000).

The practice of not having overlapping fruit crops on trees, will remove fruit containing pycnidiospores as a source of inoculum (Kotzé, 1981). However, this is not always possible for all citrus types.

Removal of leaf litter or chemical treatment of leaf litter are known effective management strategies for other ascigerous tree pathogens such as *V. inaequalis* with a similar life cycle to *P. citricarpa* (Truter, 2010; Gonzalez-Dominguez et al., 2017). However, in CBS, this strategy has been less effective. McOnie (1967) evaluated several chemicals for treatment of leaf litter to reduce ascospore inoculum. However, none of the treatments were effective in reducing the primary inoculum to a level where disease incidence was reduced (McOnie, 1967). In Brazil, leaf litter removal significantly reduced the initial amount of disease and disease progress rate relative to plots where leaf litter was not removed. In these trials there was no overlapping of fruit crops on the trees, which could provide a source of pycnidiospore inoculum from fruit for new fruit infections. Although the disease was reduced by leaf litter removal, CBS symptoms were still present and disease incidence amounted to 100% after 120 days (Spósito et al., 2011). In South Africa, Truter (2010) found a significant reduction in disease symptoms through the management

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of leaf litter, but the disease was not eliminated. Kotzé (2000) stated that covering leaves with grass mulch during the critical period of infection can reduce CBS in South Africa.

Chemical control

Chemical control is a pivotal and very effective method for managing CBS world-wide (Makowski et al., 2014). A preventative chemical strategy is used, which is aimed at protecting fruit from infection during the fruit susceptibility period. Generally this occurs between October through to February in South Africa. However, this period can be affected by the start of the first major rains, the first major discharge of ascospores and how favourable conditions are for infection of the pathogen (Kotzé, 1981). In South Africa, spore trapping, rainfall records and pseudothecium maturation and infection models (Fourie et al., 2013) have been used to predict the onset of ascospore release and the start of the protective fungicide spray programmes. These factors are critical for determining the timing of chemical applications (Kotzé, 2000). However, these factors are not always easy to predict accurately, and therefore losses to CBS still continue to occur occasionally. Initially, only protective fungicides such as mancozeb or copper fungicides were used preventatively to control CBS (Kotzé, 1981). Tree age, tree vigour, cultivar and environmental conditions determine the number of sprays required during the fruit susceptibility period, which can last for four to five months (Kotzé, 2000). Mancozeb and other dithiocarbamate fungicides are, however, no longer used in some countries due to requirements by some export markets (Silva Junior et al., 2016). Benzimidazole fungicides were found to be very effective, but resistance eventually developed against this group of fungicides (Kotzé, 1981, 2000). Currently strobilurin fungicides (quinone outside inhibitors, QoI), such as azoxystrobin and pyraclostrobin, are used in mixtures with protectant fungicides during the fruit susceptibility period (Hincapie et al., 2014; Silva Junior et al., 2016).

POPULATION GENETIC ANALYSES OF PLANT PATHOGENS

The field of population genetics was founded about 100 years ago with the work done by the fathers of the field namely R.A. Fisher and Sewall Wright (Fisher, 1930; Wright, 1931, 1943). They integrated the principles of Mendelian genetics with Darwin's theory on natural selection. Fisher demonstrated that there is a direct correlation between a population's genetic diversity and the rate of evolutionary change by natural selection with respect to fitness. In plant pathology, the awareness of genetic diversity and evolution dates back to the earliest description of host specialization and races (Milgroom, 2015).

Population genetics is a field of biology that studies the genetic composition and diversity of biological populations. It can deal with rather long time scales and large spatial scales and also forms part of evolutionary biology. It is important to note that in population genetics, the focus is on the population and not on the individual. A population's amount of genetic variation ultimately determines the evolutionary potential of a pathogen. The latter, for plant pathologists, can be indicative of the ease with which a pathogen can be managed (McDonald and Linde, 2002). Genetic variation and population structure is studied within and between populations. It involves the examination of changes in gene diversity and genotype diversity (Milgroom and Peever, 2003).

Gene diversity is an indication of the occurrence (richness) and frequency (evenness) of alleles at a locus (Nei, 1973), and the value always ranges between 0 and 1. When the gene diversity of a population is 1 (H_e = 1), it means that any two alleles sampled at a locus will be different. Determining the number of alleles (richness) at a locus is the simplest way to measure genetic diversity, while the number of private alleles in a population is a simple way to indicate genetic distinctiveness (McDonald and Linde, 2002). It is known that allele diversity is affected by the length of time that a specific population occurs in a specific area; older populations will have a higher level of genetic diversity with more alleles and also more private alleles. Therefore, it is expected that populations in the centre of origin of the pathogen will have a higher allele and private allele richness (Castric and Bernatchez, 2003; Linde et al*.,* 2009).

Genotypic diversity is an indication of the number (richness) and frequencies (evenness) of multilocus genotypes (MLGs) in a population (McDonald and Linde, 2002; Grünwald et al., 2003). A multilocus genotype is defined as unique combination of alleles (Milgroom, 2015). The genotype evenness value is an indication of how the genotypes are distributed within a population. The evenness values can vary from zero (no evenness) to one (all MLGs have equal abundance) (Grünwald et al., 2003; Shannon and Weaver, 1949). The number of MLGs (richness) is influenced by the sample size. To overcome this problem, Hulbert (1971) invented the statistical solution of rarefraction. Genotypic diversity is measured in three ways, namely the Shannon-Wiener index (H), Stoddart and Taylor index (G) and the Simpson's index (lambda) (Stoddart and Taylor, 1988; Shannon, 2001; Grünwald et al., 2003). Although the genotypic diversity of a population is influenced by the mode of reproduction of the pathogen, caution should be taken when interpreting the results. A low genotypic diversity, which is an indication of predominant asexual reproduction, does not exclude sexual reproduction (McDonald and Linde, 2002). Therefore, other approaches are important for determining sexual reproduction as discussed below.

The amount and distribution of genetic diversity within and among populations can be measured by the fixation index (F statistics) formulated by Wright in 1951 (Wright, 1951). This index can be seen as a measurement of homozygosity - the probability that any two alleles that are randomly sampled are related by descent. The values can range between zero and one. A value of zero indicates that the populations are not differentiated from each other and that most of the genetic diversity that is found can be attributed to differences between isolates within the populations. A value of one is an indication of no gene flow between the populations. As this index was developed from diploid and sexual reproducing populations, other formulations were developed to make it applicable to haploid and asexual reproducing populations as well (Nei, 1973). Several methods and programmes are available to study the amount and distribution of genetic diversity within and among populations.

There are five evolutionary forces that ultimately affect the genetic composition (allele frequencies) of populations including the distribution and change in genotype and phenotype frequencies in populations. The evolutionary forces include natural selection, genetic drift, mutation, gene flow and reproductive systems (mating systems) (Hartl and Clark, 1997). Mutation is a change in DNA sequence at a specific locus, and is a source of new genes. In plant pathology important examples include mutations in genes that result in new virulence alleles and fungicide resistance, which creates new genotypes. Migration (gene/genotypic flow) is an indication of how freely genes can be exchanged between populations. It can take place over short and long distances. In agriculture, migration is very important since new genetic material can be introduced into new areas. Migration is a powerful force that can determine genetic variation and thus differentiation between populations (Milgroom and Peever, 2003). Natural selection (directional process leading to an increased frequency of selected alleles or genotypes) is a powerful force and along with genetic drift (random process leading to unpredictable changes in pathogen populations), determines the presence or absence of an allele. Natural selection and genetic drift influence the effective population size (Ne) (Linde at al., 2009; Möller and Stukenbrock, 2017). A low effective population size can be a result of a bottleneck and an extended period of clonal reproduction (Dlugosch and Parker, 2008; Milgroom, 2015; Möller and Stukenbrock, 2017).

The reproduction or mating system of plant pathogens is an important evolutionary force that shape population structure. It affects the way in which alleles are put together in different genotypes. Populations that are outcrossing can put together new allele combinations rapidly. In contrast, populations that mainly undergo asexual reproduction keep together existing combinations of genes leading to lower genotype diversity. Populations that have a mixed reproductive system will benefit from the advantages associated with both types of reproduction.

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To ascertain whether a haploid population reproduces sexually (random mating) or asexually, the distribution of mating types can be determined or the presence of linkage disequilibrium can be tested (Slatkin, 2008). A mating type ratio that does not deviate from a 1:1 ratio, is regarded as an indication of random mating. To infer whether the population is in linkage disequilibrium or equilibrium, the index of association I_A and the standardized version of the index of association $\bar{r}_d$ P-values can be calculated (Agapow and Burt, 2001). The I_A and $\bar{r}_d$ indices provide an indication of the degree of association of alleles at different loci, within and among populations compared to that observed in a permutated dataset. A value of zero is expected for physically unlinked loci under random mating, i.e. linkage equilibrium (null model). Linkage disequilibrium among loci is indicated by a value significantly larger than zero, which is generated when no or infrequent sexual reproduction occurs. Asexual reproduction can impact linkage disequilibrium, therefore tests should be done on clone corrected and non-clone corrected datasets, since the inclusion of clonal haplotypes in the analysis can distort estimates of allelic diversity (Balloux et al., 2003).

Population genetic studies aimed at understanding the evolutionary forces that shape and maintain genetic variation within and among populations, requires polymorphic markers for genotyping populations. Many different types of genetic markers have been developed over time, and many of the markers are not used anymore. Currently used genetic markers directly assess variants (polymorphism) in DNA sequences. The markers used in a study is determined by the questions that need to be answered and the biology of the pathogen (Thompson, 2010). Ideal genetic markers are selectively neutral, polymorphic, specific to a single locus, co-dominant, independent and allow for repeatable, unambiguous scoring. The kind of markers that comply with all these requirements only recently became available with the development of microsatellites (simple sequence repeat markers - SSRs) and single nucleotide polymorphisms (Sunnucks, 2000). It is important that markers should be able to differentiate genotypes sufficiently. The ability of markers to differentiate genotypes sufficiently, can be tested using a genotype accumulation curve (Kamvar et al., 2014).

Studies on the population structure and genetic variation in and between populations can provide valuable information on the routes of pathogen introduction into new areas and to answer questions pertaining to the epidemiology of pathogens. Spatial and temporal patterns of MLGs can shed light on how pathogens spread/move within and between orchards and/or countries and can also be indicative of sources of inoculum. Information about the evolutionary processes that shape pathogen populations in agriculture is important for understanding disease dynamics and the biology of pathogens and to develop better disease management strategies. Information on

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other factors that can affect population structure such as host species and fungicide applications can provide valuable information with regard to resistance breeding against pathogens and effective chemical control strategies (McDonald and Linde, 2002; Milgroom and Peever, 2003).

MOLECULAR MARKERS FOR STUDYING GENETIC VARIATION WITHIN *P. CITRICARPA* POPULATIONS

Several dominant polymorphic markers, which are not ideal for population genetic studies, have been used to investigate the genetic structure of *P. citricarpa.* These include Randomly Amplified Polymorphic DNA markers (RAPDs) and fluorescent amplified fragment length polymorphism markers (fAFLPs). RAPD analyses were used by Stringari et al. (2009) to determine genetic variability and population structure between *P. citricarpa, P. mangiferae* and *P. spinarum* isolates from Brazil, Japan, Mexico and South Africa. They found a high genetic variability in and among the species. Glienke et al. (2002) conducted RAPD analyses on *P. citricarpa* isolates from Brazil, which also revealed a high level of intraspecific genetic variability. fAFLP analyses were used by Baldassari et al. (2008) to determine genetic diversity in *P. citricarpa* and *P. mangiferae* isolates from Brazil. The study showed that *P. mangiferae* isolates had a higher genetic diversity than the *P. citricarpa* isolates.

In *P. citricarpa,* sequence data of individual loci have not been very useful in investigating the population genetic diversity, due to low polymorphisms that were identified in the gene regions evaluated thus far. Wickert et al. (2012) used sequence data of the ITS1-5.8S-ITS2 region to determine if *P. citricarpa* populations from different orange varieties obtained from two geographic locations within Brazil, were genetically differentiated. Their study revealed low genetic diversity in populations from different varieties and geographic areas, with the highest genetic diversity found within populations. A study by Miles et al. (2013), also using the ITS region showed high similarity among *P. citricarpa* isolates from Australia. In a study by Zavala et al. (2014), genetic variation in *P. citricarpa* isolates from Florida was mainly investigated using multi-locus sequencing of four conserved loci (ITS, translation elongation factor 1-α (*TEF1*), actin (ACT) and glyceraldehyde-3-phosphate dehydrogenase [*GADPH*]). The study included the analyses of isolates from Brazil, South Africa, Zimbabwe, and Australia. Sequence analyses of the four gene regions did not reveal any genetic variation among the Floridian isolates or the isolates from the other countries (Zavala et al., 2014). However, recently Guarnaccia et al. (2017) reported the presence of seven single nucleotide polymorphisms in sequence data of the actin (*actA*) and *gapdh* genes among 21 *P. citricarpa* isolates from various countries. These limited polymorphisms

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could compliment other co-dominant markers for studies on the genetic diversity in *P. citricarpa* populations.

Recently, Wang et al. (2016) developed the first useful polymorphic co-dominant markers for *P. citricarpa* using the genome sequence of *P. citricarpa.* Although 13 simple sequence repeat (SSR) markers were developed, only seven of the markers were polymorphic. These markers were used to genotype one population from Australia, and several populations collected over different years from Florida (USA). The markers seemed to have low polymorphisms, and only identified two to four alleles per locus and 12 multilocus genotypes among 24 Australian isolates. The Florida populations were all clonal, consisting of a single multilocus genotype (Wang et al., 2016). Recently, Guarnaccia et al. (2017) used the SSR markers from Wang et al*.* (2016) along with SSR markers developed in the current study (Chapter 2), to show that *P. citricarpa* populations in Europe (Malta, Italy and Portugal) were all clonal. Interestingly, these populations were all obtained from citrus leaf litter in orchards where no CBS symptoms were present (Guarnaccia et al., 2017). Tran et al. (2017) used a selection of the markers of Wang et al. (2016) to show that new recombinant genotypes, and thus genetic recombination, were present in the F1 progeny of *P. citricarpa* isolates of opposite mating types that were paired in artificial mating studies.

CONCLUSION

Citrus black spot is a fungal disease currently influencing global citrus production and trade. From the literature review it is evident that the epidemiology of *P. citricarpa* has been unravelled in many countries including South Africa. In South Africa the epidemiology was unravelled by Kotzé, McOnie and Truter. However, despite all the research conducted in South Africa, limited information is available on the pathogen's population structure, mode of reproduction and possible introduction pathways. This information could support the current knowledge on the epidemiology of the disease, or perhaps bring new insights. The available molecular markers are able to identify only low levels or no polymorphisms in *P. citricarpa* populations. The best markers to date are the SSR markers published by Wang et al. (2016). Therefore, more informative additional markers are required to conduct population genetics studies.

The first objectives of this study were to investigate the reproductive mode of the pathogen and to develop informative markers to determine the distribution of genetic variation in global *P. citricarpa* populations. Subsequently, the study focused on the population structure of *P. citricarpa* in South Africa. In the first research chapter, the goals were to develop additional informative markers (SSR markers) and to investigate the population structure of *P. citricarpa* at a global

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scale. The markers were used to genotype global populations (China, South Africa, Australia, Florida and Brazil) in order to infer introduction pathways and possibly the centre of origin of the pathogen. The mode of reproduction of *P. citricarpa* on a global scale was also investigated. In the second research chapter, the effects of distance (spatial) and season (temporal) on *P. citricarpa* population structure were investigated at the orchard scale. *P. citricarpa* populations from two South African lemon orchards were studied over two seasons. In the third and last research chapter, the correlation between production region and population structure was investigated within the five citrus producing provinces in South Africa where CBS occurs. The effect of *Citrus* spp. (lemons vs. oranges) on the population structure of *P. citricarpa* in South Africa was investigated.

The knowledge acquired by conducting this study will better aid the understanding of the biology of the pathogen and may lead to improved control practices for the CBS disease in citrus orchards to enhanced local and global citrus production. This will be the first study to embark on using a population genetics approach to better understand the biology and epidemiology of the disease at global-, regional- and orchard scales.

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CHAPTER 2

A Global Perspective on the Population Structure and Reproductive System of *Phyllosticta citricarpa*[*]

ABSTRACT

The citrus pathogen *Phyllosticta citricarpa* was first described 117 years ago in Australia, subsequently from the summer rainfall citrus-growing regions in China, Africa, South America and recently the United States. Limited information is available on the pathogen's population structure, mode of reproduction and introduction pathways, which were investigated by genotyping 383 isolates representing 12 populations from South Africa, USA, Australia, China and Brazil. Populations were genotyped using seven published and eight newly developed polymorphic simple sequence repeat (SSR) markers. The Chinese and Australian populations had the highest genetic diversities, whereas populations from Brazil, USA and South Africa exhibited characteristics of founder populations. The U.S. population was clonal. Based on principal coordinate and minimum spanning network analyses the Chinese populations were distinct from the other populations. Population differentiation and clustering analyses revealed high connectivity and possibly linked introduction pathways between South Africa, Australia and Brazil. With the exception of the clonal U.S. populations that only contained one mating type, all the other populations contained both mating types in a ratio that did not deviate significantly from 1:1. Although most populations exhibited sexual reproduction, linkage disequilibrium analyses indicated that asexual reproduction is important in the pathogen's life cycle.

INTRODUCTION

Phyllosticta citricarpa (McAlpine) Aa, which causes citrus black spot (CBS), is a good example of a recently introduced plant pathogen that constrains the global trade of a high value fruit crop. Officially, CBS was first recorded and described from Australia on Valencia oranges in 1895 (Benson (1895). Soon after this report, McAlpine (1899) described the asexual stage of the fungus. The sexual, pseudothecial stage, was later described by Kiely (1948). The first official record of the disease from citrus areas outside of Australia was from China (Lee, 1920). Later, reports of the disease were published from Argentina (Marchionatto, 1928), South Africa (Doidge,

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[*] Carstens, E., Linde, C.C., Slabbert, R., Miles, A.K., Donovan, N.J., Hongye. L., Dewdney. M.M., Glienke. C., Schutte. G.C., Fourie, P.H. and McLeod. A. 2017. A Global Perspective on the Population Structure and Reprodcutive System of *Phyllosticta citricarpa*, a pathogen of citrus. Phytopathology 107: 758-768.

1929) and Brazil (Robbs et al., 1980). The most recent report was the introduction of the pathogen into Florida in the United States in 2010 (Schubert et al., 2012).

Today, global expansion of citrus production and the associated movement of propagation material have resulted in *P. citricarpa* being introduced into most of the major citrus producing countries around the world that have a hot, wet/humid summer rainfall climate (Paul et al., 2005; Carstens et al., 2012, Yonow et al., 2013). The disease is absent and has never been reported from regions/countries with a Mediterranean, winter rainfall climate (Paul et al., 2005; Carstens et al., 2012; Yonow et al., 2013). Even within the same country, such as South Africa, Australia and China, the disease is only present in the summer rainfall production areas (Miles et al*., 2008; Carstens et al., 2012; Wang et al., 2012). Although *P. citricarpa* has been reported as being present in New Zealand, which has an unsuitable climate for CBS (Paul et al., 2005; Yonow et al., 2013), this was an incorrect report due to the misidentification of *Phyllosticta capitalensis* as *P. citricarpa* (Everett and Rees-George, 2006). These two species are morphologically very similar, but can be differentiated molecularly. *Phyllosticta capitalensis* is an endophytic non-pathogenic species in citrus with a wide geographic distribution and host range (Baayen et al*., 2002; Stringari et al., 2009; Glienke et al*, 2011; Wikee et al., 2013a) and is of no phytosanitary concern in the world trade of fresh citrus fruit*.

Despite the importance of CBS, little is known about the origin of *P. citricarpa,* but it has been hypothesized that the pathogen is native to areas that are believed to be the primary centre of origin of its host *Citrus.* These include South- and Southeast Asia, specifically in the regions of Northeast India, south-eastwards through the Malayan Archipelago to China and Japan, but also southwards to and including Australia (Scora, 1975; Malik et al*., 2013; Hynniewta, et al*., 2014). The hypothesis for South- and Southeast Asia being the origin of *P. citricarpa* is supported by the *Phyllosticta* species diversity, with several new species recently being described from these regions including *P. citriasiana* (Wulandari et al., 2009) and *P. citrichinaensis* (Wang et al., 2012) from citrus in China, and *P. citrimaxima* from Thailand (Wikee et al*., 2013b).

To manage a disease and prevent further incursions, it is important to understand the disease cycle of the pathogen, inoculum sources and dissemination pathways. *Phyllosticta citricarpa* produces two types of spores, namely waterborne conidia (pycnidiospores) and aerially dispersed ascospores. Symptoms can develop on leaves, twigs and fruit, but symptoms on fruit are the most obvious. Waterborne, short-lived asexual pycnidiospores, are produced in pycnidia on fruit, leaves and twigs (Kiely, 1948; McOnie, 1965; Kotze, 1981; 2000; Spósito et al., 2007). Free water is required for the short-distance downward dispersal of pycnidiospores from symptomatic fruit within the tree (Kiely, 1948; Spósito et al., 2007; 2008; 2011). Ascospores are

sexually produced in pseudothecia that develop on leaf litter, but never on fruit (Kiely 1948; Kotzé, 1981; 2000). Pseudothecia mature over an extended period of time and require mild to warm temperatures and alternate wetting and drying (Kiely, 1948; Kotzé, 1981; Fourie et al., 2013). Ascospores are forcibly discharged from mature pseudothecia and are dispersed for distances up to 25 m (Spósito et al., 2007). Given the relatively short distance of dispersal of ascospores and pycnidiospores, latently infected propagation material is the most likely inoculum source that contributes towards the long distance dissemination of the pathogen into new regions (Kiely, 1948; Wager, 1953; McOnie, 1964a, b; 1965; Kotzé, 1981; 2000; Spósito et al., 2007; 2011).

Recently, the occurrence of two opposite mating types *(MAT 1-1-1* and *MAT 1-2-1*) has been reported in *P. citricarpa* populations, supporting the hypothesis that the fungus is heterothallic and will require mating between opposite mating types in order to produce ascospores (Wang et al., 2013; Amorim et al., 2016; Wang et al., 2016). Limited information is available on the mating type distribution of *P. citricarpa* worldwide, because the MAT loci were only characterized recently (Amorim et al*.,* 2016; Wang et al., 2016). In Australia and Brazil, MAT genotyping of populations revealed an almost equal mating type distribution (Zhang et al., 2015; Amorim et al*.,* 2016; Wang et al*.,* 2016). However, in Florida in the USA, only one mating type has been identified, which implies an absence of sexual reproduction and thus ascospores (Wang et al., 2016).

Recent pathogen movement around the globe should be detectable in population genetic signals. For example, recent founder populations will be characterized by low genotype and allelic diversity (Dlugosch and Parker, 2008; Linde et al., 2009). On the other hand, more established populations or populations with a long co-evolutionary history with their host, should display more allelic and genotype diversity. Information on population structure and diversity will also help to elucidate routes of pathogen introduction and migration. Furthermore, investigating the life-history and evolutionary processes that shape pathogen populations in agriculture is important for understanding disease dynamics and to develop disease control and management strategies. For such studies, codominant neutral genetic markers are essential. Simple sequence repeat (SSR) markers for *P. citricarpa* were developed recently from a published genome sequence of *P. citricarpa* and consist of seven polymorphic loci (Wang et al., 2016). These markers were used to genotype one population from Australia, and several populations collected in different years from Florida in the USA. The markers identified two to four alleles per locus in the Australian population, and 11 multilocus genotypes (MLGs) among 24 Australian isolates. The Florida populations were all clonal, consisting of a single MLG (Zhang et al., 2015; Wang et al., 2016). Therefore more

informative markers are needed to examine the population structure and migration pathways of *P. citricarpa*.

In *P. citricarpa,* sequence data of individual loci have not been very useful for investigating the population structure due to low levels of polymorphisms. Wickert et al*.* (2012) used sequence data of the internal transcribed spacer (ITS) 1-5.8S-ITS2 region to determine if *P. citricarpa* populations from different orange varieties obtained from two geographic locations within Brazil were genetically differentiated. Their study revealed low genetic diversity in populations from different varieties and geographic areas. Similarly, a study from Australia showed high ITS sequence similarity among isolates of *P. citricarpa* (Miles et al*.* 2013). In a study by Zavala et al*.* (2014), genetic variation in *P. citricarpa* isolates from Florida was investigated relative to 18 global isolates (Brazil, South Africa, Zimbabwe, and Australia) using multilocus sequencing of four conserved loci (ITS, elongation factor 1-alpha, actin and glyceraldehyde-3-phosphate dehydrogenase). The analysis did not reveal any genetic variation among the investigated isolates.

On a global scale, almost no information is available on the population structure, routes of introduction and putative origin of *P. citricarpa*. The first objective of this study was to develop additional SSR markers for population genetic studies using next generation sequencing data of a South African *P. citricarpa* isolate. These SSR markers and published markers (Wang et al., 2016) were used to genotype *P. citricarpa* populations from South Africa, Brazil, Australia, USA and China in order to infer introduction routes, the possible centre of origin of the pathogen and founder populations. Lastly, the mating type distribution, frequencies and mode of reproduction were also investigated in the aforementioned populations. In combination with genotype diversities and linkage disequilibrium analyses, mating type distribution will provide valuable insights into the mode of reproduction of *P. citricarpa*. Information gained from this study will be valuable in decision-making for disease management strategies to enhance global citrus production.

MATERIALS AND METHODS

Collection and isolation of *P. citricarpa* isolates

Phyllosticta citricarpa isolates from South Africa were sampled in 2011 and 2012 from a citrus orchard in each of the following provinces: Limpopo, Mpumalanga, North West, Eastern Cape and KwaZulu-Natal. In 2011, a single orchard was sampled in Brazil, whereas three orchards were sampled in the Unites States and two orchards in China. In Australia, one orchard was

sampled in 2010 and two orchards were sampled in 2011 (Table 1). For each orchard in South Africa, Brazil and Australia, 40 to 50 randomly selected CBS symptomatic fruits were collected. In the United States, 23 to 35 fruits were randomly sampled from each of the three orchards and, in China 15 fruits were randomly selected from each of the two orchards. Fruit were surface sterilized with a 1.25% sodium hypochlorite solution or 70% ethanol for 5 minutes followed by rinsing in sterile water. Small fragments of the lesions obtained from fruit with hard spot symptoms were placed onto potato dextrose agar (PDA) (Biolab, Midrand, South Africa) containing chloramphenicol (0.01%), and incubated for 10 to14 days at 25°C. One isolate was selected per fruit and a single spore culture was prepared for subsequent storage at 17°C in sterile distilled water and at -85°C in 30% glycerol. Isolates from the USA were kept on dried sterile filter paper on desiccant at -20°C for long term storage.

Confirming the species identity of *P. citricarpa* isolates

Due to *P. citricarpa* being morphologically very similar to *P. capitalensis,* the species identity of isolates was confirmed using morphological and molecular analyses. For morphological analyses, putative *P. citricarpa* single- spored isolates were transferred to oatmeal agar (OMA) (Biolab, Midrand, South Africa) plates and incubated at 25°C to differentiate *P. citricarpa* from other *Phyllosticta* species. The plates were examined for the presence of a yellow halo around colonies after 7 days, since *P. citricarpa* is the only *Phyllosticta* species that produces this halo (Baayen et al*.,* 2002; FAO, 2014).

Molecular identification was conducted using species-specific primers. DNA was isolated from 2-week old mycelia growing on PDA plates. The fungal growth was scraped from the agar plates and lyophilized for DNA extraction using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilde, Germany). Species-specific primers (IDT, Iowa, United States of America) (Meyer et al., 2006; Peres et al*.,* 2007) were used to amplify diagnostic fragments specific for *P. citricarpa* and *P. capitalensis*, following conditions as described previously (Meyer et al., 2006; Peres et al*.,* 2007). DNA of *P. citricarpa* (PPRI 9827) and *P. capitalensis* (PPRI 9065) (obtained from the National Collection of Fungi, Agricultural Research Council-Plant Protection Research Institute, Pretoria, South Africa) were included as positive species controls in the PCR assays. PCR products were analysed by electrophoresis at 100 V for 1 h in a 1 % (w/v) agarose gel and visualized under UV light using a Genegenius Gel Documentation and Analysis System (Syngene, Cambridge, United Kingdom) after ethidium bromide staining.

Ion Torrent genome sequencing and SSR marker development

Genomic DNA for sequencing with the Ion PGM (Life Technologies, Carlsbad, California) system was extracted from a South African *P. citricarpa* isolate (STEU-7794) according to Goodwin et.al*.* (1992). A low coverage whole genome shot-gun sequencing was performed using two Ion 318 chips and Ion sequencing V1 200 bp sequencing chemistry. These sequences (1,650kb – 12,896 kb) were assembled into 65 contigs using the Torrent Suite 2.2 and were sorted according to size using Galaxy (Giardine et al., 2005; Blankenberg et al., 2010; Goecks et al., 2010). Sequences containing at least four to six tandem repeat units of di- to hexa-nucleotides were mined for simple sequence repeats using BatchPrimer3 version 1 (Rozen and Skaletsky, 1998). Fifty-seven primer pairs were designed using this online tool. These primer pairs were tested for amplification success and levels of polymorphism using a subset of 10 isolates, representing three countries (South Africa, Australia and China). The PCR reaction for amplification with the different primer pairs contained 20 ng of template DNA, 1 x KAPA Readymix (KAPA Biosystems, Cape Town, South Africa) and 0.2 µM of each primer. Cycling was performed in a Veriti thermal cycler (Life Technologies, Carlsbad, California) using the following conditions: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 40 s followed by a final extension step at 72°C for 5 min. The fragment lengths of PCR amplicons were analysed on a 2100 Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA) using the DNA High Sensitivity Kit. Loci were considered polymorphic if two or more alleles were observed among the ten evaluated isolates. Primers from the eight selected loci were labelled with FAM, PET, NED and VIC fluorescent dyes and amplified in three multiplex reactions (Table 2). The PCR reaction and cycling conditions were as described above. Electrophoresis was performed using the 3730XL Genetic Analyser (Life Technologies, Carlsbad, California). The SSR alleles were scored using Genemapper software (version 4; Life Technologies, Carlsbad, California).

Seven published polymorphic loci (PC12, PC19, PC20, PC32, PC37, PC6, PC7) (Wang et al., 2016) were also used to genotype the *P. citricarpa* populations. Primers from the selected loci were labelled with FAM, PET, NED and VIC fluorescent dyes and amplified in two multiplex reactions (Table 2). The PCR reaction for amplification with the different primer pairs contained 10 ng of template DNA, 2 x KAPA2G Fast Multiplex Mix (KAPA Biosystems, Cape Town, South Africa) and 0.5 µM of each primer. Cycling was performed in a GeneAmp PCR System 9700 (Applied Biosystems) using the following conditions: 95°C for 5 min followed by 35 cycles of 94°C

for 30 s, 57°C for 30 s and 72°C for 60 s followed by a final extension step at 72°C for 5 min. Electrophoresis and SSR allele scoring were done as described above.

SSR data analyses

Selective neutrality of markers was evaluated with a Ewens-Watterson test in POPGENE (Yeh et al*.,* 2000). Isolates with the same alleles at all loci were considered clones or MLGs. To determine the genotypic diversities in individual and regional populations, the number of MLGs, the expected number of MLGs after rarefaction (*eMLG*) to account for different sample sizes and the evenness index (*E5*) estimating the equitability in the distribution of the sampling units, was determined in the R package Poppr (Kamvar et al. 2014; R Core Team, 2013). The equitability index *E5* varies from zero (no evenness) to one (all MLGs have equal abundance) (Grunwald et al. 2003; Shannon & Weaver, 1949).

All allele-based population genetic analyses were conducted using a per population clone-corrected dataset unless otherwise stated, since the inclusion of clonal haplotypes in the analysis can distort estimates of allelic diversity (Balloux et al. 2003). To determine the genetic diversity of populations, the following indices were calculated in GenAlEx version 6.5 (Peakall & Smouse, 2012): number of alleles, number of effective alleles, number of private alleles, number of polymorphic loci and Nei's measure of gene diversity (Nei, 1973). The values for Nei's gene diversity vary between zero and one. A zero value is an indication that there is no genetic diversity within the population, i.e. no allelic variation. The allele and allele richness for each population was determined using HP-RARE (Kalinowski, 2005) to account for populations with different sample sizes. To assess whether the 15 SSR markers were able to discriminate between unique individuals (MLGs) in the complete dataset a genotype accumulation curve was generated using the R package *Poppr* (Kamvar et al. 2014; R Core Team, 2013).

To estimate the distribution of variation within and among populations and regions, an analysis of molecular variance (AMOVA) was conducted. The statistical significance was tested using 999 permutations. Two identical MLGs from the USA population were included in the dataset to enable the performance of this analysis. To further assess genetic relatedness among populations, a principal coordinate analysis (PCoA) and a discriminant analysis of principal components (DAPC) were also conducted. The AMOVA and PCoA analyses were performed in GenAlEx version 6.5 (Peakall & Smouse, 2012), whereas the DAPC analysis was performed in the R package *adegenet* (Jombart, 2008).

To visualize the relationships among MLGs in the 12 populations and to infer introduction pathways, SSR data were used to construct a minimum spanning network based on Bruvo's

distance (Bruvo et al., 2004) using the R package Poppr on nonclone-corrected data. The network was visualized using the package igraph (Csardi and Nepusz, 2006).

Mode of reproduction

MAT-specific primer pairs (Wang et al., 2016) were used to determine the mating type of 196 isolates that represented a clone-corrected dataset. The PCR reaction contained 10 ng of template DNA, 0.2 µM of each primer, 1× reaction buffer, 0.01 µl Taq DNA polymerase (Promega Corporation, Madison, USA), 2 mM $MgCl_2$ and 0.2 mM of each dNTP. Amplification of the MAT *1-1-1* allele was performed using the following conditions: denaturation step at 95°C for 5 min followed by 30 cycles at 95°C for 30 s, 55°C for 45 s and 72°C for 45 s followed by a final extension step at 72°C for 10 minutes. The same conditions were followed for the MAT *1-2-1* allele, except that the annealing temperature was 60°C. PCR products were analysed by electrophoresis at 100 V for 1 h in a 1 % (w/v) agarose gel and visualized under UV light using a Genegenius Gel Documentation and Analysis System (Syngene, Cambridge, United Kingdom) after ethidium bromide staining. The primer pair for *MAT1-1-1* yielded a fragment of 630 bp and the primer pair for MAT *1-2-1* yielded a fragment of 500 bp. A chi-square test (Fisher and Yates, 1963) was used to determine whether the populations deviated from the null hypothesis of a 1:1 ratio of the mating types.

To infer the reproduction strategy (clonal or sexual) used by *P. citricarpa,* linkage disequilibrium analyses were performed on a SSR nonclone-corrected and clone-corrected dataset in the R package *Poppr* by calculating the index of association I_A and the standardized version of the index of association $\bar{r}_d$. *P*-values were obtained after 999 permutations (Agapow & Burt, 2001). The I_A and $\bar{r}_d$ indices provide an indication of the degree of association of alleles at different loci, within and among populations compared to that observed in a permutated dataset. A value of zero is expected for physically unlinked loci under random mating, i.e. linkage equilibrium (null model). Linkage disequilibrium among loci is indicated by a value significantly larger than zero, which is generated when no or infrequent sexual reproduction occurs.

RESULTS

Collection, isolation and identification of *P. citricarpa* isolates

A total of 383 *P. citricarpa* isolates representing 12 populations from five countries were obtained for analyses (Table 1 and Fig. 1). The species identity of all the isolates was confirmed to be *P. citricarpa* since all the isolates produced a 580 bp (Meyer et al., 2006) or a 300 bp amplicon

(Peres et al., 2007) with the *P. citricarpa* specific PCR primers, and olivaceous-grey colonies with a yellow halo on oat-meal agar.

Ion Torrent genome sequencing and SSR marker development

Only 8 of the 57 primer pairs that were evaluated on the subset of 10 isolates were polymorphic, and were used to genotype all the isolates. Sequence alignment of the newly developed SSR loci sequences with the Wang et al. (2016) loci, showed that there were no sequence similarities between these loci.

SSR data analyses

In addition to the eight newly developed polymorphic SSRs, the seven published loci (Wang et al., 2016), were also polymorphic in the analysed populations. The 15 polymorphic primer pairs revealed a total of 68 alleles across the 15 loci in 383 isolates, ranging from two to 18 alleles (Table 3). The markers were all selectively neutral according to the Ewens-Watterson test (data not shown). Two of the loci (Pc236 and Pc849) were polymorphic only in the Chinese populations. Locus Pc117 and locus Pc20 were the most polymorphic and identified 18 and 8 alleles in the 12 populations, respectively. On a regional scale, the populations from China contained the greatest number of alleles (n = 54) followed by Australia (n = 38) and South Africa (n = 35) (Table 3). Furthermore, when the data were corrected for sample size and rarefactioned to a sample size of 20 isolates, the private allelic richness was the highest in the two Chinese populations (0.37 and 0.28). Of all the other analysed populations, one private allele was found only in one population from Australia (Queensland 1) and one population from South Africa (Kwazulu-Natal). None of the populations showed 100% polymorphism for all 15 loci and none of the loci were polymorphic in the population from the USA. The populations from China (He = 0.324 (SE = 0.062)) and Australia (0.254 (SE = 0.050)) had the highest average gene diversities. The population from Brazil (0.144 (SE = 0.056)) and the United States (0) had the lowest gene diversity (Table 3).

Among the 383 isolates that were analysed, 149 MLGs were identified using the 15 SSR markers (Table 4). This provided a better resolution of MLGs than when only the seven published SSR markers (45 MLGs) or the eight newly developed SSR markers (85 MLGs) were used. The genotype accumulation curve showed a linear increase as the number of loci increased, confirming the importance of using all 15 loci for discriminating MLGs (Fig. 2). However, the genotype accumulation curve did not reach a plateau, which indicated that additional loci would further improve discrimination among individuals. Nevertheless, given that the variation in number of MLGs identified decreased dramatically with the addition of the 15th locus, and that at least the

83 isolates from the USA are likely to be true clones, the 15 loci used here were deemed sufficient to discriminate amongst most individuals in the 12 populations analysed (Fig. 2).

The number of MLGs varied among populations with the highest number (n = 60) identified in the Australian populations, followed by the South African populations (n = 54) and Chinese populations (n = 43). Only one MLG was identified in the USA population, whereas Brazil contained six MLGs (Table 4). To account for different sample sizes, the $eMLG$ value for each population and each country were determined. The $eMLG$ values also indicated that the Australian populations have the highest number of MLGs (n = 21.40), which agrees with the high genotypic diversity (D = 0.975). Based on the $eMLG$ values, the highest numbers of MLGs in South Africa were found in the populations from North West (n = 14.75) and KwaZulu-Natal (n = 14.09) and the lowest (n = 6) in the Eastern Cape province (Table 4). The evenness values ($E5$) are an indication of the relative abundance of different genotypes in populations. The high evenness in populations from Australia ($E5$ = 0.782) and China ($E5$ = 0.870) indicated that genotypes are equally abundant in the populations and also are an indication of higher genotype diversity (Table 4). The lower genotype evenness in populations from Brazil ($E5$ = 0.694) and South Africa ($E5$ = 0.626) are an indication that these populations are dominated by a smaller number of genotypes (clones).

Various MLGs were shared among populations from South Africa, Australia, Brazil and USA, but none were shared with populations from China. The USA MLG was shared with Australia (populations from Queensland 1 and New South Wales) and South Africa (populations from Mpumalanga, KwaZulu-Natal and North West) but not with Brazil or China. The population from Brazil shared MLGs with all three populations from Australia and all five populations from South Africa. The South African population from the Eastern Cape only shared MLGs with the Queensland 2 population from Australia. The South African population from the Limpopo province shared MLGs with the Queensland 2 and the New South Wales populations from Australia. The North West population shared MLGs with the Queensland 1 and New South Wales populations from Australia. The South African populations from the Mpumalanga and KwaZulu-Natal provinces shared MLGs with all three the Australian populations (data not shown).

The AMOVA analysis comparing the 12 populations from the five countries showed that 53% of the total variance was distributed among countries. A relatively low level of variance of 7% was distributed among the 12 populations, whereas a higher proportion of the variation (41%) was distributed within populations. There was significant (P = 0.001) genetic variation among countries ($PhiRT$ = 0.528) and within populations ($PhiPT$ = 0.593). The genetic variation among populations was low ($PhiPR$= 0.138) (Table 5).

Pairwise *PhiPT* values indicated that the genetic differentiation varied significantly amongst some, but not all countries (Table 6). The USA population was not significantly differentiated from the Australian ($P = 0.438$), the South African ($P = 0.481$) or the Brazilian ($P = 0.365$) populations. The South African population was not significantly differentiated from Brazil ($P = 0.318$) but was differentiated from the Australian population ($P = 0.001$). The Chinese population was significantly ($P = 0.001$) differentiated from all other populations (*PhiPT* = 0.694 - 0.700).

A principal coordinate analysis (PCoA) clearly divided the isolates into two groups (Fig. 4). The one group included the populations from China and the second group the populations from South Africa, Brazil, the USA and Australia.

The minimum spanning network revealed that the MLGs from China are distinct from all the other populations analysed and have a separate evolutionary history (Fig. 3). The MLGs from South Africa, Australia and Brazil were distributed all across the network, but distant from the Chinese MLGs. The DAPC analysis also divided the isolates from the 12 populations into two groups, with the isolates from China clearly separated from those from South Africa, Brazil, the USA and Australia (Supplementary Fig. S1). A DAPC analysis to assess the relationship between the populations in the main group (Australia, Brazil, South Africa and the USA) also showed that there is no significant differentiation between the populations from these four countries (Supplementary Fig. S2).

Mode of reproduction

A positive PCR amplification with either one of the MAT primer pairs was achieved for all isolates analysed. In all the populations from Australia, Brazil, China and South Africa, the mating-type frequencies did not deviate significantly from a 1:1 ratio ($\chi^2 = 1.14 – 2.26$; $P > 0.1$) based on Chi-square analyses (Table 7). In contrast, all the isolates obtained from the USA contained only a single MAT locus (*MAT1-2).*

Linkage disequilibrium analyses were performed to infer the reproductive strategy. For the non-clone-corrected dataset, the I_A and $\bar{r}_d$ indices in the association tests differed significantly from zero in five (Queensland 1, Brazil, China (Jiangxi), KwaZulu-Natal and Limpopo) of the 12 populations (Table 8). This is an indication of linkage among loci and thus a deviation from random mating. In the other seven populations the hypothesis of random mating could not be rejected. For the clone-corrected dataset, the association tests differed significantly from zero in only three (Queensland 1, Brazil and China (Jiangxi)) of the 12 populations (Table 8). Significant linkage disequilibrium may be achieved by no or infrequent sexual reproduction.

DISCUSSION

In this study, using a population genetics approach, a worldwide collection of *Phyllosticta citricarpa* isolates originating from five continents were analysed. The study revealed some novel and important aspects regarding the relatedness of populations from different continents, possible pathways of introduction and the reproductive system of *P. citricarpa*. China and Australia were shown to have had a longer evolutionary history of *P. citricarpa* compared to South Africa, Brazil and the USA. This is in agreement with the documented history of first reports of the pathogen in these countries. The USA population was confirmed to be clonal, using eight newly developed SSR markers. Populations from South Africa, Australia, Brazil and the USA shared some MLGs, which is indicative of long distance human-mediated dispersal. Investigations into the reproductive system of the pathogen showed that all populations, except the U.S. population, contained both MAT, and that asexual reproduction may play a more important role in the epidemiology of the disease than previously thought. Population genetic inferences were strengthened by the development of eight new polymorphic SSR markers, which were used in combination with seven previously published SSRs (Wang et al., 2016). Some of the new markers (loci Pc117 and Pc440) were highly polymorphic and identified five to 18 alleles in the 12 analysed populations that allowed for a better resolution of MLGs, as was evident from the genotype accumulation curve.

Knowledge of the origin of *Citrus* spp. (the host of *P. citricarpa*) could aid in the development of hypotheses on the origin of the pathogen. However, the origin, domestication, distribution and botanical classification or taxonomy of edible / true *Citrus* are characterized by a history of controversy and interesting folklore. Yet, it is accepted that *Citrus* most likely originated from certain parts of South-east Asia possibly China, India and the Malay Archipelago (Gmitter and Hu, 1990). More recently, Liu et al. (2012) and Carbonell-Caballero et al. (2015) considered Australia, New Caledonia and New Guinea to be the centre of origin. In the current study, *P. citricarpa* populations were sampled from some of the regions hypothesized as being the centre of origin of *Citrus.* Two of the major citrus production areas in China were sampled, which included the Jiangxi area where citrus has been produced since the Xia dynasty (21st - 17th century BC) (AQSIQ, 2014). However, in Australia only commercial citrus production areas in Queensland and New South Wales were sampled; but not regions close to New Caledonia and New Guinea. Therefore, future analyses of populations from New Caledonia and New Guinea, as well as the Malay Archipelago will be instrumental in better understanding the origin of *P. citricarpa*.

Combined with the known native distribution of *Citrus* hosts in China and Australia, the high levels of gene and genotypic diversities of *P. citricarpa* populations identified in China and Australia, as well as the presence of more private alleles in these populations, indicate that the pathogen has had a long co-evolutionary history with its host in these countries. It is known that allele diversity is affected by the length of time that a specific population occurs in a specific area, with older populations having a higher level of genetic diversity with more alleles and also more private alleles (Linde et al*.,* 2009). This finding is also consistent with a co-evolutionary relationship between the pathogen on its wild host.

The low genetic diversity of the five South African *P. citricarpa* populations and the population from Brazil is an indication of these populations being founder populations that have undergone genetic drift. Further support for this hypothesis is gained from low numbers of private alleles in the South African populations compared to populations from Australia and China, and the lack of private alleles in the Brazilian population. In Brazil, the low genetic diversity can be attributed to a more recent introduction of *P. citricarpa* (Robbs et al., 1980). Similarly, the low genetic diversity in the South African populations may also be attributed to a relatively recent introduction (Doidge, 1929).

In South Africa, the population from the KwaZulu-Natal province had the highest level of gene and genotypic diversity, whereas the Eastern Cape population had the lowest. This correlates with the history of CBS in South Africa. The recorded first discovery of CBS in South Africa was in 1929 in the humid coastal regions of the KwaZulu-Natal province, but the disease only became severe in 1940. During surveys in 1946 the disease was also found in the North West, Limpopo and Mpumalanga provinces (previously known as the Western Transvaal, Northern Transvaal and Eastern Transvaal, respectively) (Wager, 1952). The disease was first discovered in the Eastern Cape province in the 1970s (C. Kellerman, personal communication).

The *P. citricarpa* populations from the five continents differed in their connectivity and differentiation from each other, which provided clues as to possible introduction pathways. Principal coordinate analysis (PCoA), a discriminant analysis of principal components (DAPC) and a minimum spanning network based on genetic distances between MLGs, revealed that there is little connectivity between the Chinese populations and the populations from the other countries (South Africa, Brazil, Australia and the USA). This was also supported by the pairwise *PhiPT* comparisons. Population differentiation and clustering analyses showed that there are high levels of connectivity between South Africa, Australia and Brazil, as well as between South Africa, Australia and the USA. No connectivity was evident between the USA population and the population from Brazil. The high levels of connectivity among the *P. citricarpa* populations in South

Africa, Australia and Brazil is most likely due to exchanges of plant material and the associated *P. citricarpa* genotypes, either between these countries directly, or between un-sampled populations that share the same *P. citricarpa* populations. Sharing of plant material and possibly *P. citricarpa,* may date back to the establishment of the citrus industries in these countries. The recorded history of the South African citrus industry goes back as far as 1654 when the first trees were brought from the Island of St. Helena, a stopover for ships from the East (Scora, 1975). Additional records prior to the 1900s indicated that citrus trees were also brought to South Africa from Brazil (1850) and India (Powell, 1930; Allwright, 1957). The commercial citrus industry in Australia was established using trees imported in 1788 from the Cape of Good Hope (South Africa) and Brazil (Rio de Janeiro) (Scora, 1975). The citrus industry in Brazil was founded with trees brought from Europe in 1540 by the Portuguese explorers (http://irrec.ifas.ufl.edu; Navarro, de Andrade, 1933).

The mating type analysis and genotyping showed that only a single clonal mating type (*MAT 1-2-1*) was present in the U.S. population, which suggests a human introduction, a founder effect and subsequent asexual reproduction of the pathogen in Florida, as was also reported by Wang et al. (2016). The finding of only one mating type in the USA population will result in atypical disease cycles, compared to other countries. In this population, the primary inoculum is pycnidiospores, whereas in populations from other continents the primary inoculum is sexually produced ascospores. Asexual overwintering of the pathogen might occur endophytically as latent infections in leaves or twigs, in leaf or twig lesions and on infected out-of-season fruit on the tree (Kiely, 1948; Wager, 1952; Whiteside, 1967; Spósito et al., 2007, 2011).

Very little is known about the reproductive system of *P. citricarpa* based on genetic data. It was only recently that Wang et al. (2016) and Amorim et al. (2016) were able to clone the MAT genes, supporting the hypothesis that *P. citricarpa* is heterothallic. Mating type analyses of the global populations showed that both mating types were present in populations from Australia, Brazil, China and South Africa at similar frequencies. This supports frequency-dependent-selection of mating types and hence the occurrence of regular sexual reproduction in the life cycle of the pathogen. However, the association tests (I_A and $\bar{r}_d$), indicated significant linkage disequilibrium in clone-corrected populations, suggesting no or infrequent sexual reproduction in some of the populations (Queensland 1, Brazil and China (Jiangxi)). The low genotype evenness, which indicates that only a few MLGs are present, in most of the South African populations and in the Brazilian population further indicates that these populations have frequent clonal reproduction. This finding is surprising considering that historical data from epidemiological studies have shown that pycnidiospores play a relatively minor role in the epidemiology compared

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with ascospores (Kiely, 1948; Kotzé, 1981; Wager, 1952; Whiteside, 1967; McOnie, 1964b; Spósito et al., 2007, 2008, 2011). However, our findings as well as the recent identification of a single clonal genotype causing epidemics in Florida (Wang et al., 2016; this study) suggest a more significant contribution of asexual reproduction to the epidemiology of the disease, as was concluded by Spósito et al. (2011) for areas with highly suitable climates.

The importance of asexual reproduction to disease development may be management and climate dependent. Management practices in citrus types (lemons) that is known to produce multiple crops, or where the fruit remain on the trees during fruit set of the next crop may likely result in symptomatic fruit that can produce pycnidiospores that coincided with the young susceptible fruitlets. It was shown in Brazil that under high rainfall conditions, and particularly under management practices with overlapping fruit set, pycnidiospores played an important role in disease spread within trees (Spósito et al., 2007, 2011). Our data indicates that asexual reproduction in some populations is more important than previously thought. In the future, a structured within-orchard sampling strategy will shed more light on the role of asexual reproduction and pycnidiospores within and between trees in orchards where both mating types occur.

Our study on the global population genetic structure of *P. citricarpa* provides novel and important insights into historical dissemination of the pathogen, the genetic structure of the global population and the reproductive system of the pathogen. Information on the introduction pathways of *P. citricarpa* to date has been based on historical records of first reports and speculations. The historical records can be incorrect and misleading especially since the pathogen can be present in a latent form (cryptic infection) for a long period of time (Kiely, 1948, Wager, 1952; Kotzé, 1981). The source of the South African population could be from the Far East or Australia. We also identified either Australia or South Africa as a likely source of the Brazilian population. Australia or South Africa can also be the source of the USA population, although it cannot be ruled out that the USA's MLG remained un-sampled elsewhere. Our study, however, does not provide a complete picture of the introduction pathways, as additional populations from other countries in Asia and from New Caledonia and New Guinea need to be analysed to determine whether the Oceanian countries, China or another country in Asia was the source population from which dispersal of the pathogen to other continents took place. Knowledge on the reproductive system of *P. citricarpa* has only been based on epidemiological studies, which has concluded that sexual reproduction frequently occurs in this pathogen system (Kiely, 1948; McOnie, 1965; Kotzé, 1981). Our data supports the importance of a sexual reproductive system, but further indicate that in some populations asexual (clonal) reproduction may also be important.

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Table 1. Geographic origin, collection year and host of *Phyllosticta citricarpa* populations analysed in this study.

Country	Province or State/County	Year	Citrus Type	No. of isolates
Australia	Queensland 1(Qld1)	2011	Lemon	25
	Queensland 2 (Qld2)	2011	Soft Citrus	29
	New South Wales (NSW)	2010	Navels	25
Brazil	Sao Paulo	2011	Lemons	24
China	Jiangxi	2011	Soft Citrus	35
	Zhejiang	2011	Soft Citrus	23
South Africa	Eastern Cape (SA-EC)	2012	Lemons	20
	KwaZulu Natal (SA-KZN)	2011	Grapefruit	30
	Limpopo (SA-LIM)	2011	Valencias	30
	Mpumalanga (SA-MP)	2011	Valencias	29
	North West (SA-NW)	2012	Lemons	30
Unites States of America	Florida/Collier (USA)	2011	Valencias	83

Table 2. Characteristics of 15 simple sequence repeat markers used to genotype *Phyllosticta citricarpa* populations and the allele size ranges present in a collection of 383 isolates.

Locus [a]	Multiplex reaction nr.	Size range (bp)	Primer sequence (5' – 3')	Labelling Dye	Repeat MOTIF
Pc117*	3	146-250	F: GCG AAA AAT AAG TCT GCA CT R: AGA TAG CTC CGT CTT GGA TA	VIC	(GATT)
Pc179.1*	3	141-153	F: ATT TTG CTG ACT GAC TGG AC R: CTA AAT CTC CTG CTT GTG CT	PET	(CAGA)
Pc236*	3	137-143	F: CTG ATG CGT GAC CTT CTC R: CTA GCC CAG TTC ATG TCT TC	FAM	(GTC)
Pc440*	1	270 -282	F: CCT CTC TCG TCA AGA AAC AAG R: TCC TGC ATG GTA AGA CAG AC	FAM	$(CAT)_n$
Pc849*	1	290-293	F: CAA TGA CGA TAG CGA AGA AG R: GCT CGA ACA GAA CCA TGA C	VIC	$(GAA)_n$
Pc1007*	2	147-151	F: AGA GTC GTC GGT TTT GAA G R: CTG GCA GGC TAA TAG ATT GA	FAM	$(TG)_n$
Pc2073*	2	157-177	F: GAC AGG ACA GAT GGA TGA AT R: AGA AGC GCT AGA ATT GAG TG	NED	$(GCCTG)_n$
Pc3011*	2	157-160	F: TGA GCA GGT CCA TAC AAG A R: ACC GAA GAC AAC CTC TCT G	PET	$(TCC)_n$
Pc6	4	117-135	F: GGC CTG CAG TAC GAT TTT A R: ATA TCC ACG TCC ATC AAC TC	FAM	$(CAA)_n$
Pc7	4	173-185	F: AAG GTG GTC GTG GTC ATC R: CAA GTT CTT GGG AGT ACA TCA	VIC	$(GTG)_n$
Pc12	4	167-187	F: TAA AGT AAT GAC GCT CGA CTC R: GAG AGA AAG GAG ACG TGA CA	FAM	$(ACC)_n$
Pc19	4	154-160	F: GCA GGC ACT ACC TTA GAC C R: GTC GAG GAT GAC AGT ACC C	NED	$(ACC)_n$
Pc20	5	187-222	F: GTT TCG GCA TCT TTG TTT T R: GAT TCC TAA ACC TGC TGT TG	NED	$(CTG)_n$
Pc32	5	155-163	F: TGTCTGAGGCTAAGAGTTCTG R: AGA AGG GAG AAG AGA GTT GAA	PET	$(GGCT)_n$
Pc37	5	144-147	F: GCA TCT CTT CTC CTT CTT CTT R: AAA TCG AGA CTG TGC TAT TTG	FAM	$(CTC)_n$

[a] Loci followed by * were developed in the current study, whereas the remaining loci were previously published by Wang et al., (2016).

Table 3. The number of alleles, private alleles and gene diversity of 15 simple sequence repeat loci in clone corrected *Phyllosticta citricarpa* populations from Australia, Brazil, China, South Africa, and the United States.

Country States/Province	#Isolates	# Alleles (Private Alleles)															Ne	Gene Diversity[a]
		Pc 117	Pc 179.1	Pc 236	Pc 440	Pc 849	Pc 1007	Pc 2073	Pc 3011	Pc 12	Pc 19	Pc 20	Pc 32	Pc 37	Pc 6	Pc 7		
Australia																		
NSW	25	4	1	1	2	1	3	2	2	2	2	1	2	1	3	2	1.37	0.216
Queensland 1	25	4(2)	2	1	3	1	2	1	2	3	2	1	2	2	3	1	1.48	0.243
Queensland 2	29	5	2	1	3	1	2	2	2	2	2	2	2	2	2	2	1.42	0.252
Total	**79**	**8(2)**	**2**	**1**	**3**	**1**	**3**	**2**	**2**	**3**	**2**	**2**	**2**	**2**	**3**	**2**	**1.43**	**0.254**
Brazil																		
Sao Paulo	24	2	1	1	2	1	2	2	1	1	1	1	1	1	1	2	1.27	0.144
Total	**24**	**2**	**1**	**1**	**2**	**1**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1.27**	**0.144**
China																		
Jiangxi	35	8(4)	2	2(1)	2	2	2	2	2	4(2)	3(1)	3(2)	1	1	3(1)	1	1.60	0.309
Zhejiang	23	10(2)	2	2	3(1)	2	3	1	2	2(1)	1	5(4)	1	1	2	2(1)	1.53	0.213
Total	**58**	**15(6)**	**2**	**3(1)**	**3(1)**	**2**	**3**	**2**	**2**	**5(3)**	**3(1)**	**7(6)**	**1**	**1**	**3(1)**	**2(1)**	**1.79**	**0.324**
South Africa																		
EC	20	1	1	1	2	1	1	2	2	1	1	1	1	1	2	2	1.17	0.107
KZN	30	2(1)	1	1	2	1	2	2	2	2	2	2	2	1	2	2	1.34	0.202
LIM	30	5	2	1	3	1	2	2	2	1	2	1	1	1	2	2	1.33	0.190
MP	29	3	1	1	3	1	2	2	2	1	2	1	1	2	1	1	1.25	0.153
NW	30	4	2	1	2	1	2	2	2	1	1	1	1	1	2	2	1.31	0.179
Total	**139**	**7(1)**	**2**	**1**	**3**	**1**	**3**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**1.35**	**0.210**
USA																		
Collier	83	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.00	0
Total	**83**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1.00**	**0**
Total	**383**	**18**	**3**	**3**	**5**	**2**	**3**	**3**	**2**	**6**	**3**	**8**	**2**	**2**	**4**	**4**	**1.37**	**0.184**

[a]Nei's gene diversity, H_e (Nei, 1973); *Ne* - effective population size

Table 4. Summary of genotypic diversity indices for 12 populations of *Phyllosticta citricarpa* from Australia, Brazil, China, South Africa and the United States.

Country/ States/Province	#Isolates	#MLGsª	*eMLG*	SE	*E5*	*D*
Australia						
Queensland 1	25	23	18.73	0.654	0.961	0.954
Queensland 2	29	25	17.82	0.965	0.883	0.951
NSW	25	23	18.73	0.654	0.961	0.954
Total	**79**	**60**	**21.40**	**1.39**	**0.782**	**0.975**
Brazil						
Sao Paulo	24	6	5.64	0.530	0.694	0.684
Total	**24**	**6**	**6.00**	**0.530**	**0.694**	**0.684**
China						
Jiangxi	35	24	15.84	1.289	0.859	0.945
Zhejiang	23	20	17.64	0.662	0.904	0.941
Total	**58**	**43**	**20.90**	**1.38**	**0.870**	**0.970**
South Africa						
KZN	30	19	14.09	1.249	0.789	0.920
LIM	30	15	11.70	1.183	0.712	0.880
MP	29	14	11.38	1.101	0.746	0.880
NW	30	20	14.75	1.237	0.820	0.929
EC	20	6	6.00	0.000	0.735	0.710
Total	**139**	**54**	**16.90**	**1.930**	**0.626**	**0.952**
USA						
Collier	83	1	1.00	0.000	-	0
Total	**83**	**1**	**1.00**	**0.000**	**-**	**0**
Total	**383**	**149**	**14.59**	**1.917**	**0.256**	**0.930**

ªMLGs = Multilocus genotype, *eMLG* = expected number of MLGs after rarefraction, SE = Standard error based on *eMLG*, *E5* = Evenness and *D* = Genotypic diversity

Table 5. Analysis of molecular variance (AMOVA) for *Phyllosticta citricarpa* populations in five countries using 15 simple sequence repeat loci.

Source	d.f.	SS	% Variation	AMOVA statistics	*P*
Among countries	4	319.260	53%	*PhiRT* = 0.528	0.001
Among populations	7	46.994	7%	*PhiPR* = 0.138	0.001
Within populations	185	318.711	41%	*PhiPT* = 0.593	0.001
Total	196	684.964	100%		

Table 6. Estimates of pairwise *PhiPT* values (below the diagonal) averaged over 15 microsatellite loci of *Phyllosticta citricarpa* populations in five countries (Australia, Brazil, China, South Africa and the United States). Significance values indicated above the diagonal.

	Australia	Brazil	China	South Africa	USA
Australia	-	0.011	0.001	0.001	0.438
Brazil	0.097	-	0.001	0.318	0.365
China	0.649	0.659	-	0.001	0.001
South Africa	0.165	0.013	0.700	-	0.481
USA	0.000	0.013	0.674	0.000	-

Table 7. Mating-type (MAT) composition and χ^2 testing for expected 1:1 MAT ratios in clone corrected *Phyllosticta citricarpa* populations from five countries.

Country	Province or State/ County	Populations	Number of A1 mating-type isolates	Number of A2 mating-type isolates	Number of MLGs[a]	χ^2 value	*P*-value
Australia	Queensland 1	1	12	11	23	0.04	0.80
	Queensland 2	1	14	11	25	0.36	0.50
	New South Wales	1	14	9	23	1.09	0.30
Total		**3**	**40**	**31**	**71**	**1.14**	**0.30**
Brazil	Sao Paulo/Parana	1	4	2	6	0.67	0.50
China	Jiangxi	1	14	10	24	0.67	0.50
	Zhejiang	1	13	7	20	1.80	0.20
Total		**2**	**27**	**17**	**44**	**2.26**	**0.10**
South Africa	Eastern Cape	1	3	3	6	-	-
	KwaZulu Natal	1	12	7	19	1.32	0.30
	Limpopo	1	5	10	15	1.67	0.20
	Mpumalanga	1	6	8	14	0.29	0.70
	North West	1	6	14	20	3.20	0.10
Total		**5**	**32**	**42**	**74**	**1.35**	**0.30**
USA	Florida/Collier	1	0	1	1	-	-

[a] The total number of Multilocus genotypes (MLG) per population in clone corrected *Phyllosticta citricarpa* populations from Australia, Brazil, China, South Africa and the USA.

Table 8. Linkage disequilibrium analyses for 12 populations of *Phyllosticta citricarpa* from Australia, Brazil, China, South Africa, and the United States with corresponding significance level (*P*-value).

Country/ States/Province	#Isolates	#MLGs	eMLG	Non-clone-corrected populations				Clone-corrected populations			
				I_A	P value	$\bar{r}_d$	P value	I_A	P value	$\bar{r}_d$	P value
Australia											
Queensland 1	25	23	18.73	0.275	0.046	0.310	0.047	0.240	0.061	0.027	0.063
Queensland 2	29	25	17.82	-0.164	0.924	-0.014	0.923	-0.267	0.992	-0.023	0.992
NSW	25	23	18.73	-0.008	0.480	-0.001	0.480	-0.065	0.651	-0.007	0.651
Total	**79**	**60**	**21.40**	**0.885**	**0.138**	**0.008**	**0.135**				
Brazil											
Sao Paulo	24	6	5.64	1.336	0.001	0.350	0.001	0.311	0.153	0.078	0.185
Total	**24**	**6**	**6.00**	**1.336**	**0.001**	**0.350**	**0.001**				
China											
Jiangxi	35	24	15.84	1.068	0.001	0.100	0.001	0.960	0.001	0.089	000.1
Zhejiang	23	20	17.64	-0.202	0.911	-0.213	0.909	-0.352	0.990	-0.037	0.990
Total	**58**	**43**	**20.90**	**0.912**	**0.001**	**0.079**	**0.001**				
South Africa											
KZN	30	19	14.09	0.186	0.072	0.020	0.646	-0.034	0.546	-0.004	0.545
LIM	30	15	11.70	0.001	0.456	0.000	0.456	-0.231	0.925	-0.030	0.920
MP	29	14	11.38	-0.079	0.732	-0.013	0.732	-0.316	0.981	-0.534	0.979
NW	30	20	14.75	-0.056	0.656	-0.008	0.657	-0.213	0.953	-0.313	0.951
EC	20	6	6.00	-0.081	0.648	-0.021	0.646	-0.472	0.960	-0.118	0.955
Total	**139**	**54**	**16.90**	**0.129**	**0.010**	**0.013**	**0.010**				
USA											
Collier	83	1	1.00	-	-	-	-	-	-	-	-
Total	**83**	**1**	**1.00**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Total	**383**	**149**	**14.59**	**5.758**	**0.001**	**0.414**	**0.001**	**4.645**	**0.001**	**0.332**	**0.001**

MLGs = Multilocus genotype, *eMLG* = expected number of MLGs after rarefraction, I_A = Index of Association; $\bar{r}_d$ = Standardized index of association

56
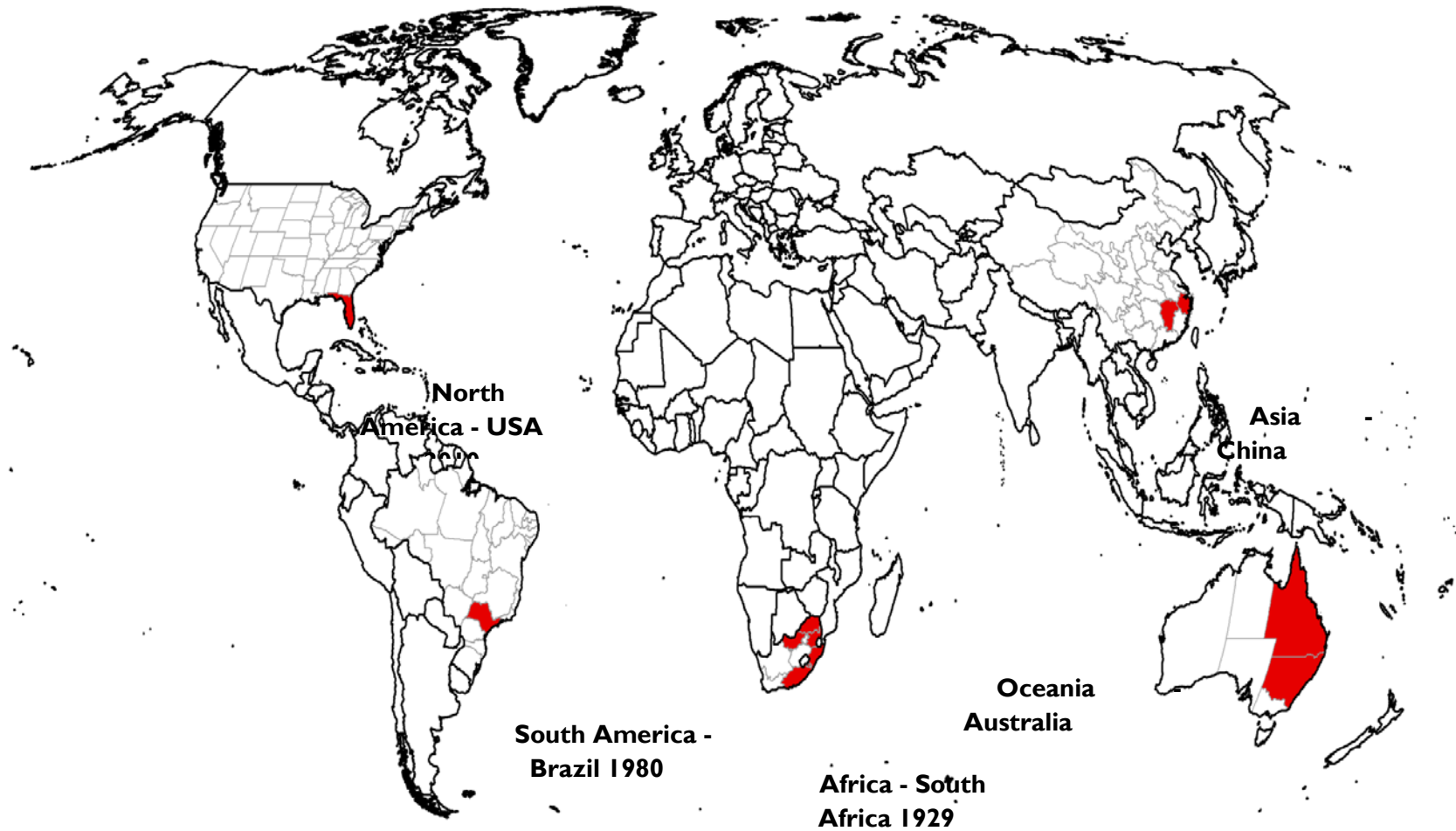
Figure 1. Geographic distribution of the *Phyllosticta citricarpa* isolates indicating the five countries in which the isolates were collected. Dates indicated are those that are known as the earliest record of *Phyllosticta citricarpa*.
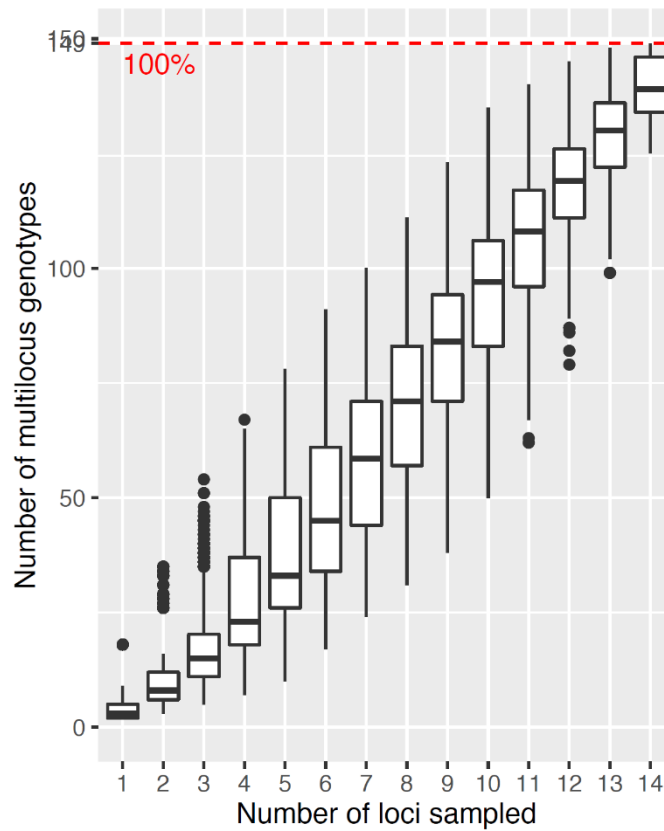
Figure 2. Genotype accumulation curve for 15 microsatellite loci in 12 *Phyllosticta citricarpa* populations. The numbers of observed multilocus genotypes (MLGs) are denoted by the vertical axis, from 0 to the observed 149 MLGs in the *P. citricarpa* populations. The numbers of loci that were randomly sampled without replacement are denoted on the horizontal axis. The boxplots each contain 1000 random samples representing different possible combinations of *n* loci. A MLG resolution of 100% is indicated by the horizontal red dashed line.
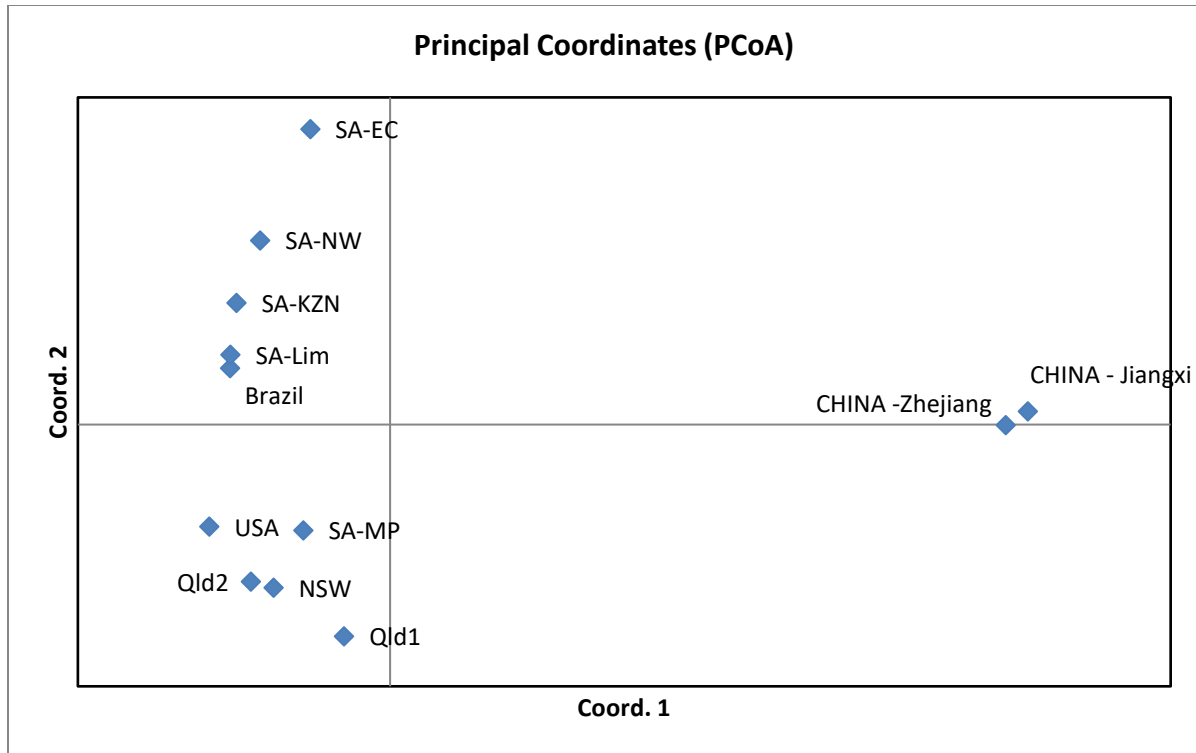
Figure 3. Principal coordinate analysis (PCoA) for 12 *Phyllosticta citricarpa* populations collected in five different countries.

POPULATION

■ Australia
■ Brazil
■ China
■ South Africa
■ USA

0.033   0.033   0.058   0.067   0.333
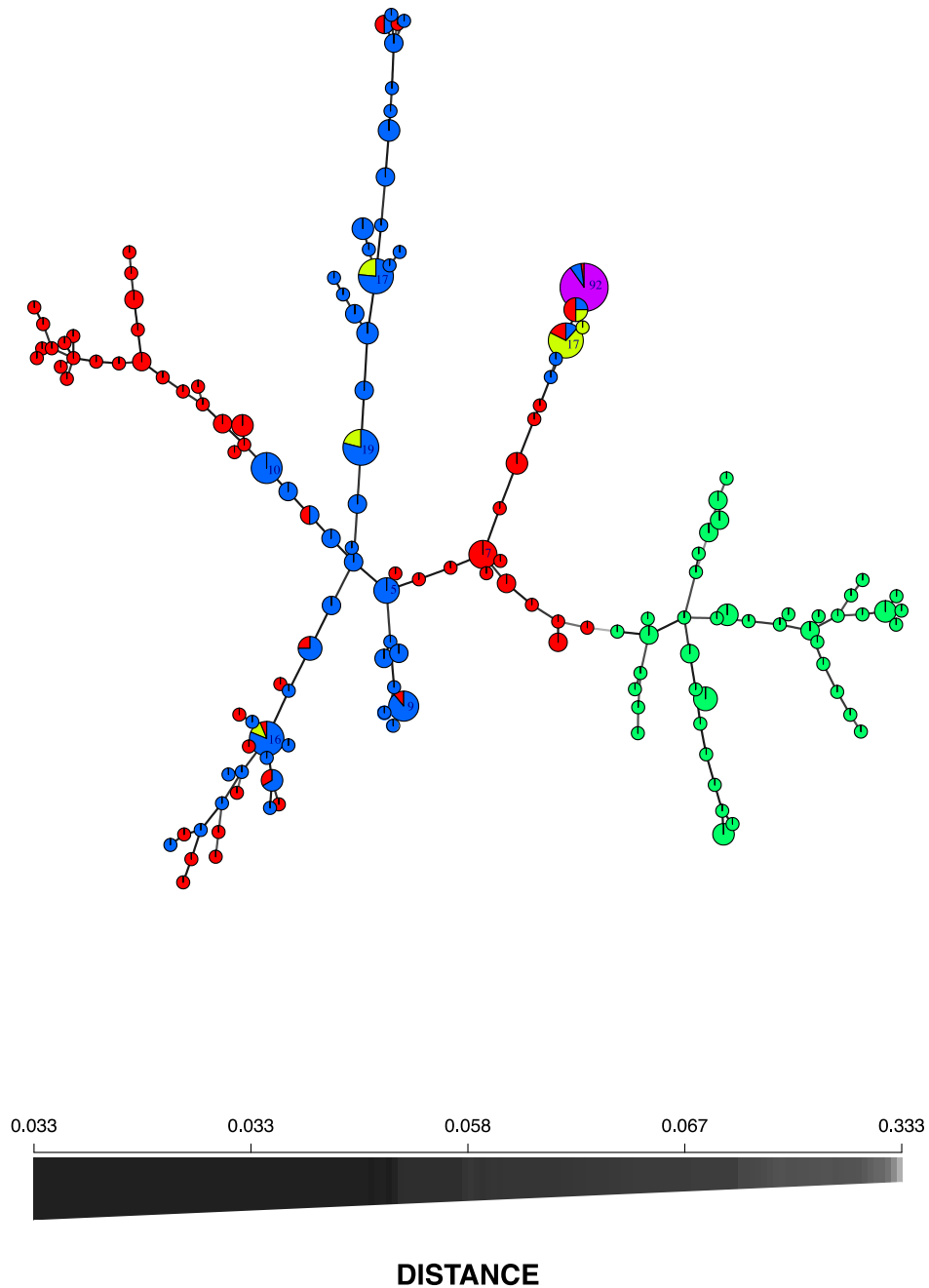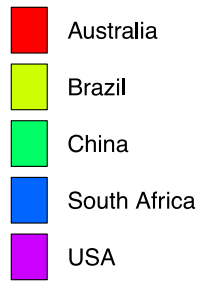
DISTANCE

Figure 4. Minimum Spanning Network based on Bruvo Genetic Distances. In total, 149 multilocus genotypes were observed in *Phyllosticta citricarpa* populations from five countries. Node colors represent population membership proportional to the pie size. Node sizes are relatively scaled to $\log_{1.75} n$, where *n* is the number of samples in the nodes to reduce node overlap. Edges (lines) represent minimum genetic distance between individuals.
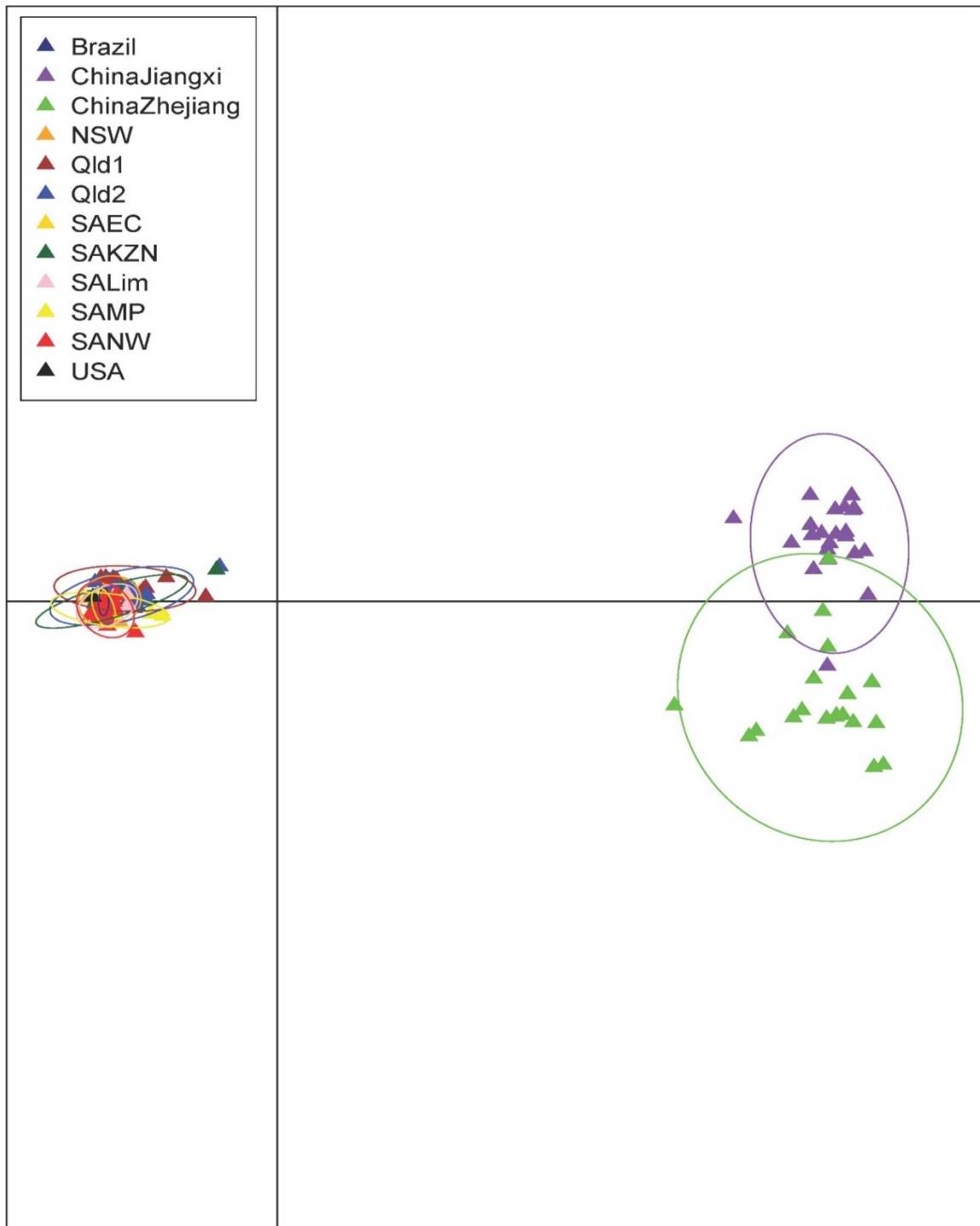
Supplementary Figure S1. Discriminant analysis of principal components (DAPC) analysis of worldwide *Phyllosticta citricarpa* populations (clone-corrected) sampled from five different continents. Populations are indicted by different colours and shapes. The number of axes retained for the principal component analysis was 30 and 3 for the discriminant analysis.
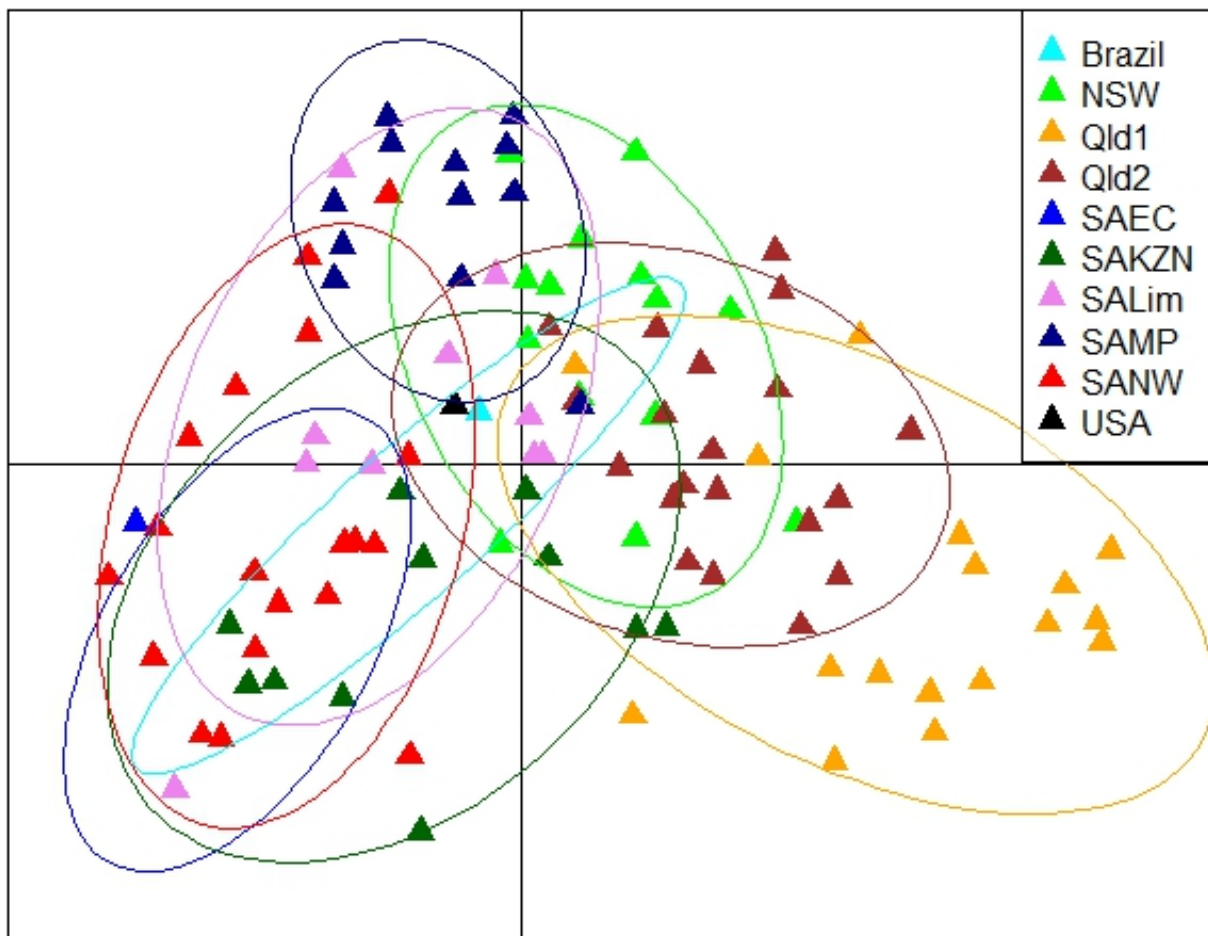
Supplementary Figure S2. Discriminant analysis of principal components (DAPC) analysis of *Phyllosticta citricarpa* populations (clone-corrected) sampled from Australia, Brazil, South Africa and the United States of America. Populations are indicted by different colours and shapes. The number of axes retained for the principal component analysis was 20 and 2 for the discriminant analysis.

CHAPTER 3

Spatial and temporal analysis of *Phyllosticta citricarpa* populations in two South African citrus orchards

ABSTRACT

Citrus Black Spot (CBS), caused by *Phyllosticta citricarpa,* is a disease that influences citrus industries world-wide. No information is available on the population structure of *P. citricarpa* at the orchard spatial (distance) - and temporal (seasonal) scales. These aspects, as well as the reproductive system of the pathogen were investigated in South Africa. *Phyllosticta citricarpa* populations were genotyped from two lemon orchards differing in climate; one in the province of Mpumalanga (sub-tropical) and the other in the North West province (semi-arid). Each orchard was sampled in each of two years (2012 and 2013). Spatial analyses at the orchard scale indicated that subpopulations separated by a short distance (within 200 m) were typically not significantly genetically differentiated, but those that were separated by larger distances (200 m to 400 m) were sometimes significantly differentiated. Temporal analyses in the North West orchard showed that seasonal populations were not significantly genetically differentiated. In contrast, seasonal populations from the Mpumalanga orchard were significantly differentiated. In both orchards, linkage disequilibrium analyses indicated that populations were sexual. The exception was the population from Mpumalanga in one season, although only the non-clone corrected and not the clone corrected population was in linkage disequilibrium. Sexual reproduction was supported by the clone corrected mating type ratios not deviating significantly from a 1:1 ratio. Asexual clonal reproduction was evident from low genotype evenness values for seasonal- and some subpopulations. In each orchard, two dominant multilocus genotypes (MLGs) were identified in most of the subpopulations, as well as in the seasonal populations. Therefore, pycnidiospores are important in the development of CBS over time and space in South African orchards. On a regional scale, the Mpumalanga seasonal populations were significantly genetically differentiated from the North West populations.

INTRODUCTION

In South Africa, *Phyllosticta citricarpa* (McAlpine) Aa is a globally distributed fungal pathogen that causes citrus black spot (CBS). The pathogen primarily causes fruit lesions that only affect the rind of fruit. Although the lesions do not cause decay, the cosmetic damage caused by the lesions results in the downgrading of fruit in local markets (Kotzé, 1981, 2000). Lesions on fruit can result in the rejection of consignments of exported fruit, since *P. citricarpa* is considered a regulated pest by some of South Africa's trade partners (Carstens, et al., 2012). Various types of fruit symptoms have been described, but hard spot is the most typical symptom (Kotzé, 1981, 2000; De Goes, 2000, 2001; Aguilar-Vildoso et al., 2002). Twig symptoms are rare and are characterised by small, round, sunken necrotic spots with grey centres, surrounded by a dark brown ring. Leaf lesions seldom occur, and have only been found on leaves of lemons (*Citrus limon*) and less often on Valencia oranges (*Citrus sinensis*). The lesions on leaves consist of small round sunken necrotic spots and at times a yellow halo may be present around these lesions (Kiely, 1948; Kotzé, 2000; FAO, 2014).

Phyllosticta citricarpa was first identified in South Africa in 1929 from the former Natal province (KwaZulu-Natal) and subsequently in four other provinces (Doidge, 1929; Wager, 1952). Today, CBS is present in citrus producing regions of the Limpopo, Mpumalanga, North West and Eastern Cape provinces, but absent from the citrus producing regions of the Western Cape and Northern Cape provinces (Paul et al., 2005; Carstens et al., 2012). The restricted occurrence of the pathogen to only five of the seven citrus producing provinces in South Africa is attributed to the disease favouring citrus producing areas with warm, humid, summer rainfall climates (Paul et al., 2005; Carstens et al., 2012; Fourie et al., 2013; Yonow et al., 2013; Magarey et al., 2015; Guarnaccia et al., 2017).

The epidemiology of *P. citricarpa* is not only highly influenced by climatic conditions, but by leave and fruit age too. The fruit infection period starts at fruit set, with the young fruitlet remaining susceptible for 4 to 5 months after fruit set (Kiely, 1948; Kotzé, 1981). After this period, natural resistance to infection sets in. Following infection, *P. citricarpa* remains in a quiescent state and most often becomes visible after fruit colour break (Kotzé, 2000). Leaves, while on the tree, are susceptible to infection for up to 10 months (Truter, 2010). Pycnidia and pycnidiospores may be produced on such infected leaves (Kiely, 1948; Kotzé, 2000; FAO, 2014). In fallen leaves (leaf litter) the availability and discharge of sexual ascospores, formed within pseudothecia, are influenced by climatic conditions. The required conditions for pseudothecium maturation and ascospore development include recurrent wet and dry periods at temperatures of between 15°C to 35°C, for a period of 40 to 180 days after leaf fall (Kotzé, 1981; Reis et al., 2006; Truter, 2010; Fourie et al., 2013, Dummel et al., 2013). Under

favourable conditions, ascospores can be produced during the entire year if both mating types are present to enable sexual reproduction. The optimal conditions for the released ascospores to germinate and infect susceptible plant parts are 15 hours of wetness at 27°C (Kiely, 1948; Kotzé, 1981; McOnie, 1964a; Huang and Chang, 1972). Conditions for asexual pycnidiospores to germinate and infect susceptible plant parts include a minimum wet period of 12 hours at 24°C (Noronha, 2002; Wang and Dewdney, 2014).

Previous molecular studies on *P. citricarpa* include a microsatelite-based population genetics structure and reproductive system study at a global scale (Australia, China, Brazil, USA (Florida) and South Africa). The South African populations, as well as those from Australia, Brazil and China were shown to reproduce sexually, based on linkage disequilibrium analyses and the co-occurrence of mating types (MAT *1-1-1* and MAT *1-2-1*) at a 1:1 ratio. In populations from South Africa and Brazil, a low level of genotype evenness was identified along with some non-clone corrected populations showing significant linkage disequilibrium. This suggest frequent clonal reproduction. Pycnidiospores, may thus play a more important role in the spatial distribution of the pathogen in South Africa (Carstens et al., 2017) than previously understood (McOnie, 1965; Kotzé, 1981). Furthermore, in Florida (USA), only one mating type is present (Wang et al., 2016; Carstens et al., 2017) and pycnidiospores are likely the only determinant of the spatial distribution of the pathogen (Hendricks et al, 2017).

Ascospores and pycnidiospores likely play a different role in the spatial distribution of CBS within orchards and trees based on published studies using spore traps, inoculation studies and other conventional approaches. Pycnidiospores, which are exuded in a gelatinous mass, are dispersed over short distances and are the source for distribution of the pathogen within trees in South Africa, Australia and Brazil (Kiely, 1948; McOnie, 1964b; Kotzé, 1981; 2000; Spósito et al., 2008, 2011). Ascospores are forcibly discharged and dispersed over distances of up to 25 m (Spósito et al., 2007) and are seen as the primary source of between-tree pathogen dispersal in orchards in Australia and South Africa (Kiely, 1948; Kotzé, 1981). However, in Brazil where fruit set, climate (higher rainfall), cultural and management practices differ from South Africa and Australia, pycnidiospores are considered to be more important in the spatial distribution of the pathogen (Spósito et al., 2007, 2008, 2011). Furthermore, in Florida, pycnidiospores are the only spore type present and are solely responsible for dispersal and disease expansion over longer distances, including between trees and across rows (Hendricks et al., 2017). Studies on the spatial structure of CBS in orchards outside South Africa and Australia concluded that the CBS pathogen disperse over a relatively short distance, and that the disease has an aggregated dispersal pattern at the orchard tree scale, as well as within trees. Unfortunately the aforementioned population genetic studies all used a random sampling strategy, and were thus unable to make inferences on the importance of

sexual versus asexual reproduction within and across seasons in orchards. This study therefore aims to investigate the spatial within orchard structure of *P. citricarpa* populations.

The objectives of the current study were to further investigate the reproductive mode of *P. citricarpa* in South Africa, and to determine the effect of distance (spatial) and season (temporal) on the population structure of *P. citricarpa.* Important aspects that were investigated were: (i) contribution of sexual and asexual spores to disease, (ii) whether dominant multilocus genotypes (MLGs) persist over time and space, and (iii) whether population structure differ in orchards which differ in climate. The study was conducted in two lemon orchards situated in the Mpumalanga province (subtropical) and North West province (semi-arid) in South Africa, over two seasons (2012 and 2013). A better understanding of the reproductive mode of the CBS pathogen and the contribution of sexual and asexual spores to disease may lead to improved control strategies in orchards. Currently, a preventative control strategy is applied that focuses on targeting ascospore infections during the fruit susceptibility period (October through to February).

MATERIALS AND METHODS

Orchard locations

Two commercial lemon orchards, situated in the Mpumalanga and North West provinces (Fig. 1), with contrasting climates and a history of CBS, were included in the study. The site in the North West province was a 15-year-old Eureka lemon orchard located near Brits, with a BSh Köppen-Geiger climate classification (StepSA; CGA, 2017). BSh climates are described as semi-arid; low relative humidity, warm summers and mild winters. The Mpumalanga site was a 9-year-old Eureka lemon orchard, located near Mbombela (Nelspruit), with a Cwa Köppen-Geiger classification. Cwa climates are described as humid, subtropical; the summers are hot and the winters are dry. The two orchards were separated by approximately 400 km.

Phyllosticta citricarpa isolates were collected according to a structured orchard sampling strategy over two consecutive production seasons (2012 and 2013). The number of sampling sites and the layout varied between the orchards (Fig. 2). In the North West orchard, four sampling sites were selected. The distances of sample sites varied based on their relative location to each other. Sample sites were separated at distances ranging from 50 m (sampling sites 1 vs. 2; 2 vs. 3; 3 vs. 4), to 100 m (sampling sites 1 vs. 3; 2 vs. 4), or 200 m (sampling sites 1 vs. 4) (Fig. 2A). The number of rows of trees between the samples sites varied with the highest number (34 rows) being between sample sites 1 and 4. In the Mpumalanga orchard, three sampling sites were selected. Sample sites were separated by 200 m (sampling sites 1 vs. 2), 300 m (sampling sites 1 vs. 3), or 400 m (sampling site 2 vs. 3) (Fig. 2B). The number of rows of trees between the sampling sites varied with the highest number (48 rows) being

between sample sites 2 and 3, and the lowest being 35 rows between sampling sites 1 and 2. A sampling site consisted of a total of five trees located in three different rows; three of the sampled trees were in the middle row, and the other two trees were in the adjacent rows (cross figure). At each sampling site, ten CBS symptomatic fruits were randomly collected from each of the five trees for isolation studies.

Phyllosticta citricarpa isolations and genotyping

Isolation of *P. citricarpa* from CBS lesions on lemons, verification of species identity and DNA extraction were performed as described by Carstens et al. (2017). Fifteen polymorphic SSR markers (Pc117, Pc179.1, Pc236, Pc440, Pc849, Pc1007, Pc2073, Pc3011, PC12, PC19, PC20, PC32, PC37, PC6, PC7) were used to genotype the *P. citricarpa* populations (Wang et al., 2016; Carstens et al., 2017). Primer labelling as well as PCR reaction and amplification conditions were as previously described (Carstens et al., 2017). Electrophoresis was performed using the 3730XL Genetic Analyzer (Life Technologies) and the SSR alleles were scored using GeneMapper software version 4 (Life Technologies).

SSR data analyses

Population genetic diversity

Isolates with the same alleles at all loci were considered clones or members of the same multilocus genotype (MLG). All allele based population genetic analyses were conducted using a per population clone corrected dataset unless otherwise stated, since the inclusion of clonal haplotypes in the analysis can distort estimates of allelic diversity (Balloux et al. 2003). For the spatial analysis, each sample site was considered a subpopulation. For the temporal analysis, seasons within an orchard were considered as populations, hereafter referred to as seasonal populations. For the gene and genotypic analyses on a spatial scale, the subpopulations were grouped per orchard and for the temporal and regional analysis, the seasonal populations were grouped per orchard (Table 2). The number of alleles (Na), effective population size, number of polymorphic loci and Nei's measure of gene diversity (H_e) (Nei, 1973) were calculated using GenAlEx version 6.5 (Peakall and Smouse, 2012). The number of MLGs, the expected number of MLGs after rarefaction (*eMLG*) to account for different sample sizes, and the evenness index (*E5*), was determined using the R package (Kamvar et al. 2014; R Core Team, 2013). The *eMLGs* were calculated for three different groupings of data: (i) for spatial analyses by comparing the subpopulations per orchard e.g. four subpopulations of North West in 2012, (ii) for temporal analyses by comparing the two seasons per orchard with each other, e.g. North West 2012 season population with North West 2013 season population, and (iii) for regional analyses by comparing the North West seasonal population with the Mpumalanga seasonal population, e.g. North West orchard 2012 with

Mpumalanga orchard 2012. The equitability index $E5$ is an indication of the evenness of MLGs present in subpopulations and populations, and varies from zero (no evenness) to one (all MLGs have equal abundance) (Grünwald et al., 2003; Shannon and Weaver, 1949).

Mode of reproduction

To infer the reproduction strategy (clonal or sexual) used by *P. citricarpa,* linkage disequilibrium analyses were performed on a SSR non-clone corrected and clone corrected dataset in the R package Poppr by calculating the index of association I_A and the standardized version of the index of association $\bar{r}_d$. *P* values were obtained after 999 permutations (Agapow and Burt, 2001). The I_A and $\bar{r}_d$ indices provide an indication of the degree of association of alleles at different loci, within and among populations compared to that observed in a permutated dataset. A value of zero is expected for physically unlinked loci under random mating, i.e. linkage equilibrium. A value significantly larger than zero is an indication of linkage disequilibrium, which is generated when no or infrequent sexual reproduction occurs. Most fungi are able to reproduce both sexually and asexually. Thus, the presence of clones indicate asexual reproduction, but may mask the signal of sexual reproduction. Therefore, both non-clone corrected and clone corrected datasets were analysed to test the null hypotheses of random mating in populations.

PCR analyses with mating type specific primers (Wang et al., 2016) were used to determine the presence of the MAT*1-1-1* and MAT*1-2-1* alleles in 203 isolates that represented a clone corrected dataset, as previously described (Carstens et al., 2017). A chi-square test was used to determine whether the populations deviated from the null hypothesis of a 1:1 ratio of the mating types (Fisher and Yates, 1963).

Effect of distance (spatial) and season (temporal) on distribution of genetic variation

For each orchard, the effects of distance (between sampling sites of an orchard) and season (temporal) on genetic differentiation of populations were investigated using three approaches. The genetic differentiation of populations and subpopulations within and among orchards were analysed using analysis of molecular variance (AMOVA). The statistical significance was tested using 999 permutations. Secondly, genetic differentiation using a pairwise *PhiPT* analyses was also determined for subpopulations and seasonal populations. These analyses were performed in GenAlEx version 6.5 (Peakall and Smouse, 2012). Thirdly, to determine the inter-relationship between the subpopulations within orchards, a discriminant analysis of the principal components (DAPC) was performed in the R package adegenet (Jombart, 2008).

Comparison between the North West and Mpumalanga orchards (regional sale)

Weather data, provided by the Soil, Climate and Water Business Unit, Agricultural Research Council, South Africa, were collected from 2011 to 2013. The prevailing weather data (rainfall, wind speed, humidity and temperatures) in the two orchards during the fruit susceptibility period were evaluated for each season.

The 2012 seasonal population from the North West orchard was compared with the 2012 population from the Mpumalanga orchard, using AMOVA analyses and repeated for the 2013 season.

RESULTS

Phyllosticta citricarpa **isolations**

In total, 599 *P. citricarpa* isolates were collected over a 2-year period from the Mpumalanga and North West orchards for SSR analyses (Table 1). Of these, 373 isolates were obtained from the North West orchard (200 in 2012 and 173 in 2013). The remaining 226 isolates were from the Mpumalanga orchard (130 in 2012 and 96 in 2013). The species identity of all the isolates were confirmed as being *P. citricarpa* as described by Carstens et al. (2017).

Population genetic diversity of sub-populations within each orchard (spatial scale)

In both orchards and years, locus Pc117 was the most polymorphic (with a maximum of 8 alleles). None of the populations showed 100% polymorphism for all 15 loci (Table 1). In the North West orchard, the 15 polymorphic loci revealed a total of 30 to 31 alleles in the different subpopulations in both seasons, ranging from 1-5 different alleles per locus. The gene diversity (*He*) for the four subpopulations within each season in the North West orchard ranged from 0.180 to 0.227 (Table 1).

In the Mpumalanga orchard, a total of 38 alleles were present in the different subpopulations in 2012, ranging from 1-8 different alleles per locus. In the 2013 season, a total of 31 alleles were present in the subpopulations, ranging from 1-7 alleles per locus. The gene diversity (*He*) for the subpopulations within each of the seasons in the Mpumalanga orchard ranged from 0.178 to 0.234. (Table 1).

The number of *e*MLGs varied slightly (*e*MLG = 20 to 24.8) in the North West orchard subpopulations for each of the seasons (Table 2). The genotypic diversity (*D*) was high and varied from 0.915 to 0.942 in the different subpopulations within each season. The evenness values (*E5*), which are an indication of the relative abundance of different genotypes in the subpopulations within each season, varied in 2012 from 0.682 to 0.832, and in 2013 from 0.718 to 0.789 (Table 2).

In the Mpumalanga orchard, the *e*MLG numbers for subpopulations were higher in 2012 (18.9 to 28.0) than for the 2013 subpopulations (16.0 to 17.0). The genotypic diversity (*D*) in the subpopulations was high in both seasons (0.846 to 0.940). The evenness values (*E5*) varied in 2012 from 0.677 to 0.733, and in 2013 from 0.558 to 0.721 (Table 2).

Population genetic diversity of populations in the 2012 and 2013 seasons (temporal scale)

The gene diversity was low in the North West orchard for both seasonal populations, but was higher, although not significantly so, in 2012 (*He* = 0.221; SE = 0.057) than in 2013 (*He* = 0.203; SE = 0.057) (Table 1). In the Mpumalanga orchard, gene diversity was low in both years but was significantly higher in 2012 (*He* = 0.238; SE = 0.048) than in 2013 (*He* = 0.212; SE = 0.053) (Table 1).

In the North West orchard, based on *eMLG* values (which account for the different sample sizes), 55.5 and 49.0 MLGs (SE = 1.80), which differed significantly from each other, were identified in 2012 and 2013 respectively. Genotypic diversity (0.947 and 0.944) was high in the 2012 and 2013 populations, regardless of time. The evenness values (*E5*) were 0.587 and 0.638 in the two seasons (Table 2).

In the Mpumalanga orchard, based on the *eMLG* values, a significantly higher number of MLGs was identified in 2012 (45.1; SE = 2.23) than in 2013 (39.0; SE = 0.00). The genotypic diversity was higher in 2012 (*D* = 0.942) than in 2013 (*D* = 0.881). The evenness values (*E5*) were low in both seasons (*E5* = 0.533 and 0.425) (Table 2).

Occurrence of prevalent MLGs within each orchard

In the North West orchard, there were two dominant multilocus genotypes (MLG81 and MLG50) that were equally abundant and represented 23% and 24% of the 2012 and 2013 season populations respectively. MLG81 represented 12% of the 2012 and the 2013 populations. MLG50 constituted 11% and 12% of the 2012 and 2013 season populations respectively (Fig. 3A). Both MLGs occurred in all four subpopulations at the four different locations within the orchard, representing 6 to 15% of each subpopulation. Different dominant MLGs were detected in subpopulations in the 2012 season, but not in the 2013 season (data not shown).

In the Mpumalanga orchard across the two seasons, there were also two MLGs (MLG53 and MLG83) that represented 24% and 40% of the 2012 and 2013 season populations, respectively. The most dominant MLG (MLG83) represented 17% and 29% of the 2012 and 2013 season populations. The second most prevalent MLG (MLG53) represented 7% and 11% of the 2012 and 2013 season populations, respectively (Fig. 3B).

The dominant MLG83, but not MLG53, occurred in each of the three subpopulations in each season representing 12 to 34% of each subpopulation (data not shown).

Mode of reproduction

Linkage disequilibrium analyses for the non-clone corrected and the clone corrected data set of each of the four subpopulations in the North West orchard, indicated that the index of association did not differ significantly ($P > 0.05$) from zero in any of the subpopulations in the two seasons (Table 3). Similarly, in the total population of both seasons in the North West, for the non-clone corrected and clone corrected dataset, the index of association (I_A) and standardized index of association $(\bar{r}_d)$ did not differ significantly from zero. This is an indication that the loci are not linked and that the hypothesis of random mating occurring could not be rejected.

In the Mpumalanga orchard, the index of association differed significantly ($P < 0.050$) from zero in the 2013 season in the non-clone corrected dataset for subpopulation 3, and in the 2013 seasonal population (Table 3). All the other populations, including all the clone corrected populations, were not in significant linkage disequilibrium (Table 3).

Amplification of the mating type loci yielded a positive PCR amplification for either one of the mating type primer pairs for all analysed isolates. The mating type frequencies were found to not deviate significantly from a 1:1 ratio in the North West orchard ($\chi^2 = 1.00 - 1.07$; $P = 0.30$) and the Mpumalanga orchard ($\chi^2 = 0.02 - 1.27$; $P = 0.90$; $P = 0.20$) (Table 4).

Effect of distance on distribution of genetic variation (spatial)

The AMOVA showed that the total genetic variance attributable to differences among isolates within the subpopulations in both orchards was high (93-99%). For all populations, *PhiPT* was low (0.013-0.068) and non-significant except for the Mpumalanga 2012 seasonal population ($P = 0.021$) (Table 5).

In the North West orchard, spatially significant population structuring was only observed in the 2012 season, and only between subpopulations 1 and 4, based on pairwise *PhiPT* values. These populations were located at the largest distance (200 m) from each other (*PhiPT* = 0.113; $P = 0.011$) in this orchard. Subpopulations that were separated by 50 or 100 m were not significantly differentiated (Fig. 2A; Table 6). In the 2013 season, subpopulations 1 to 4 in all possible combinations showed no significant population sub-structuring (*PhiPT* = 0 to 0.070; $P = 0.091$ to 0.465) (Table 6). These populations were spaced at distances ranging from 50 m to 200 m.

In the Mpumalanga orchard, spatial population structuring was observed in the 2012 and 2013 seasons based on pairwise *PhiPT* values. In both seasons, subpopulations 2 and 3, which were separated by 400 m (Fig. 2B) were significantly genetically differentiated from each other

($PhiPT$ = 0.090; P = 0.007; $PhiPT$ = 0.082; P = 0.042). Furthermore, subpopulations 1 and 3, which were separated by 300 m (Fig 2B) were also significantly genetically differentiated from each other ($PhiPT$ = 0.068; P = 0.037; $PhiPT$ = 0.117; P = 0.021) (Table 7). However, in both seasons, subpopulation 1 was not significantly differentiated from subpopulation 2 ($PhiPT$ = 0.000; P = 0.386; $PhiPT$ = 0.000; P = 0.390), which were separated by the shortest distance (200 m) from each other in this orchard (Fig. 2B; Table 7).

The DAPC, which reveals the variation between populations, in general supported the pairwise $PhiPT$ analyses. Analyses in the North West orchard only showed separate clustering of subpopulation 1 and 4 in the 2012 season but complete overlap and thus no differentiation in the next season (Fig. 4). In the Mpumalanga orchard, DAPC also revealed differentiation between some of the subpopulations in both seasons (Fig. 5). The eigenvalues chosen for both orchards and both seasons in the orchards represented 80% of the total variation.

Effect of season on distribution of genetic variation (temporal)

In the North West orchard, the AMOVA analysis comparing the populations over the two seasons showed that 100% of the genetic variance was attributable to differences among isolates within the populations, while there was no variance among the populations (Table 8). Pairwise $PhiPT$ values confirmed the absence of a temporally differentiated population structure in this orchard (0.000; P = 0.445).

In the Mpumalanga orchard, the AMOVA analysis showed that 97% of the total variance was attributed to differences between isolates within the populations and 3% to differences among populations ($PhiPT$ = 0.027; P = 0.013) (Table 8). Based on pairwise $PhiPT$ values, temporally population differentiation was observed in this orchard (0.027; P = 0.016).

Comparison between the North West orchard and Mpumalanga orchard (regional sale)

Weather data

Lemon fruit in the North West and Mpumalanga provinces are susceptible to *P. citricarpa* infections between the beginning of October and the end of February (C. Kellerman, Pest Management Services, Nelspruit, South Africa, personal communication). The monthly weather parameters (minimum and maximum temperatures, relative humidity, wind speed and rainfall) differed in the two orchards during the fruit susceptibility period (October through to February) in the 2012 and 2013 seasons (Supplementary Table 1). Mpumalanga had a higher total rainfall in both seasons (2012 season 765.04 mm; 2013 season: 950.96 mm) than the North West (2012 season: 407.93 mm; 2013 season: 476.52 mm). In both seasons, the maximum temperatures were higher in the North West orchard than in Mpumalanga orchard; approximately 2 to 3°C for 2012 season, and 3 to 5 °C for the 2013 season. However, the minimum temperatures for Mpumalanga were higher (approximately 2.5°C) in both seasons

than in the North West. The relative humidity was higher in Mpumalanga (91.36 %) during these periods than in North West (88.60 %) (Supplementary Table 1).

Population structure

AMOVA analyses and pairwise *PhiPt* values showed that there was significant differentiation between the two orchards in both seasons. In 2012, 48% (*PhiPT* = 0.479; *P* = 0.001) of the variation was due to differences between the orchards and in 2013, it was 54% (*PhiPT* = 0.543; *P* = 0.001) (Supplementary Table 2).

DISCUSSION

The current study investigated the effect of distance (spatial) and season (temporal) at the orchard scale, on the population structure and mode of reproduction of *P. citricarpa* in South Africa. The populations were studied over two seasons in two lemon orchards; one in the sub-tropical Mpumalanga province and the other in the semi-arid North West province. The study has shown for the first time that distance and season have an effect on the distribution of genetic variation of *P. citricarpa*. Distance affected population structure in both orchards, since subpopulations that were within 200 m were typically not significantly genetically differentiated, but those that were separated by larger distances of 200 to 400 m were often significantly differentiated. The effect of season (temporal) differed in the two orchards. In the North West orchard (semi-arid), season did not affect population structure, whereas in the Mpumalanga orchard (subtropical) the populations from the 2012 and 2013 seasons were genetically differentiated. In both orchards, populations were sexual. However, the persistence of two dominant MLGs in populations over space and seasons in both orchards, indicated strong asexual reproduction. On a regional scale, the Mpumalanga seasonal populations were both significantly differentiated from the North West seasonal populations. The genotypic diversity and genotype evenness were comparable between the two orchards in the 2012 season. However, in the 2013 season the Mpumalanga orchard had a lower genotypic diversity and genotype evenness than the North West orchard, but a higher number of *eMLGs*.

Previous studies on *P. citricarpa* populations in Brazil, South Africa, Australia and China, where both mating types occur, have shown that the pathogen reproduces sexually and asexually (Carstens et al., 2017). Similar results were found in the current study in both of the orchards investigated over the two seasons. Mating type frequencies did not deviate significantly from a 1:1 ratio in both of the orchards and seasons. Furthermore, the index of association tests (I_A and $\bar{r}_d$) did not differ significantly from zero in all of the North West populations, and for most of the Mpumalanga populations. The exception was for one non-clone corrected 2013 Mpumalanga subpopulation and also the non-clone corrected 2013

seasonal population in the Mpumalanga orchard. This indicated the importance of asexual reproduction in the orchard. The occurrence of asexual reproduction in the 2013 season in the Mpumalanga population was likely driven by the two dominant MLGs (clones) that increased from 24% in the 2012 season to 40% in the 2013 season. In the North West orchard, a similar situation was found in that two dominant MLGs were present, but these represented the same percentage of the populations in both seasons (23 to 24%). The occurrence of asexual reproduction was also supported by low genotype evenness. The genotype evenness was low for the seasonal populations over time in both orchards ($E5$ = 0.425 to 0.638), compared to that of the subpopulations ($E5$ = 0.677 to 0.832). This indicated that the abundance of genotypes were more equal in subpopulations than across the total population. The occurrence of asexual and sexual reproduction indicates that the CBS pathogen can use both reproductive systems to adapt and survive in changing environments. An important environmental factor that may have affected asexual reproduction in the Mpumalanga orchard in the 2013 season was a higher rainfall (951 mm) during the fruit susceptibility period than in the 2012 season (765 mm). Similarly, in Brazil, under high rainfall conditions, pycnidiospores play an important role in disease development (Spósito et al., 2007, 2011).

Clonal reproduction of *P. citricarpa* has been reported in citrus orchards where only one mating type occurs. In Florida in the USA, only one clonal mating type population exists (Wang et al., 2016; Carstens et al., 2017). In the orchards, the disease occurs in a clustered pattern supporting short-distance dispersal by pycnidiospores as the major inoculum source (Hendricks et al., 2017). In these Florida orchards, the spread of CBS by pycnidiospores was observed at distances further than the 80 cm reported in Brazil (Spósito et al., 2011), and occurred at least across tree rows, approximately 6.7 m apart. It was hypothesized that the spread of pycnidiospores was most likely by wind-driven rain and/or through the spread of infected twigs and leaves from diseased trees during hurricanes and tropical storms that occur in Florida (Hendricks et al., 2017). The clonal survival and reproduction of *P. citricarpa* were recently reported in Europe by Guarnaccia et al. (2017). The finding by Guarnaccia et al. (2017) of *P. citricarpa* populations, consisting of a single mating type and a single clone per country, in citrus leaf litter in Italy, Malta and Portugal was unexpected since no CBS symptoms were found in the orchards nor were CBS symptoms ever reported from these countries (Paul et al., 2005; Yonow, et al., 2013; Fourie et al., 2013; Magarey et al. 2015; Guarnaccia et al., 2017;).

The ability of *P. citricarpa* to spread and persist clonally is supported by reports that MLGs can be shared between countries. Carstens et al. (2017) reported that MLGs were shared among populations from South Africa, Australia, Brazil, and the United States. For example, MLGs present in Brazil were shared with all three populations from Australia and all

five populations from South Africa. No MLGs from the aforementioned four countries were shared with China, one of the putative origins of the pathogen (Carstens et al., 2017). Guarnaccia et al. (2017) furthermore reported that the single *P. citricarpa* MLG from Portugal, found in leaf litter, was shared with the three Australian populations from the Carstens et al. (2017) study. Malta and Italy shared the same single *P. citricarpa* MLG, which was not present in Portugal or any of the populations studied by Carstens et al. (2017) (Guarnaccia et al., 2017). In both of the aforementioned studies, and in the current study, MLGs were identified using 15 SSR markers. A genotype accumulation curve of the markers showed that the curve did not reach a plateau, suggesting that additional loci might improve discrimination among MLGs (Carstens et al., 2017). It might therefore be possible that these markers are not sufficiently polymorphic to identify true clones. It has been a challenge finding polymorphic SSR markers in *P. citricarpa* (Wang et al., 2016; Carstens et al., 2017). Previously sequence data were not useful for identifying polymorphisms in *P. citricarpa* (Wickert et al., 2012, Miles et al., 2013, Zavala et al., 2014). However, recently the presence of seven single nucleotide polymorphisms in sequence data of the actin gene (*actA*) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) genes among 21 *P. citricarpa* isolates from various countries was reported (Guarnaccia et al., 2017). In future studies, sequence data from these genes could be used to ascertain whether the clonal MLGs identified thus far in the various studies are true clones. However, considering that (i) all *P. citricarpa* clones identified in the current study, and those identified by Guarnaccia et al. (2017) consist of the same single mating type and (ii) that Zavala et al. (2014) found no polymorphisms in the *actA* gene and *gapdh* genes in the clonal population from Florida, supports the fact that the 15 SSR markers may identify true clones with a high level of probability.

In the current study, two dominant MLGs were identified in subpopulations and populations from both seasons in each of the two South African lemon orchards. This suggests that pycnidiospores play a role in the epidemiology of the pathogen in South Africa at spatial and temporal scales, which has not been reported previously. This contradicts the current understanding of the minor epidemiological importance of pycnidiospores in disease development over seasons and space in South African and Zimbabwean orchards (Kiely, 1948; Wager, 1952; McOnie, 1964b, 1965; Whiteside, 1967; Kotzé, 1963, 1981; Truter, 2010). However, the number of peer reviewed studies published to provide evidence for this are limited, and data may have been interpreted incorrectly. In South Africa, McOnie (1964b) concluded that pycnidiospores were not important based on experiments using spore trapping, fruit bagging, and staggered spray experiments. These experiments all showed that initial fruit infection coincided with the earliest and highest ascospore discharge (McOnie, 1964b). However, the species identity of the trapped ascospores could not be ascertained as being *P.*

citricarpa, due to the similarity in ascospore morphology of *P. citricarpa* and the saprophyte *P. capitalensis* (Meyer et al., 2001). McOnie (1964b) furthermore found that pycnidiospores released from dead leaves were not important since their release from dead leaves did not coincide with fruit infections. Lastly, in South Africa, except for lemons, fruit do not overlap within trees. It was therefore concluded that pycnidiospores from fruit on trees in general are not a source of inoculum for new fruit infections (McOnie, 1964b; Kotzé, 1981). Wager (1949) and Whiteside (1967), in neighbouring Zimbabwe, demonstrated the short-distance wash-down dispersal of pycnidiospores from pycnidia in fruit lesions and concluded that the epidemiological contribution of these spores would be much more limited than the airborne ascospores.

In Australia, where populations were recently shown to be sexual (Carstens et al., 2017; Tran et al., 2017), pycnidiospores have in the past not been considered as being important in the epidemiology of the pathogen but new studies are underway to determine the roles of the different spores in Australia (N.T. Tran, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Australia, personal communication and unpublished data). Similar to South Africa, limited peer reviewed literature is currently available to support the prominent role of ascospores in CBS epidemiology (Kiely, 1948). Spore trap studies conducted by Kiely (1948) showed that ascospores could frequently be trapped between tree rows, and around the boundary of an orchard containing severe fruit infections. However, the species identity of the ascospores could not be confirmed. Pycnidiospores were rarely trapped between rows, and not at all at the boundary of the orchard. Pycnidiospores were, however, frequently identified in water sampled from a filter funnel placed at the bottom of trees (Kiely, 1948) and the author concluded that pycnidiospores are a source of inoculum within trees, but are not disperse between trees. This data, however, is not conclusive since rain dispersed pycnidiospores will be difficult to trap onto slides, as opposed to the wind dispersed ascospores.

Pycnidiospores are important in the epidemiology of CBS in Brazil and Florida (Spósito et al., 2011; Hendricks et al., 2017), although the pathogen also does reproduce sexually in Brazil (Amorim et al., 2017; Carstens et al., 2017). The subtropical and tropical climates in these two countries are considered to be much more favourable for CBS development than in Africa and Australia (Yonow et al. 2013; Magarey et al., 2015). The importance of pycnidiospores in Brazil has been shown by three studies. Firstly, Spósito et al. (2007, 2008) reported that the aggregation of symptomatic fruits in all sectors and heights in trees provided support for the importance of pycnidiospores that were likely splash-dispersed. Secondly, Spósito et al. (2011) showed that the placement of *P. citricarpa* inoculum consisting of infected fruits or dead twigs in trees in a CBS-free orchard were able to cause new fruit infections.

Thirdly, fruit on trees were furthermore considered as an important inoculum source, since fruit infections were more severe when there was an overlap between young and old fruit on trees. Fourthly, the removal of all possible leaf litter as ascospore inoculum source from the orchard floor, did not prevent disease development when there was no overlap in fruit crops on trees (Spósito et al., 2011).

The difference in the perceived role of pycnidiospores in Brazil compared to Australia and South Africa, is thought be due to differences in cultural and environmental conditions. In Australia and South Africa, in contrast to the predominant production of late maturing Valencia oranges for juicing in Brazil, the period of fruit infection is restricted to four to five months. Lanza et al. (2018), however, recently reported from Brazil that although fruit susceptibility declined after 4 to 5 months, fruits are likely to be susceptible for up to 7 months. There is less overlapping of old and new fruit in orchards in South Africa and Australia. Furthermore, less frequent pruning of dead twigs, a source of pycnidiospore inoculum in orchards managed for juice fruit production, are conducted in Brazil than in South Africa and Australia (Spósito et al., 2011).

Spatial analyses at the orchard scale in the current study in South Africa showed that in both orchards, distance affected population structure. In the North West orchard, where subpopulations were spatially separated by relative short distances of 50 m, 100 m, or 200 m, the subpopulations were not significantly sub-structured in both seasons (at a 95% confidence level interval). The only instance in the North West orchard where two subpopulations were significantly differentiated was for two subpopulations that were the most distant from each other (200 m) in the 2012 season. In the Mpumalanga orchard, where subpopulations were separated further away from each other (200 m or 300 m or 400 m), significant differentiation occurred between the subpopulations; the exception, however, in the Mpumalanga subpopulations was where subpopulations were separated at the shortest distance from each other (200 m). Thus, in both orchards, the genetic structure of the subpopulations revealed distance limitations in the dispersal of propagules. DAPC analyses supported population sub-structuring at the orchard spatial scale by distance. In Brazil, Spósito et al. (2011) showed, based on a spatial analyses of CBS-symptomatic trees, that trees were aggregated in small foci with a maximum radius of 24.7 m, and that dispersal was thus likely limited to this distance. *Venturia inaequalis,* another ascomycete tree pathogen, also produces ascospores in leaf litter, which are dispersed at an effective distance of up to 33 m from the source, but could be dispersed up to 45 m (Holb et al., 2004). Yet in *Mycosphaerella fijensis,* a pathogen of banana and plantain, ascospores were wind-dispersed at a mean distance of 104 m to 613 m from source (depending on the wind direction), although the disease gradient declined sharply 100 m from the source (Rieux et al., 2014).

The current study indicated that *P. citricarpa* pycnidiospores were important, as evidenced by the MLGs that were shared between spatially separated subpopulations within the same orchard, but their dispersal would not most likely not have been evident over the short period of the two studied seasons. This is due to the fact that *P. citricarpa* pycnidiospores are dispersed over short distances (only 0.80 m or up to 6.7 m) (Spósito et al., 2011; Hendricks et al., 2017). Therefore, the dispersal of pycnidiospores within orchards will rather occur over multiple seasons in the long term. The epidemiological role of asexual reproduction can be hypothesized to be at least sufficient for clonal survival (under less favourable conditions for CBS), but also contributing to CBS disease (under more favourable conditions). The former was particularly evident from the findings of Guarnaccia et al. (2017) that *P. citricarpa* apparently persists under climatic conditions unsuitable for CBS disease development. The role of pycnidiospores in CBS disease development is clear from the studies in Florida (Wang et al., 2016; Hendricks et al., 2017) and Brazil (Spósito et al., 2007, 2008, 2011).

Season is not expected to affect the population structure of *P. citricarpa* in citrus orchards, especially in lemon orchards. This is due to citrus trees being evergreen and leaves dropping throughout the year, with the lifespan of a leaf ranging from 2 to 3 or even more years (Kelley and Cummins, 1920; Wallace et al., 1954). Since leaves are susceptible to infection for the first 10 months (Truter, 2010), leaves on a tree can over time potentially be infected with a range of *P. citricarpa* genotypes. Infected leaves on the tree are therefore an important inoculum reservoir of a variety of genotypes, which can contribute to sexual reproduction over a 2 to 3 year period once leaves drop from the tree. In lemon orchards, fruit crops overlap on trees, which may further contribute to populations not being differentiated between seasons since a single genotype can infect different fruit crops and can contribute to asexual reproduction over a prolonged period. The occurrence of CBS symptoms on green leaves on the tree is more common in lemons (Kiely, 1948; Kotzé, 2000), which will prolong the potential contribution of asexual spores produced in these lesions. In the current study, it was found that *P. citricarpa* populations in the North West orchard populations were not significantly genetically differentiation based on AMOVA analysis. In contrast, the seasonal populations in the Mpumalanga orchard, were significantly differentiated as indicated by AMOVA analysis. This could be due to a higher rainfall which can favour asexual reproduction (Kotzé, 1963; Huang and Chang, 1972; Truter, 2010), a higher disease incidence (subtropical climate), larger distances between sub-populations in the Mpumalanga than the North West orchard (reduced ability of sub-populations to interbreed) or cultural practices.

In summary, the current study on the population structure of *P. citricarpa* in two orchards in two different production regions in South Africa showed that distance at the scale of the orchard influenced population structure. The effect of season on population structure

was small and orchard dependent. Asexual reproduction and clonal reproduction were evident in both orchards and played a major role in the epidemiology of CBS. Airborne ascospores may play an important role in the spatial distribution of the pathogen at the orchard scale. Even though sexual and clonal reproduction was evident in both of the studied lemon orchards, this might not be true for other citrus types as the periods of flowering and fruiting are different in citrus types. Lemons can have up to three fruit sets in one production cycle, whilst most other citrus types are managed to have one fruit set only. The observation that pycnidiospore play an important role in CBS in South Africa, require changes to the current management strategies, which only targets ascospores during fruit susceptibility periods and weather conditions conducive for their release and infection. Future studies should re-investigate the role of pycnidiospores and ascospores in South Africa. Conventional inoculation (fruit and twigs) and leaf removal studies should be conducted in citrus orchards, combined with population genetic analyses of the trials. New ascospore trap data should be generated to determine the specific differentiation of *P. citricarpa* from *P. capitalensis* ascospores. Additional studies should be conducted in orange orchards, since the relative importance of ascospore and pycnidiospore inoculum sources might differ between citrus types.

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Table 1. Gene diversity and number of alleles of 15 microsatellite loci across time (between 2012 and 2013 seasons) and space (within orchard between subpopulations separated by defined distances) between clone corrected *Phyllosticta citricarpa* populations in two lemon orchards in the North West and Mpumalanga provinces of South Africa.

| Province | #Isolates | #MLGs[a] | # Alleles | | | | | | | | | | | | | | | Ne | H_e [b] |
			Pc 117	Pc 179.1	Pc 236	Pc 440	Pc 849	Pc 1007	Pc 2073	Pc 3011	Pc 12	Pc 19	Pc 20	Pc 32	Pc 37	Pc 6	Pc 7		
North West																			
2012																			
Subpopulation 1	52	14	4	2	1	4	1	2	2	2	1	2	1	1	2	3	2	1.34	0.200
Subpopulation 2	50	21	3	2	1	3	1	2	2	2	1	2	1	1	2	3	2	1.37	0.212
Subpopulation 3	50	15	4	1	1	2	1	3	2	2	1	2	1	1	2	3	2	1.42	0.227
Subpopulation 4	48	10	3	1	1	2	1	2	2	2	1	1	1	1	1	3	2	1.36	0.183
Seasonal	**200**	**60**	**5**	**2**	**1**	**3**	**1**	**3**	**2**	**2**	**1**	**2**	**1**	**1**	**2**	**3**	**2**	**1.40**	**0.221**
2013																			
Subpopulation 1	41	14	3	2	1	2	1	2	2	2	1	2	1	1	1	3	2	1.36	0.201
Subpopulation 2	46	13	4	1	1	2	1	2	2	2	1	2	1	1	1	3	2	1.38	0.185
Subpopulation 3	46	15	3	1	1	3	1	2	2	2	1	2	1	1	1	3	2	1.33	0.183
Subpopulation 4	40	7	2	1	1	2	1	2	2	2	1	2	1	1	1	2	2	1.29	0.180
Seasonal	**173**	**49**	**5**	**2**	**1**	**3**	**1**	**2**	**2**	**1**	**2**	**1**	**1**	**1**	**1**	**3**	**2**	**1.37**	**0.203**
Mpumalanga																			
2012																			
Subpopulation 1	45	14	4	2	2	2	1	2	2	2	2	1	1	1	2	3	2	1.34	0.216
Subpopulation 2	41	21	5	1	1	3	1	2	2	2	1	2	1	1	2	3	1	1.41	0.223
Subpopulation 3	44	20	5	3	2	3	2	2	2	2	1	2	1	1	2	3	2	1.36	0.227
Seasonal	**130**	**55**	**8**	**3**	**3**	**3**	**2**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**2**	**3**	**2**	**1.37**	**0.238**
2013																			
Subpopulation 1	32	13	5	2	1	3	1	2	2	2	1	1	1	1	2	2	1	1.36	0.188
Subpopulation 2	32	15	6	2	1	3	1	2	2	2	1	2	1	1	2	2	1	1.38	0.178
Subpopulation 3	32	11	3	2	1	2	1	2	2	2	2	2	1	1	2	2	1	1.39	0.234
Seasonal	**96**	**39**	**7**	**2**	**1**	**3**	**1**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**2**	**2**	**1**	**1.38**	**0.212**

[a] #MLGs = Multilocus genotypes; [b]Nei's gene diversity, H_e (Nei, 1973); *Ne* - effective population size

TABLE 2. A summary of genotypic diversity indices for populations of *Phyllosticta citricarpa* across space (within orchard between subpopulations separated by defined distances) and a summary of the genotypic diversity indices for populations of *Phyllosticta citricarpa* across time (between 2012 and 2013 seasons) in two lemon orchards in the North West and Mpumalanga provinces of South Africa.

Province/Year	#Isolates	#MLGs[a]	*eMLG*[b]	SE[b]	*E5*[c]	*D*[d]
North West						
2012						
Subpopulation 1	52	26	24.8	0.887	0.768	0.936
Subpopulation 2	50	25	24.4	0.628	0.832	0.942
Subpopulation 3	50	24	23.4	0.653	0.682	0.915
Subpopulation 4	48	22	22.0	0.000	0.751	0.921
Seasonal	**200**	**60**	**55.5**	**1.80**	**0.587**	**0.947**
2013						
Subpopulation 1	41	22	21.7	0.455	0.786	0.930
Subpopulation 2	46	23	20.9	1.095	0.718	0.918
Subpopulation 3	46	23	21.9	1.059	0.789	0.931
Subpopulation 4	40	20	20.0	0.00	0.768	0.917
Seasonal	**173**	**49**	**49.0**	**0.00**	**0.638**	**0.944**
Mpumalanga						
2012						
Subpopulation 1	45	20	18.9	0.853	0.677	0.896
Subpopulation 2	41	28	28.0	0.000	0.733	0.940
Subpopulation 3	44	25	23.8	0.819	0.728	0.930
Seasonal	**130**	**55**	**45.1**	**2.23**	**0.533**	**0.942**
2013						
Subpopulation 1	32	17	17.0	0.00	0.558	0.850
Subpopulation 2	32	16	16.0	0.00	0.721	0.885
Subpopulation 3	32	16	16.0	0.00	0.579	0.846
Seasonal	**96**	**39**	**39.0**	**0.00**	**0.425**	**0.881**

[a] MLGs = Multilocus genotype

[b] *eMLG* = expected number of MLGs after rarefraction, SE = Standard error based on *eMLG*. The *eMLGs* values for subpopulations were calculated separately for each orchard and season. The *eMLG* value for the seasonal populations were calculated separately for each orchard using the total populations of the 2012 and 2013 seasons.

[c] *E5* = Evenness

[d] *D* = Genotypic diversity

TABLE 3. Linkage disequilibrium analyses for populations of *Phyllosticta citricarpa* across space and time within two lemon orchards in South Africa with corresponding significance level (*P*-value).

Province/Year	#Isolates	#MLGs	*eMLG*	Non-clone-corrected populations				Clone-corrected populations			
				I_A	*P* value	$\bar{r}_d$	*P* value	I_A	*P* value	$\bar{r}_d$	*P* value
North West/2012											
Subpopulation 1	52	26	24.8	0.001	0.454	0.000	0.454	-0.349	0.982	-0.045	0.978
Subpopulation 2	50	25	24.4	0.038	0.318	0.005	0.317	-0.104	0.749	-0.012	0.749
Subpopulation 3	50	24	22.7	0.130	0.088	0.017	0.088	-0.329	0.983	-0.042	0.982
Subpopulation 4	48	22	19.9	0.015	0.387	0.003	0.388	-0.086	0.610	-0.015	0.609
Seasonal	**200**	**60**	**55.5**	**0.019**	**0.330**	**0.002**	**0.328**	**-0.214**	**0.999**	**-0.025**	**0.999**
North West/2013											
Subpopulation 1	41	22	21.7	-0.045	0.660	-0.006	0.662	-0.300	0.960	-0.038	0.957
Subpopulation 2	46	23	20.9	0.043	0.267	0.007	0.264	0.041	0.364	0.007	0.365
Subpopulation 3	46	23	21.1	-0.060	0.743	-0.009	0.745	-0.349	0.988	-0.051	0.987
Subpopulation 4	40	20	20.0	-0.109	0.872	-0.017	0.874	-0.248	0.795	-0.035	0.788
Seasonal	**173**	**49**	**49.0**	**-0.056**	**0.880**	**-0.008**	**0.884**	**-0.268**	**1.000**	**-0.035**	**1.000**
Mpumalanga/2012											
Subpopulation 1	45	20	18.9	0.147	0.124	0.014	0.123	-0.255	0.922	-0.026	0.911
Subpopulation 2	41	28	28.0	0.150	0.069	0.019	0.068	-0.109	0.789	-0.016	0.789
Subpopulation 3	44	25	23.8	-0.068	0.708	-0.007	0.709	-0.315	0.979	-0.029	0.978
Seasonal	**130**	**55**	**45.1**	**0.108**	**0.060**	**0.010**	**0.058**	**-0.162**	**0.974**	**-0.015**	**0.975**
Mpumalanga/2013											
Subpopulation 1	32	17	17.0	0.210	0.062	0.032	0.062	-0.188	0.845	-0.027	0.838
Subpopulation 2	32	16	16.0	0.219	0.074	0.029	0.076	0.283	0.091	0.036	0.094
Subpopulation 3	32	16	16.0	0.325	0.022	0.038	0.022	-0.199	0.808	-0.022	0.805
Seasonal	**96**	**39**	**39.0**	**0.233**	**0.007**	**0.028**	**0.007**	**-0.137**	**0.914**	**-0.016**	**0.914**

MLGs = Multilocus genotype, *eMLG* = expected number of MLGs after rarefraction, I_A = Index of Association; $\bar{r}_d$ = Standardized index of association

TABLE 4. Mating type composition and χ² testing for expected 1:1 mating-type ratios in clone corrected *Phyllosticta citricarpa* populations collected in the 2012 and 2013 seasons from two lemons orchards in the North West and Mpumalanga provinces of South Africa.

Province and Year	Number of A1 mating type isolates	Number of A2 mating type isolates	Number of MLGs[a]	χ² value	*P*-value
North West					
2012	26	34	60	1.07	0.300
2013	21	28	49	1.00	0.300
Mpumalanga					
2012	27	28	55	0.02	0.900
2013	23	16	39	1.27	0.200

[a] The total number of multilocus genotypes (MLG) per population in clone corrected *Phyllosticta citricarpa* populations.

TABLE 5. Analysis of molecular variance (AMOVA) of *Phyllosticta citricarpa* subpopulations per season in each of the two orchards (North West and Mpumalanga) using 15 simple sequence repeat loci.

Source	d.f.	SS	% Variation	AMOVA statistics	*P*
North West 2012					
Among subpopulations	3	6.519	2		
Within sub populations	56	93.614	98	*PhiPT* = 0.020	0.163
Total	**59**	**110.133**	**100**		
North West 2013					
Among subpopulations	3	5.339	1		
Within subpopulations	45	69.171	99	*PhiPT* = 0.013	0.284
Total	**48**	**74.510**	**100**		
Mpumalanga 2012					
Among subpopulations	2	8.597	5		
Within subpopulations	52	118.676	95	*PhiPT* = 0.047	0.021
Total	**54**	**127.273**	**100**		
Mpumalanga 2013					
Among subpopulations	2	9.969	7		
Within subpopulations	36	92.390	93	*PhiPT* = 0.068	0.059
Total	**38**	**102.359**	**100**		

TABLE 6. Estimates of pairwise *PhiPT* values (below the diagonal) averaged over 15 simple sequence repeat loci of *Phyllosticta citricarpa* subpopulations within the lemon orchard in the North West province, for two consecutive years. Significance values indicated above the diagonal.

2012	Subpopulation1	Subpopulation 2	Subpopulation 3	Subpopulation 4
Subpopulation 1	-	0.431	0.131	0.011
Subpopulation 2	0.000	-	0.461	0.067
Subpopulation 3	0.032	0.000	-	0.392
Subpopulation 4	0.113	0.057	0.005	-
2013	Subpopulation1	Subpopulation 2	Subpopulation 3	Subpopulation 4
Subpopulation 1	-	0.100	0.465	0.461
Subpopulation 2	0.050	-	0.063	0.091
Subpopulation 3	0.000	0.058	-	0.435
Subpopulation 4	0.000	0.070	0.000	-

TABLE 7. Estimates of pairwise *PhiPT* values (below the diagonal) averaged over 15 simple sequence repeat loci of *Phyllosticta citricarpa* subpopulations within the lemon orchard in the Mpumalanga province, for two consecutive years. Significance values indicated above the diagonal.

2012	Subpopulation 1	Subpopulation 2	Subpopulation 3
Subpopulation 1	-	0.386	0.037
Subpopulation 2	0.000	-	0.007
Subpopulation 3	0.068	0.090	-
2013	Subpopulation 1	Subpopulation 2	Subpopulation 3
Subpopulation 1	-	0.390	0.021
Subpopulation 2	0.000	-	0.042
Subpopulation 3	0.117	0.082	-

TABLE 8. Analysis of molecular variance (AMOVA) of *Phyllosticta citricarpa* populations between 2012 and 2013 for each of the two lemon orchards (North West and Mpumalanga) using 15 simple sequence repeat loci.

Source (2012/2013)	d.f.	SS	% Variation	AMOVA statistics	*P*
North West					
Among populations	1	1.149	0		
Within populations	107	173.860	100	*PhiPT* = 0.000	0.732
Total	**108**	**175.009**	**100**		
Mpumalanga					
Among populations	1	3.959	3		
Within populations	92	159.967	97	*PhiPT* = 0.027	0.013
Total	93	163.926	**100**		
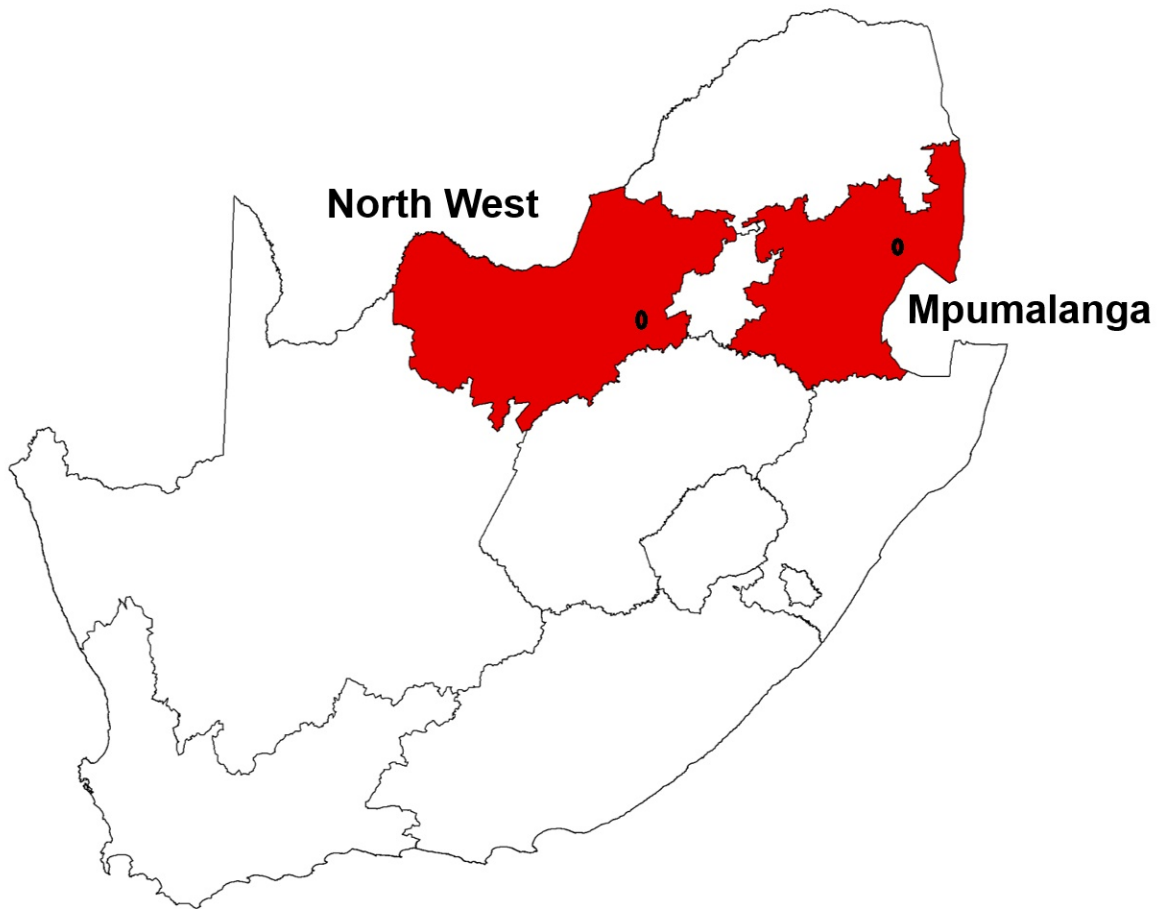
Figure 1. Location of two orchards in the Mpumalanga and North West provinces in South Africa where the population structure of *Phyllosticta citricarpa* was studied at the orchard spatial- and temporal scales.
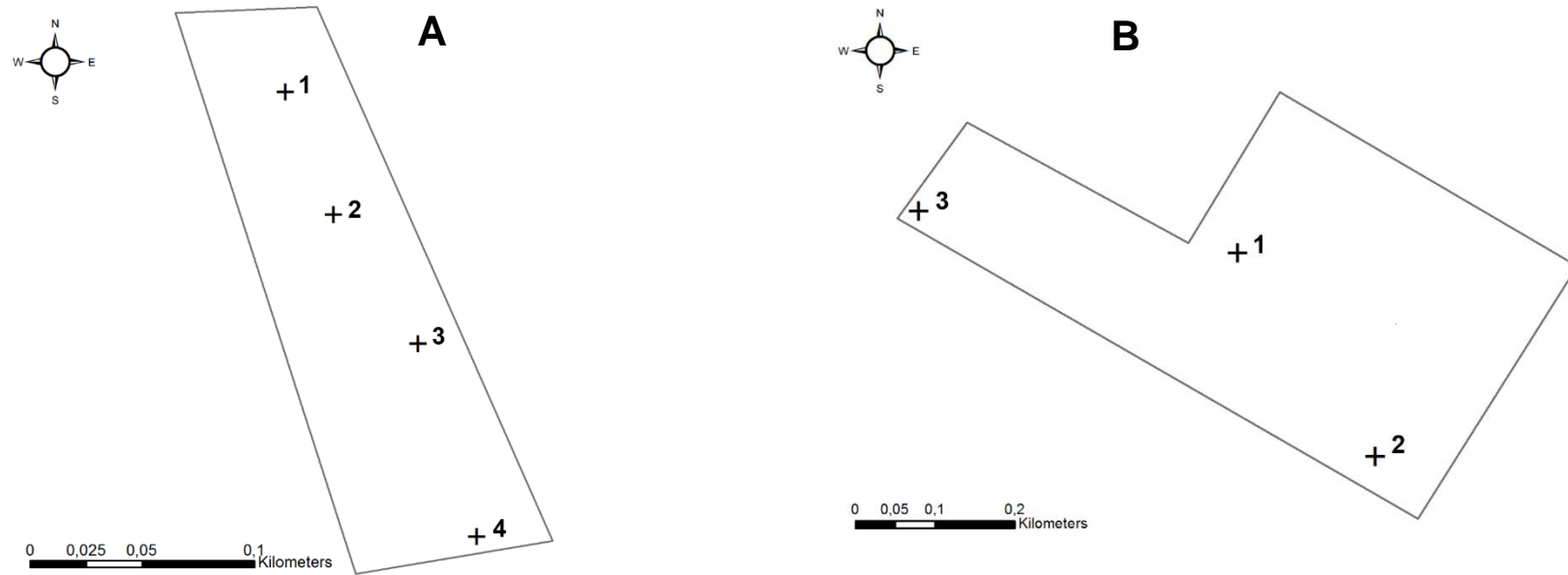
Figure 2. Sampling strategy used for studying the population structure of *Phyllosticta citricarpa* at the spatial- and temporal scales in two lemon orchards situated in the (A) North West and (B) Mpumalanga provinces in South Africa. In each orchard, the selected sampling sites are indicated by "+" along with the *P. citricarpa* subpopulation numbers. At each of the sampling sites five trees located in a cross over two rows were selected, from which 10 citrus black spot fruits were randomly sampled for subsequent *P. citricarpa* isolation and genotyping.
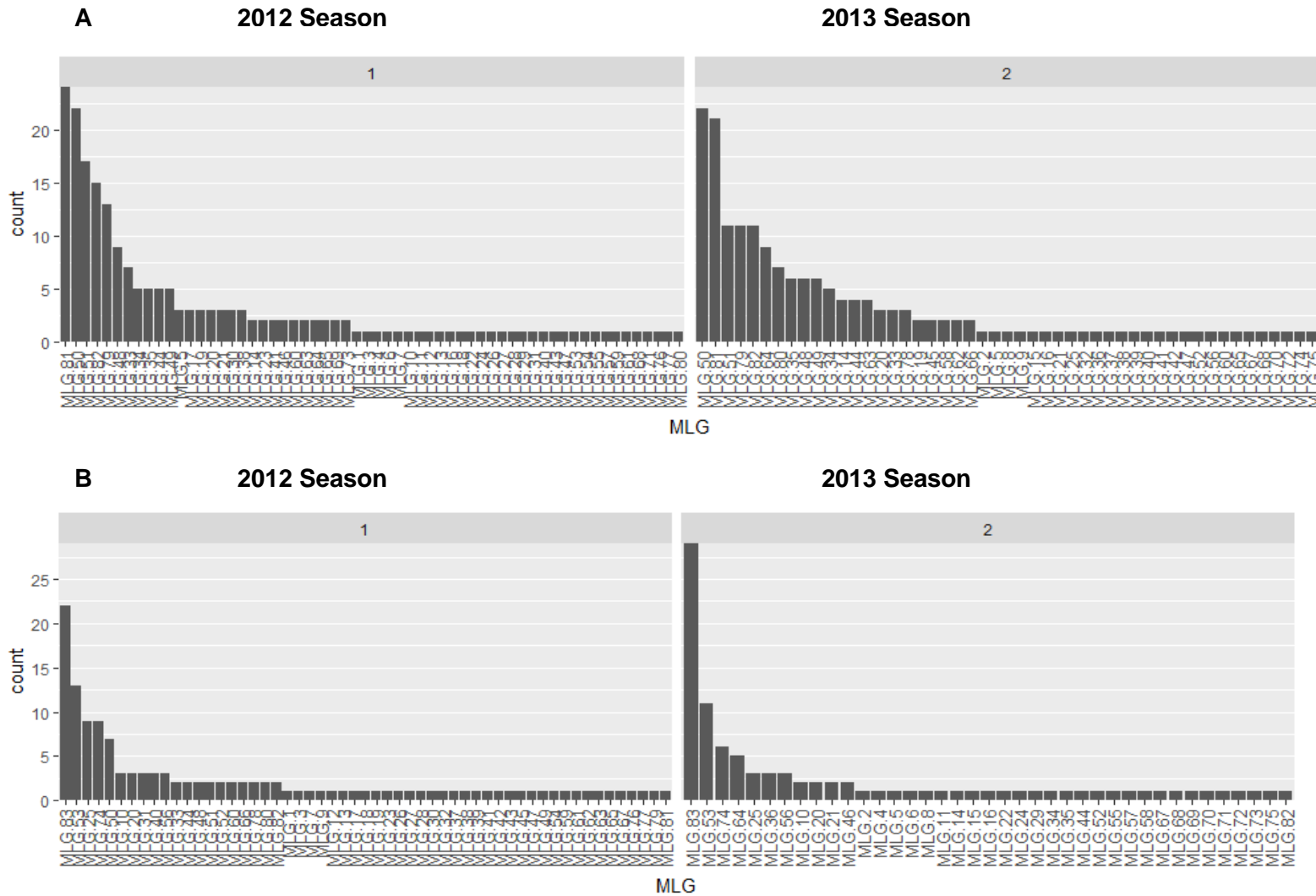
Figure 3. Occurrence of *Phyllosticta citricarpa* multilocus genotypes (MLGs) in two citrus orchards situated in the (A) North West and (B) Mpumalanga provinces in South Africa. In each orchard, the occurrence (count) of MLGs was investigated in the 2012 and 2013 season.
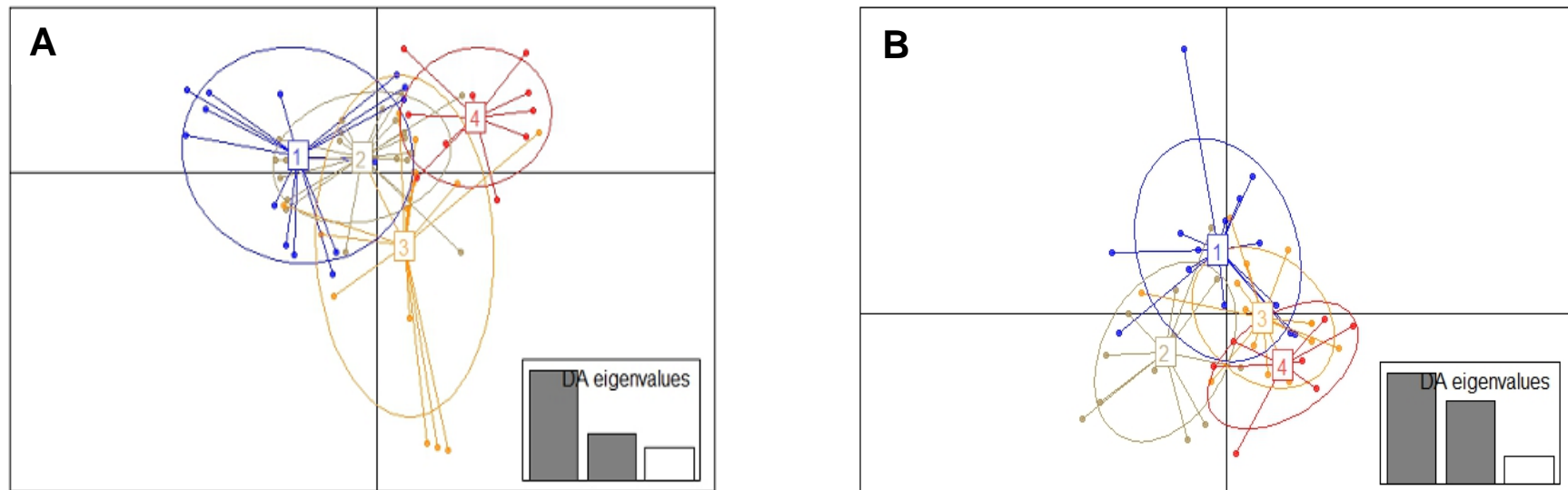
Figure 4. A discriminant analysis of principal components (DAPC) of *Phyllosticta citricarpa* subpopulations (clone corrected) sampled from a lemon orchard in the North West province in the (A) 2012 and (B) 2013 season. Subpopulations are indicted by numbers and different colours. The number of axes retained for the principal component analysis was 16 and 2 for the discriminant analysis. The eigenvalues represented 80% of the variation in both seasons.
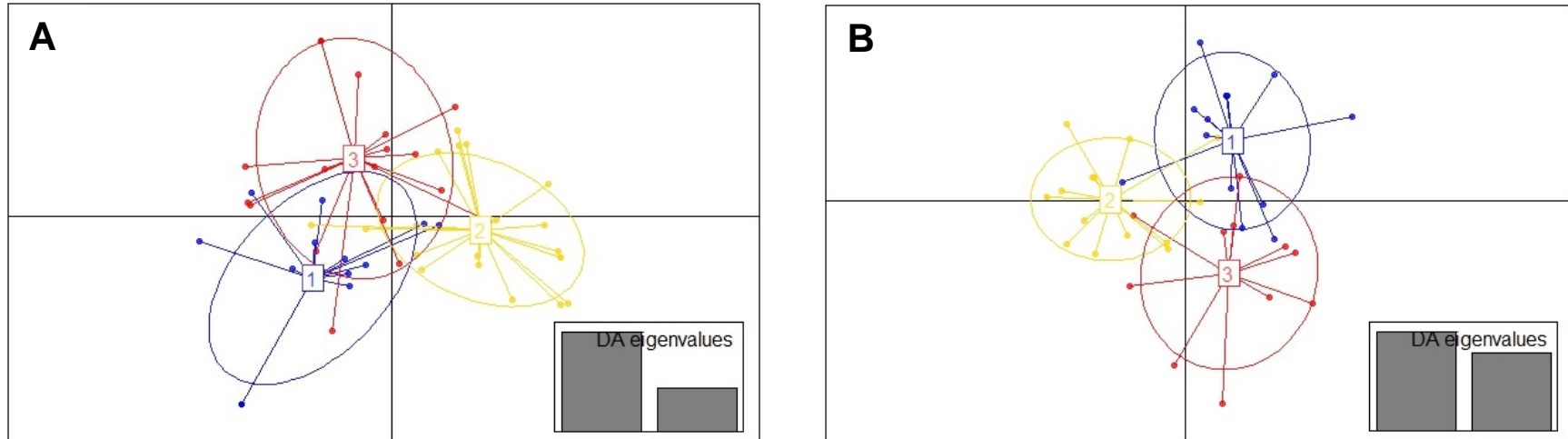
Figure 5. A discriminant analysis of principal components (DAPC) of *Phyllosticta citricarpa* subpopulations (clone corrected) sampled from a lemon orchard in the Mpumalanga province in the (A) 2012 and (B) 2013 season. Subpopulations are indicted by numbers and different colours. The number of axes retained for the principal component analysis was 24 and 2 for the discriminant analysis. The eigenvalues represented 80% of the variation in both seasons.

Supplementary Table 1. Monthly weather conditions prevailing in two regions (North West and Mpumalanga) where *Phyllosticta citricarpa* populations were studied. Weather conditions are summarized according to the fruit infection period (October to February).

| Weather parameter	Month	North West		Mpumalanga	
		2012 season	**2013 season**	**2012 season**	**2013 season**
Total Rainfall (mm)	October	61.98	126.75	117.35	168.89
	November	67.56	76.71	59.95	134.11
	December	129.29	145.8	217.16	201.17
	January	96.52	83.06	264.67	272.3
	February	52.58	44.2	105.91	174.49
Average maximum humidity (%)	October	84.29	88.3	90.91	90.23
	November	84.57	87.49	90.22	90.68
	December	91.18	94.23	90.78	91.32
	January	92.76	90.77	92.23	92.08
	February	90.31	92.24	91.3	92.51
Average maximum temperature (°C)	October	31.33	30.19	27.5	26.67
	November	32.58	31.77	28.91	26.98
	December	30.47	30.37	28.91	28.14
	January	32.67	32.46	28.83	28.95
	February	33.51	34.04	31.31	29.21
Average minimum temperature (°C)	October	11.6	12.42	14.5	15.21
	November	14.39	14.44	16.75	16.12
	December	16.4	16.18	18.4	18.44
	January	16.73	17.43	18.52	18.79
	February	17.02	15.87	19.59	18.54
Average wind speed (ms)	October	0.95	1.06	0.9	0.85
	November	0.94	1.01	0.83	0.96
	December	0.86	0.84	0.58	0.85
	January	0.93	0.84	0.69	0.8
	February	0.79	0.85	0.79	0.8

Supplementary Table 2. Analysis of molecular variance (AMOVA) for *Phyllosticta citricarpa* populations across space between the North West and Mpumalanga orchards using 15 simple sequence repeat loci.

Source	d.f.	SS	% Variation	AMOVA statistics	*P*
2012					
Among populations	1	93.644	48		
Within populations	112	196.391	52	*PhiPT* = 0.479	0.001
Total	**113**	**290.035**	**100**		
2013					
Among populations	1	83.450	54		
Within populations	86	136.459	46	*PhiPT* = 0.543	0.001
Total	**87**	**219.909**	**100**		

CHAPTER 4

Population structure of *Phyllosticta citricarpa* on a regional scale in South Africa, and the influence of *Citrus* species on the population structure

ABSTRACT

Citrus black spot (CBS) caused by *Phyllosticta citricarpa,* is a disease that negatively affects the South African citrus industry. All commercial *Citrus* species are susceptible to the disease, with oranges (*Citrus sinensis*) and lemons (*Citrus limon*) being the most susceptible. The population structure of ten *P. citricarpa* populations were investigated, representing the five citrus producing provinces (North West, Eastern Cape, Mpumalanga, Limpopo and KwaZulu-Natal) in which CBS occurs in South Africa. The effect of *Citrus* spp. (orange vs lemon) on population structure was also investigated. AMOVA analysis showed that most genetic variation (88%) was distributed within populations and only 2% among citrus provinces. Populations from the five provinces were not significantly genetically differentiated ($P = 0.094$). The Eastern Cape was confirmed as being the province into which the last introduction of *P. citricarpa* occurred as was evident from low gene and genotypic diversities of all populations within this province. The KwaZulu-Natal (only one population sampled) and Limpopo provinces had higher gene and genotypic diversities than the North West and Mpumalanga provinces. The Limpopo province had the highest private allele richness, followed by the KwaZulu-Natal province. Therefore, the KwaZulu-Natal or Limpopo provinces are the regions where the pathogen was likely first introduced. There might have been at least two separate introductions of the pathogen into South Africa, based on principal coordinate analyses, pairwise *PhiPT* analyses and the sharing of multilocus genotypes (MLGs) between populations. All ten populations reproduced sexually based on linkage disequilibrium analyses; I_A and $\bar{r}_d$ did not differ significantly from zero in any of the clone corrected populations. Asexual reproduction was evident from low genotype evenness values for some populations, which furthermore indicates clonal reproduction. All orchards had at least one dominant clonal MLG that represented 10% to 48% of the population. Despite lemon trees having overlapping fruit crops, which potentially provide increased opportunities for clonal reproduction, *Citrus* spp. (lemon vs. oranges) did not have an effect on population structure as not all lemon populations were significantly genetically differentiated from all orange populations.

INTRODUCTION

The South African citrus industry was founded in 1654 when the seafarer, Jan van Riebeeck of the Dutch East India Company, planted the first orange trees in the Cape Colony on his farm and in the Company's Garden (Chapot, 1975). Today, the range of citrus types grown in South Africa has expanded to an area of 70 055 ha. About 60% of the crop is sweet oranges (Valencias 38% and navels 22%), 16% soft citrus, 13% lemons and limes and 11% grapefruit. Citrus is produced throughout South Africa, with the Limpopo and Eastern Cape provinces being the largest. South Africa is the tenth largest producer of fresh citrus fruit, and the second largest exporter of fresh citrus fruit. Seventy six percent of the fresh fruit is exported, 18% is for processing and only 6% is consumed locally (CGA, 2017).

International citrus market access is significantly impacted by CBS, since some of South Africa's trade partners are free from the disease. (Paul et al., 2005; Carstens et al., 2012). CBS causes losses on local markets due to fruit symptoms that result in the downgrading of fruit (N. Wentzel, Perishable Product Export Control Board, South Africa, personal communication).

Citrus black spot, caused by *Phyllosticta citricarpa* in South Africa, only occurs in five of the seven citrus producing provinces (KwaZulu-Natal, Mpumalanga, Limpopo, Eastern Cape and North West) (Paul et al., 2005; Carstens et al., 2012). CBS was first detected in 1929 nearby Pietermaritzburg in KwaZulu-Natal (then known as the Natal province), but it only became a disease of concern in 1940 (Doidge, 1929; Wager, 1952). By 1950 the disease was noticed in other areas in KwaZulu-Natal and in other citrus production regions in the North West, Limpopo and Mpumalanga (then known as Western Transvaal, Northern Transvaal and Eastern Transvaal, respectively) (Wager, 1952). In the Eastern Cape, symptoms of the disease was first found in the early 1970's (C. Kellerman, Citrus Consulting Association, personal communication).

In South Africa, the population structure of *P. citricarpa* was investigated by Carstens et al. (2017) using 15 simple sequence repeat (SSRs) markers. The five South African populations investigated (one from each province) were related to each other based on principal coordinate analysis (PCoA) and discriminant analysis of principal components (DAPC). Gene and genotypic analyses of the populations supported the documented history of CBS in South Africa; with the Eastern Cape population having the lowest gene and genotypic diversity, whereas the KwaZulu-Natal population had the highest. The low genetic diversity in populations suggested that the South African populations were founder populations. This is in agreement with the relative young history of CBS in South Africa. The South African populations were sexual and contained both

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mating types required for sexual reproduction of the pathogen. Some of the populations were in linkage disequilibrium, indicating clonal reproduction (Carstens et al., 2017).

All *Citrus* spp. including sweet oranges (*Citrus sinensis*), lemons (*Citrus limon*), grapefruit (*Citrus paradisi*), mandarins (*Citrus reticulata*) and their hybrids are susceptible to *P. citricarpa* (Kiely, 1948). Oranges and lemons are the most susceptible citrus types, with mandarins and grapefruit being less sensitive. According to Kiely (1948) and Kotzé (1981), CBS symptoms will first be noticed on lemons in a new area. Vegetative plant parts can be infected but infections mostly remain asymptomatic and lesions develop mainly on fruit after colour break; however their appearance varies, which has resulted in some confusion regarding their characterization and description. The lesions furthermore differ in the ability to produce pycnidia containing pycnidiospores (Kotzé, 1981, 2000; De Goes et al., 2000; De Goes, 2001; Aguilar-Vildoso et al., 2002; Marques et al., 2012; FAO, 2014). CBS leaf lesions in orchard trees have only been reported for oranges and lemons. The leaf lesions, however, are rare for both host species, more so for oranges than for lemons (Kiely, 1948; Kotzé, 2000; De Oliveira Silva et al., 2017). In all *Citrus* spp., leaves on trees can contain latent infections without any visible symptoms. Pseudothecia, in which sexual ascospores are produced, will only develop and mature once the infected leaves have fallen from the tree onto the orchard floor and following suitable conditions over a period of 40 to 180 days (Kiely 1948; Kotzé, 1981; 2000; Fourie et al., 2013). Pseudothecia have never been recorded from fruit (Kiely, 1948; McOnie, 1964; Kotzé, 1981, 2000). The pathogen can infect living branches asymptomatically (De Oliveira Silva et al., 2017). Symptoms are found on dead twigs, but these are rare (Kiely, 1948).

Worldwide, almost no information is available on how the genetic diversity and population structure of *P. citricarpa* are affected by different *Citrus* spp. or varieties. Only one study was conducted, in Brazil, to determine whether the host type affected the population structure. Populations from different orange varieties were investigated using sequence data of the ITS region. It was concluded that the population structure was not significantly affected by the different varieties (Wickert et al., 2012). However, the ITS region contains almost no polymorphisms in *P. citricarpa* (Guarnaccia et al., 2017), thus confounding population structure inferences.

A recent study in South Africa, investigated *P. citricarpa* populations in two lemon orchards over two seasons. Although populations were reproducing sexually, high clonal reproduction was found in both orchards and over two seasons (Chapter 3). This contradicts the current understanding that pycnidiospores are of minor importance as a contributor to disease development over time and space within orchards under South African climatic conditions and production practices (McOnie, 1964; Kotzé, 1981). Chapter 3 showed that each lemon orchard

contained at least two dominant multilocus genotypes (MLGs) representing 23% to 40% of the populations within each season, and that populations furthermore had low evenness values. It was hypothesized that this phenomenon could be specific to lemons, since lemons, unlike other citrus types, can have up to three fruit sets in one production cycle, and thus can have overlapping fruit crops. This could contribute to higher levels of asexual reproduction (Chapter 3). It is therefore important to further investigate whether *Citrus* spp. will influence the relative importance of asexual reproduction of *P. citricarpa,* and if populations from different *Citrus* spp. differ in genetic variability on an orchard population scale.

The first objectives of the current study was to investigate (i) whether *P. citricarpa* population structure differed in the five provinces in South Africa, (ii) if domninant MLGs were present, (iii) if MLGs were shared between orchards and provinces and (iv) the mode of reproduction in *P. citricarpa* populations. The second objective was to determine if *Citrus* spp. [(specifically *C. sinensis* (oranges) vs. *C. limon* (lemons)] affected the genetic diversity in *P. citricarpa* populations. The ten investigated *P. citricarpa* populations were either newly isolated (four populations) or were from two previous studies conducted in South Africa (Carstens et al., 2017; Chapter 3). Population analyses were conducted using genotyping data obtained from 15 SSR loci (Wang et al., 2016; Carstens et al., 2017). The frequency of the two mating type alleles (MAT *1-1-1* and MAT *1-2-1*) was investigated in all populations. The comparisons of populations from different provinces and their mode of reproduction will allow for a better understanding of the biology of the pathogen, while knowledge of the effects of host species can influence disease management strategies.

MATERIALS AND METHODS

Phyllosticta citricarpa populations used in population genetic analyses

A total of ten *P. citricarpa* populations were investigated to determine if the population structure of the pathogen differed in the five provinces where CBS occurs in South Africa (Table 1; Fig. 1). Four populations were from the Eastern Cape, two from Mpumalanga, two from Limpopo, and one from each of the North West and KwaZulu-Natal provinces. Six of the populations (ECLE2, LIMOR, MPLE, MPOR, KZN1, NW) were from two previous studies (Carstens et al., 2017; Chapter 3), and were isolated between 2011 and 2012. The other four populations (ECL1, ECOR1, ECOR2 and LIMLE) were newly isolated in 2016 (Table 1; Fig. 1). The new populations were obtained by randomly selecting 40 CBS fruits with lesions in each orchard. Only one isolate per fruit was used. Isolation of *P. citricarpa* from CBS lesions using procedures for obtaining pure

cultures and verification of species identity were performed as described by Carstens et al. (2017). In total, 105 isolates were obtained from the four new orchards, which were used in the population analyses (Table 1).

To study the effect of *Citrus* spp. (orange and lemon) on the population structure of *P. citricarpa*, nine populations (20 to 30 isolates per population) were investigated which were also used to compare populations from the different provinces. One lemon and one orange orchard each were included for the Limpopo (LIMLE and LIMOR) and Mpumalanga provinces (MPLE and MPOR), and one lemon population from the North West (NW). In the Eastern Cape, two orange (ECOR1 and ECOR2) and two lemon (ECLE1 and ECLE2) populations were included (Table 1; Fig. 1).

Genotyping of populations

DNA extraction from the four new populations (ECL1, ECOR1, ECOR2 and LIMLE) (Table 1) was done as previously described by Carstens et al. (2017). The fifteen published polymorphic SSR markers (Pc117, Pc179.1, Pc236, Pc440, Pc849, Pc1007, Pc2073, Pc3011, PC12, PC19, PC20, PC32, PC37, PC6, PC7) were used to genotype the populations (Wang et al., 2016; Carstens et al. 2017). Primer labelling as well as PCR reaction and amplification conditions were as previously described (Carstens et al., 2017). Electrophoresis was performed using the 3730XL Genetic Analyzer (Life Technologies) and the SSR alleles were scored using Genemapper software version 4 (Life Technologies). The other six *P. citricarpa* populations were previously genotyped with the 15 SSR markers (Carstens et al., 2017; Chapter 3).

The mating type alleles (MAT *1-2-1* or MAT *1-1-1*) present within all 105 isolates of the four new populations (ECL1, ECOR1, ECOR2 and LIMLE) were determined using published PCR primers (Wang et al., 2016). PCR reaction and amplification conditions and gel electrophoresis were conducted as previously described (Carstens et al., 2017). The 169 isolates from the other six populations (ECL2, LIMOR, MPLE, MPOR, KZN1 and NW) was previously genotyped for the presence of mating type alleles (Carstens et al., 2017; Chapter 3).

SSR data analyses of ten *P. citricarpa* populations

Population genetics

Isolates with the same alleles at all loci were defined as clones or members of the same multilocus genotypes (MLGs). Per population clone-corrected datasets were used for all allele based analyses, since the inclusion of clonal haplotypes in the analysis can distort estimates of allelic diversity (Balloux et al. 2003). GenAlEx version 6.5 (Peakall and Smouse, 2012) was used to

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calculate the following indexes: number of alleles (Na); number of polymorphic loci; effective population size and Nei's measure of gene diversity (H_e) (Nei, 1973).

The number and the expected number (to account for different sample sizes) of MLGs, richness, diversity and evenness (*E5*) of genotypes were examined using the package Poppr in the R software (Kamvar et al., 2014; R Core Team, 2013). To calculate the expected number of MLGs (*eMLG*) for the five provinces, all 10 populations were grouped together. To calculate the expected number of MLGs (*eMLG*) for the *Citrus* spp., the populations were grouped together per province. Evenness is an indication of the relative abundance of a MLG in a population and richness is an indication of how many MLGs in a population (Grünwald et al., 2003; Shannon and Weaver, 1949).

Mode of reproduction

In order to access the level of sexual and asexual reproduction in the populations, analyses were conducted on SSR non-clone-corrected and clone-corrected dataset in the R package Poppr by calculating the index of association I_A and the standardized version of the index of association $\bar{r}_d$. P values were obtained after 999 permutations (Agapow and Burt, 2001). These indices provide an indication of the degree of association of alleles at different loci, within and among populations compared to that observed in a permutated dataset. A value of zero can be expected when there is random association of loci. A value significantly different from zero is an indication of linkage disequilibrium, which is generated when no or infrequent sexual reproduction occurs.

The mating type genotypes of clone corrected populations were used in a chi-square test (Fisher and Yates, 1963). The chi-square test was used to determine whether the populations deviated from the null hypothesis of a 1:1 ratio of the mating types.

***Phyllosticta citricarpa* population structure in the five citrus producing provinces**

The genetic variation within and among populations and within and among production regions (provinces) were investigated using three approaches. Firstly, an analysis of molecular variance (AMOVA) was used. The statistical significance was tested using 999 permutations. Secondly, pairwise *PhiPT* values were determined. Both analyses were performed in GenAlEx version 6.5 (Peakall and Smouse, 2012). Thirdly, to further define and visualize the genetic differences between the populations, a principal coordinates analysis (PCoA) was performed in GenAlEx version 6.5 and a discriminant analysis of the principal components (DAPC) was performed in the R package adegenet (Jombart, 2008).

Effect of *Citrus* spp. on the population structure of *P. citricarpa*

The nine populations obtained from lemon and orange orchards were investigated further using the pairwise *PhiPT* and PCoA analyses to determine whether the structure of *P. citricarpa* populations obtained from these orchards differed.

RESULTS

SSR data analyses of ten *P. citricarpa* populations

Population genetics

The gene diversity of the total Eastern Cape province was significantly lower ($P < 0.001$) ($H_e = 0.113$; SE = 0.024) than the other provinces (Table 2). The populations within the Eastern Cape had the lowest gene diversity of all the populations ($H_e = 0.106$ to 0.123; SE = 0.052 to 0.053). The gene diversity for the Limpopo, Mpumalanga and North West populations were 0.198, 0.174 and 0.179, respectively, with Mpumalanga differing significantly ($P = 0.002$) from KwaZulu-Natal (Table 2). Private allele richness was the highest in the Limpopo, which contained six private alleles, followed by KwaZulu-Natal with three private alleles; the other provinces had one to two private alleles (Table 2).

Among the total South African isolates (274), the 15 markers identified 89 MLGs. The number of MLGs and *eMLGs* varied between the provinces. The number of MLGs were rarefied to the smallest population size of 20 for all 10 populations. This showed that one of the populations from the Limpopo had the highest *eMLG* value (LIMLE - *eMLG* = 16. 2, SE = 1.147). The four populations from the Eastern Cape all had the lowest *eMLG* values (4.60 to 7.70; SE = 0.00 to 0.736) (Table 3).

The genotypic diversity for the provinces was the highest in one of the populations from the Limpopo ($D = 0.944$) and the lowest for the four populations from the Eastern Cape ($D = 0.682$ to 0.817), in comparison to the populations from the other provinces ($D = 0.880$ to 0.944). Combined, the South African populations had a high genotypic diversity ($D = 0.947$) (Table 3).

Mode of reproduction

In the analysis of the 10 populations, the association tests only differed significantly from zero in two of the non-clone corrected data sets; one orchard each in the Eastern Cape (I_A and $\bar{r}_d$; $P = 0.016$ and $P = 0.015$) and Mpumalanga (I_A and $\bar{r}_d$; $P = 0.039$). All ten South African populations that were non-clone corrected, deviated significantly from zero (I_A and $\bar{r}_{d;}$ $P = 0.002$). In all the

other non-clone corrected and all the clone corrected data sets, the I_A and $\bar{r}_d$ did not differ significantly from zero (Table 4).

All the isolates analysed gave a positive result following PCR amplification with either one of the mating type primer pairs. The mating-type frequencies were found not to deviate significantly ($P > 0.050$) from a 1:1 ratio in nine of the populations. In one Limpopo population (LIMLE), mating type frequencies deviated significantly ($P = 0.050$) from a 1:1 ratio based on a Chi-square analyses (Table 5).

The genotypic evenness for each province varied, as well as for populations within a province. Half of the populations had relatively high genotype evenness values ($E5 \geq 0.789$), whereas evenness was slightly lower ($E5 = 0.746$ to 0.670) for the rest of the populations. The Eastern Cape and populations within this province had the lowest evenness values, with the Limpopo having the highest. The total South African population had a low genotypic evenness ($E5 = 0.461$) (Table 3).

Prevalence of dominant MLGs within orchards of different citrus producing provinces

Frequent sharing of MLGs was evident among the populations. All the populations, except the populations from KZN1 (KwaZulu-Natal) and ECLE2 (Eastern Cape), shared MLG 68. MLG 68 was furthermore the dominant MLG in four populations from the Eastern Cape and Mpumalanga (ECLE1, ECOR, MPLE, MPOR), or it occurred at least twice or more in three other populations from the Eastern Cape and Limpopo (ECOR2, LIMLE, LIMOR). MLG 68 occurred only once in the North West population (NW). Another MLG frequently shared between populations was MLG 62; it occurred in all populations except the ECLE2 and LIMOR populations (Fig. 2). The ECLE2 population shared several MLGs with the KZN1 population (MLG31, 41, 38, 50), the NW population (MLG 40, 31, 41), one of the Limpopo populations (LIMOR; MLG 38, 41 and 40) and one of the Mpumalanga populations (MPLE; MLG 41) (Fig. 2).

In nine populations, one or two MLGs were dominant; the exception was the LIMLE population where all MLGs were almost equally abundant, which was evident from this population having the highest evenness value ($E5 = 0.894$) (Table 3; Fig. 2). In each of the nine populations, the most dominant MLG represented 13% to 48% of each orchard population.

***Phyllosticta citricarpa* population structure in the five citrus producing provinces**

The main source of genetic variation (88%) when comparing the ten populations was attributed to differences within the populations of the five provinces (*PhiPT* = 0.117; *P* = 0.001). Significant genetic differentiation (10%) was also found among the ten populations (*PhiPR* = 0.100; *P* =

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0.001). There was no significant genetic differentiation among populations of the different provinces (*PhiRT* = 0.020; *P* = 0.094) (Table 6).

For the PCoA analysis, the axes explained 82.19% of the variation for Coordinate 1 and 8.66% for Coordinate 2. Two groups were evident from the PCoA analyses among the 10 populations. The one group contained two populations, KZN1 and ECLE2 from KwaZulu-Natal and Eastern Cape, respectively, and the second group contained the other eight populations (Fig. 3). This was evident from DAPC analyses, although DAPC included the NW population with the KZN1 and ECLE2 populations (Fig. 4). Pairwise *PhiPT* analyses showed that there was significant and high levels of genetic differentiation between the KZN1 and ECLE2 populations and seven of the other populations; the exception was the non-significant, but lower level of genetic differentiation between the KZN1 and ECLE1 populations (*PhiPT* = 0.111; *P* = 0.058). The NW population was significantly differentiated from the KZN1 population (*PhiPT* = 0.061; *P* = 0.015), but not from the ECLE2 population (*PhiPT* = 0.042; *P* = 0.182). The two populations KZN1 and ECLE2, were not significantly differentiated from each other (*PhiPT* = 0.036; *P* = 0.214) (Table 7). Specific differences between the remaining populations on the influence of *Citrus* spp. on population structure, will be discussed in the section below.

Effect of *Citrus* spp. on the population structure of *P. citricarpa*

Since AMOVA and PCoA analyses showed there was no significant difference between regions (provinces) (Table 6; Fig. 3), lemon and orange populations were compared, irrespective of their province of origin. Pairwise *PhiPT* comparisons of lemon and orange populations showed that there were 13 highly significant differences (*P* ≤ 0.009) between and within lemon and orange populations. These differences were not always due to host differences (Table 7). The first two highly significant differences were between an orange and lemon population (MPOR and LIMLE; *PhiPT* = 0.068; *P* = 0.007) and between two orange populations (LIMOR and MPOR; *PhiPT* = 0.118; *P* = 0.007). The remaining highly significant difference (*P* ≤ 0.009) involved the Eastern Cape lemon population ECLE2 and the North West lemon population, which was already mentioned above in the comparison of the provinces. The lemon orchard ECLE2 was responsible for six of the 13 highly significant different combinations (*PhiPT* = 0.389 to 0.494; *P* ≤ 0.009). It differed significantly from three orange populations (ECOR1, ECOR2, MPOR), and from three lemon populations (ECLE1, LIMLE, MPLE) based on pairwise *PhiPT* values (Table 7). The NW lemon population was responsible for five of the 13 highly significant differences between populations (*PhiPT* =0.100 to 0.250; *P* ≤ 0.009). It differed significantly from two lemon populations (LIMLE, MPLE) and three orange populations (ECOR1, ECOR2, MPOR). The other

significant differences between and within lemon and orange populations had a low level of significance and *PhiPT* values (P = 0.014 to 0.045; *PhiPT* = 0.116 to 0.168).

The evenness values in lemon and orange populations were comparable. The lemon populations ranged between 0.735 and 0.894 and orange populations ranged between 0.670 and 0.812. The *eMLG* values between orange and lemon populations were comparable. For example in the Eastern Cape the two lemon populations (ECLE1 and ECLE2) had *eMLG* values of 4.60 and 6.00, whereas the two orange populations (ECOR1 and ECOR2) had *eMLG* values of 6.20 and 7.70 (Table 3).

As previously mentioned, each orchard contained one or two dominant MLGs (Fig. 2). There was no trend for the dominant MLG in the lemon orchards being higher than in the orange orchards. A good example is evident from the Eastern Cape province where in the orange populations (ECOR1 and ECOR2) the dominant MLG represented 30% to 48% of the populations, and in the lemon orchards (ECLE1 and ECLE2), it presented 40% to 45% of the populations (Fig. 2).

DISCUSSION

Findings from the current study support those from previous studies with regards the predominant modes of reproduction of *P. citricarpa*, provide insight into potential introductions of the pathogen into South Africa, supports the known history of introductions and is the first study to report on the effect of *Citrus* spp. on *P. citricarpa* population structure. At least two separate introductions of the pathogen likely occurred into South Africa. The pathogen was found to reproduce sexually and asexually, with a high level of clonal reproduction occurring in some populations. *Citrus* spp. did not affect population structure, which was evident from the fact that not all lemon populations were genetically significantly differentiated from orange populations.

All population genetic studies conducted to date in South Africa (Carstens *et al.*, 2017; Chapter 2), including the current study, have found that, although *P. citricarpa* reproduces sexually (based on linkage disequilibrium analyses in clone correct populations analyses [I_A and $\bar{r}_d$ do not differ significantly from zero]), some non-clone corrected populations showed linkage of alleles, which highlights the predominance of clonal reproduction. Asexual and clonal reproduction, evidenced by low genotype evenness values, was identified in the current study. This was previously reported in *P. citricarpa* populations in South Africa (Chapter 3; Carstens et al., 2017). The ability of *P. citricarpa* to persist clonally was discussed in detail in Chapter 3. In the current study, clonal reproduction was quite prominent in some orchards where the dominant MLG represented 30 to 48% of the population in four of the ten studied populations.

In the current study it is reported for the first time that a *P. citricarpa* clone corrected population (LIMLE from the Limpopo province) can have a mating type ratio that deviates significantly from 1:1, with mating type being skewed towards the MAT *1-2-1* allele. This skewed mating type distribution in the LIMLE population further rejects the assumption that the pathogen mainly reproduces sexually, and provides strong support for a mixed reproduction system of the pathogen within populations. Interestingly, the LIMLE population was the population that showed the lowest degree of clonal reproduction (two dominant MLGs each only represented 10% of population), which was evident from the high genotype evenness ($E5$ = 0.894). In previous studies, the mating type ratios in all of the studied clone corrected populations never deviated significantly from a 1:1 ratio (Amorim et al., 2017, Carstens et al., 2017; Chapter 3; Tran et al., 2017).

In the current study, the population structure of the pathogen in the different provinces mostly correlated with the known history of introductions into South Africa. The low overall gene diversity for the ten populations corresponded with the finding of Carstens et al. (2017) and supports the relatively recent introduction of *P. citricarpa* into South Africa (Doidge, 1929). Carstens et al. (2017) suggested that the latest introduction of the pathogen into South Africa occurred in the Eastern Cape, based on the analyses of a single population from this province, since the population had the lowest level of gene and genotypic diversity. The current study included an additional three populations from the Eastern Cape, which further supported this province as being the province where the latest introduction of CBS occurred. This correlates with historical records of the disease first becoming evident in the 1970s in the Eastern Cape (Kellerman, personal communication). The four populations from the Eastern Cape had the lowest gene and genotypic diversities of all the investigated populations, and the second lowest number of private alleles. Carstens et al. (2017) suggested that KwaZulu-Natal was the province where the first introduction occurred into South Africa (population had the highest level of gene and genotypic diversity), which concurs with historical records of the first detection of the disease being prior to 1929 (Doidge, 1929; Wager, 1952). However, in the current study, when the same KwaZulu-Natal population from Carstens et al. (2017) was analysed along with an additional population from the Limpopo province (LIMLE), this was no longer clear. The LIMLE population had similar high gene- and genotypic diversities as the KwaZulu-Natal population. Furthermore, the Limpopo had the highest private allele richness followed by KwaZulu-Natal. Therefore, both of these provinces could have been the place of first introduction into South Africa. According to Wager (1952), CBS was first reported in 1945 from a different province in South Africa, namely the Limpopo province (previously known as the Northern Transvaal).

Two separate introductions of *P. citricarpa* likely occurred into South Africa. PCoA and DAPC analyses, which is based on genetic distances between MLGs, suggested the presence of at least two groups among the South African populations. The one group contained a population from the Eastern Cape (ECLE2) and the population from KwaZulu-Natal (KZN1), and the second group contained the remaining eight populations from the other provinces. The two groups were supported by pairwise *PhiPT* comparisons, and to some extent by DAPC analyses. DAPC analyses grouped the NW population with the ECLE2 and KZN1 populations. No or limited connectivity was evident for the ECLE2 and KZN1 populations with most of the other eight populations. High connectivity, however, was evident between the ECLE2 and KZN1 populations. The ECLE2 and KZN1 populations were further unique in that they did not contain MLG68, which was a dominant MLG in four of the other populations and also occurred in the remaining four populations at least twice. In the Eastern Cape, more than one introduction likely occurred, one consisting of the ECLE2 population, whereas the other three Eastern Cape populations likely represent a different introduction. The fact that introductions were not province specific, was evident from AMOVA analysis which showed that only 2% of the variation was attributed to differences among provinces and that there were no significant differentiation between the provinces ($PhiRT = 0.020$; $P = 0.093$) from where the populations were sampled. The differences among the isolates within the populations were the highest (88%) ($PhiPT = 0.117$; $P = 0.001$).

The current study is the first to conduct a relatively extensive study on the population structure of *P. citricarpa* on a regional scale within a country, i.e. South Africa. This has been done to a limited extent in Brazil, China, Australia and the United States. The first studies in Australia and Brazil, however, were restricted by the molecular markers used. Populations from different regions within Brazil (Wickert et al., 2012) and Australia (Miles et al*.,* 2013) were reported to have low genetic diversity based on sequence data of the internal transcribed spacer (ITS) region. The ITS region is known to contain almost no polymorphisms in *P. citricarpa* populations (Guarnaccia et al., 2017). Carstens et al*.* (2017), using the same 15 SSRs markers used in the current study, analysed two and three populations from China and Australia, respectively. The three Australian populations were related to each other based on PCoA and DAPC analyses (Carstens et al*.,* 2017). In Florida (USA), populations from two different counties were found to contain only one mating type and was clonal based on 13 SSR markers (Wang et al., 2016). The clonality of the Florida populations, was subsequently confirmed by Carstens et al*.* (2017) using additional SSR markers.

Citrus host species did not affect *P. citricarpa* population structure in South African orchards based on the analyses of nine populations (five lemon and four orange populations)

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representing four provinces. Pairwise *PhiPT* comparisons of lemon and orange populations showed that significant genetic differences were not always due to host differences. Lemon populations were furthermore often significantly differentiated from each other, as reported in Chapter 3. Furthermore, PCoA and DAPC analyses showed high levels of connectivity between several of the orange and lemon populations. Clonal reproduction was not more pronounced in lemon populations than orange populations. For example, in a lemon and orange orchard in the Eastern Cape the same dominant MLG (MLG68) represented 40 and 48% of each population respectively. Clonal reproduction was evident from the fact that genotype evenness values were comparable between lemon and orange populations.

The current study on the population structure of *P. citricarpa,* provides further insights into historical introductions of the pathogen into South Africa. The data supports the known history of the pathogen in South Africa. However, to clarify the results further, additional populations from the five provinces need to be analysed to determine whether KwaZulu-Natal or Limpopo was the first place of introduction. Data from the additional populations will indicate whether the South African belief is true that CBS was spread from KwaZulu-Natal, via planting material from a commercial nursery, to other provinces (Kotzé, 1996) and whether there was more than one introduction into a province from different nurseries. The study further supported the importance of the mixed reproductive system (sexual and asexual) of the pathogen, along with the prominence of clonal reproduction and that clonal reproduction is not confined to lemons, but also occur in oranges. The current study is the first to show that *Citrus* spp., specifically oranges and lemons, apparently do not have an effect on *P. citricarpa* population structure. Future studies should investigate the effect of other citrus types on *P. citricarpa* population structure.

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Table 1. Origin of *Phyllosticta citricarpa* populations used to study differences in the population structure of *P. citricarpa* in five provinces in South Africa, and the effect of *Citrus* spp. on the population structure of the pathogen.

Population name[a]	Province	Citrus Type	Number isolates (*N*)	Year of isolation	Source[a]
ECLE1	Eastern Cape	Lemons	25	2014	Current study
ECLE2	Eastern Cape	Lemons	20	2012	Carstens et al. 2017
ECOR1	Eastern Cape	Oranges	27	2016	Current study
ECOR2	Eastern Cape	Oranges	23	2016	Current study
LIMLE	Limpopo	Lemons	30	2016	Current study
LIMOR	Limpopo	Oranges	30	2011	Carstens et al. 2017
MPLE	Mpumalanga	Lemons	30	2012	Chapter 3
MPOR	Mpumalanga	Oranges	29	2011	Carstens et al. 2017
KZN1	Kwazulu-Natal	Grapefruit	30	2011	Carstens et al. 2017
NW	North West	Lemons	30	2012	Carstens et al. 2017

[a] Carstens et al. 2017. A global perspective on the population structure and reproductive system of *Phyllosticta citricarpa.* Phytopathology 107: 758-768. Chapter 3 from the PhD dissertation of E. Carstens, Stellenbosch University.

Table 2. Number of alleles, private alleles, multilocus genotypes and gene diversity of 15 microsatellite loci in ten clone corrected *Phyllosticta citricarpa* populations from five provinces (Eastern Cape, Limpopo, Mpumalanga, Kwazulu-Natal and the North West) in South Africa.

Populations within provinces	#Isolates	# MLGs	# Alleles (Private alleles)															Ne	$H_e{}^a$
			Pc 117	Pc 179.1	Pc 236	Pc 440	Pc 849	Pc 1007	Pc 2073	Pc 3011	Pc 12	Pc 19	Pc 20	Pc 32	Pc 37	Pc 6	Pc 7		
Eastern Cape																			
ECLE1	25	5	2	1	1	1	1	2	2	2	2	1	1	1	1	1	1	1.19	0.117
ECLE2	20	6	1	1	1	2	1	1	2	2	1	1	1	1	1	2	2	1.17	0.107
ECOR1	27	7	3(1)	1	1	1	1	2	1	2	1	1	2	1	1	1	1	1.21	0.106
ECOR2	23	8	4(1)	2	1	1	1	2	2	2	1	1	1	1	1	1	1	1.25	0.123
Total	**95**	**26**	**5**	**2**	**1**	**2**	**1**	**2**	**2**	**2**	**2**	**1**	**2**	**1**	**1**	**2**	**2**	**1.20**	**0.113**
Limpopo																			
LIMLE	30	22	5(3)	2	3(2)	3	1	2	2	2	1	2	1	1	2	2	2	1.36	0.206
LIMOR	30	15	5(1)	2	1	3	1	2	2	2	1	2	1	1	1	2	2	1.33	0.190
Total	**60**	**37**	**8**	**2**	**3**	**3**	**1**	**2**	**2**	**2**	**1**	**2**	**1**	**1**	**2**	**3**	**2**	**1.35**	**0.198**
Mpumalanga																			
MPLE	30	16	4(1)	2	1	3	1	2	2	2	2	2	1	1	2	3	2	1.31	0.196
MPOR	29	14	3	1	1	3	1	2	2	2	1	2	1	1	2	1	1	1.25	0.153
Total	**59**	**30**	**5**	**2**	**1**	**3**	**1**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**2**	**3**	**2**	**1.28**	**0.174**
Kwazulu-Natal																			
KZN1	30	19	2(1)	1	1	2	1	2	2	2	2(1)	2	2	2(1)	1	2	2	1.34	0.202
North West																			
NW	30	20	4(1)	2	1	2	1	2(1)	2	2	1	1	1	1	1	2	2	1.31	0.179
Total (all provinces)	**274**	**132**	**14**	**2**	**3**	**3**	**1**	**3**	**2**	**2**	**3**	**2**	**2**	**2**	**2**	**3**	**2**	**1.27**	**0.158**

a = Nei's gene diversity, H_e (Nei, 1973); *Ne* - effective population size

TABLE 3. Summary of genotypic diversity indices for ten populations of *Phyllosticta citricarpa* from five provinces (Eastern Cape, Limpopo, Mpumalanga, KwaZulu-Natal and North West) in South Africa.

Populations within provinces	#Isolates	#MLGs[a]	Populations per province		All populations		E5[d]	D[e]
			eMLG[b]	SE[c]	eMLG	SE		
Eastern Cape								
ECLE1	25	5	4.60	0.554	4.60	0.554	0.819	0.682
ECLE2	20	6	6.00	0.000	6.00	0.000	0.735	0.710
ECOR1	27	7	6.20	0.736	6.20	0.736	0.670	0.705
ECOR2	23	8	7.70	0.489	7.70	0.489	0.812	0.817
Total	**95**	**18**	**9.25**	**1.462**			**0.679**	**0.864**
Limpopo								
LIMLE	30	22	22.0	0.00	16.2	1.147	0.894	0.944
LIMOR	30	15	15.0	0.00	11.7	1.183	0.712	0.880
Total	**60**	**36**	**21.8**	**1.75**			**0.735**	**0.953**
Mpumalanga								
MPLE	30	16	15.7	0.471	12.3	1.209	0.738	0.893
MPOR	29	14	14.0	0.000	11.4	1.101	0.746	0.880
Total	**59**	**26**	**16.4**	**1.730**			**0.596**	**0.903**
Kwazulu-Natal								
KZN1	30	19	-	-	14.1	1.249	0.789	0.920
North West								
NW	30	20	-	-	14.7	1.237	0.820	0.929
TOTAL (ALL)	**274**	**89**	**-**	**-**	**14.4**	**1.840**	**0.461**	**0.947**

[a]MLGs = Multilocus genotype, [b]eMLG = expected number of MLGs after rarefraction, [c]SE = Standard error based on *eMLG*,[d] *E5* = Evenness
[e] *D* = Genotypic diversity

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TABLE 4. Linkage disequilibrium analyses for populations of *Phyllosticta citricarpa* from five provinces (Eastern Cape, Limpopo, Mpumalanga KwaZulu-Natal and North West) in South Africa with corresponding significance level (*P*-value).

Province and population names	#Isolates	#MLGs	Non-clone-corrected populations				Clone-corrected Populations			
			I_A	*P* value	$\bar{r}_d$	*P* value	I_A	*P* value	$\bar{r}_d$	*P* value
Eastern Cape										
ECLE 1	25	5	0.393	0.016	0.110	0.015	-0.200	0.615	-0.050	0.614
ECLE 2	20	6	-0.081	0.657	-0.021	0.653	-0.472	0.940	-0.118	0.936
ECOR 1	27	7	0.034	0.319	0.013	0.320	-0.190	0.718	-0.064	0.706
ECOR 2	23	8	0.031	0.362	0.008	0.364	-0.230	0.825	-0.058	0.818
Limpopo										
LIMLE	30	22	0.01083	0.414	0.001	0.412	-0.106	0.741	-0.011	0.741
LIMOR	30	15	0.00014	0.488	0.000	0.448	-0.231	0.905	-0.030	0.902
Mpumalanga										
MPLE	30	16	0.3156	0.039	0.033	0.039	-0.033	0.525	-0.003	0.524
MPOR	29	14	-0.0789	0.722	-0.013	0.720	-0.316	0.987	-.0053	0.984
KwaZulu-Natal										
KZN1	30	19	0.1855	0.092	0.020	0.092	-0.034	0.565	-0.004	0.565
North West										
NW	30	20	-0.0559	0.646	-0.008	0.645	-0.213	0.962	-0.031	0.961
Total (All provinces)	**274**	**89**	0.134	0.002	0.013	0.002	**-0.082**	**0.928**	**-0.007**	**0.935**

MLGs = Multilocus genotype, *eMLG* = expected number of MLGs after rarefraction, I_A = Index of Association; $\bar{r}_d$ = Standardized index of association

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TABLE 5. Mating type composition and χ^2 testing for expected 1:1 mating-type ratios in clone corrected *Phyllosticta citricarpa* populations collected in five provinces (Eastern Cape, Mpumalanga, North Wes, KwaZulu-Natal and Limpopo) in South Africa.

Province/Type	Number of A1 mating type isolates	Number of A2 mating type isolates	Number of MLGs[a]	χ^2 value	*P*-value
Eastern Cape					
ECLE1	1	4	5	2	0.200
ECLE2	4	2	6	0.67	0.500
ECOR1	4	3	7	0.29	0.700
ECOR2	3	5	8	0.50	0.500
Mpumalanga					
MPLE	8	8	16	0	0.950
MPOR	7	7	14	0	0.950
Limpopo					
LIMLE	6	16	22	4.54	0.050
LIMOR	6	10	16	1	0.700
KwaZulu-Natal					
KZN1	11	8	19	0.47	0.500
North West					
NW	6	14	20	3.2	0.100

[a] The total number of Multilocus genotypes (MLG) per population in clone corrected *Phyllosticta citricarpa* populations.

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TABLE 6. Analysis of molecular variance (AMOVA) comparing *Phyllosticta citricarpa* populations from five citrus production regions (Eastern Cape, Mpumalanga, Limpopo, KwaZulu-Natal and North West provinces) in South Africa using 15 simple sequence repeat loci.

Source	d.f.	SS	% Variation	AMOVA statistics	*P*
Among regions	4	18.950	2	*PhiRT* = 0.020	0.094
Among populations	5	15.235	10	*PhiPR* = 0.100	0.001
Within populations	122	172.717	88	*PhiPT* = 0.117	0.001
Total	**131**	**206.902**	**100**		

TABLE 7. Estimates of pairwise *PhiPT* values (below the diagonal) with significance values (above the diagonal) averaged over 15 simple sequence repeat loci of 10 *Phyllosticta citricarpa* populations obtained from five provinces (Eastern Cape, Limpopo, KwaZulu-Natal, North West and Mpumalanga) in South Africa.

Populations[a]	ECOR 1	ECOR 2	ECLE1	ECLE2	KZN	LIMLE	LIMOR	MPLE	MPOR	NW
ECOR1	-	0.422	0.395	0.002	0.001	0.154	0.026	0.147	0.259	0.002
ECOR2	0.000	-	0.406	0.001	0.001	0.055	0.026	0.116	0.452	0.001
ECLE1	0.000	0.000	-	0.009	0.058	0.343	0.170	0.377	0.412	0.014
ECLE2	0.494	0.476	0.389	-	0.214	0.001	0.045	0.003	0.001	0.182
KZN	0.247	0.258	0.111	0.036	-	0.001	0.026	0.001	0.001	0.015
LIMLE	0.037	0.063	0.010	0.231	0.149	-	0.196	0.429	0.007	0.002
LIMOR	0.120	0.126	0.056	0.116	0.070	0.017	-	0.290	0.007	0.149
MPLE	0.039	0.046	0.000	0.222	0.096	0.000	0.010	-	0.030	0.004
MPOR	0.029	0.000	0.007	0.410	0.255	0.086	0.118	0.061	-	0.001
NW	0.230	0.242	0.168	0.042	0.061	0.100	0.029	0.098	0.250	-

[a] Population names starting with "EC" were from the Eastern Cape, "KZN" from KwaZulu-Natal, "NW" from the North West , "LIM" from Limpopo and "MP" from Mpumalanga. Populations that were sampled from lemon orchards included ECLE1, ECLE2, LIMLE, MPLE and NW. The KZN population was obtained from a grapefruit orchard. The remaining populations were obtained from orange orchards.
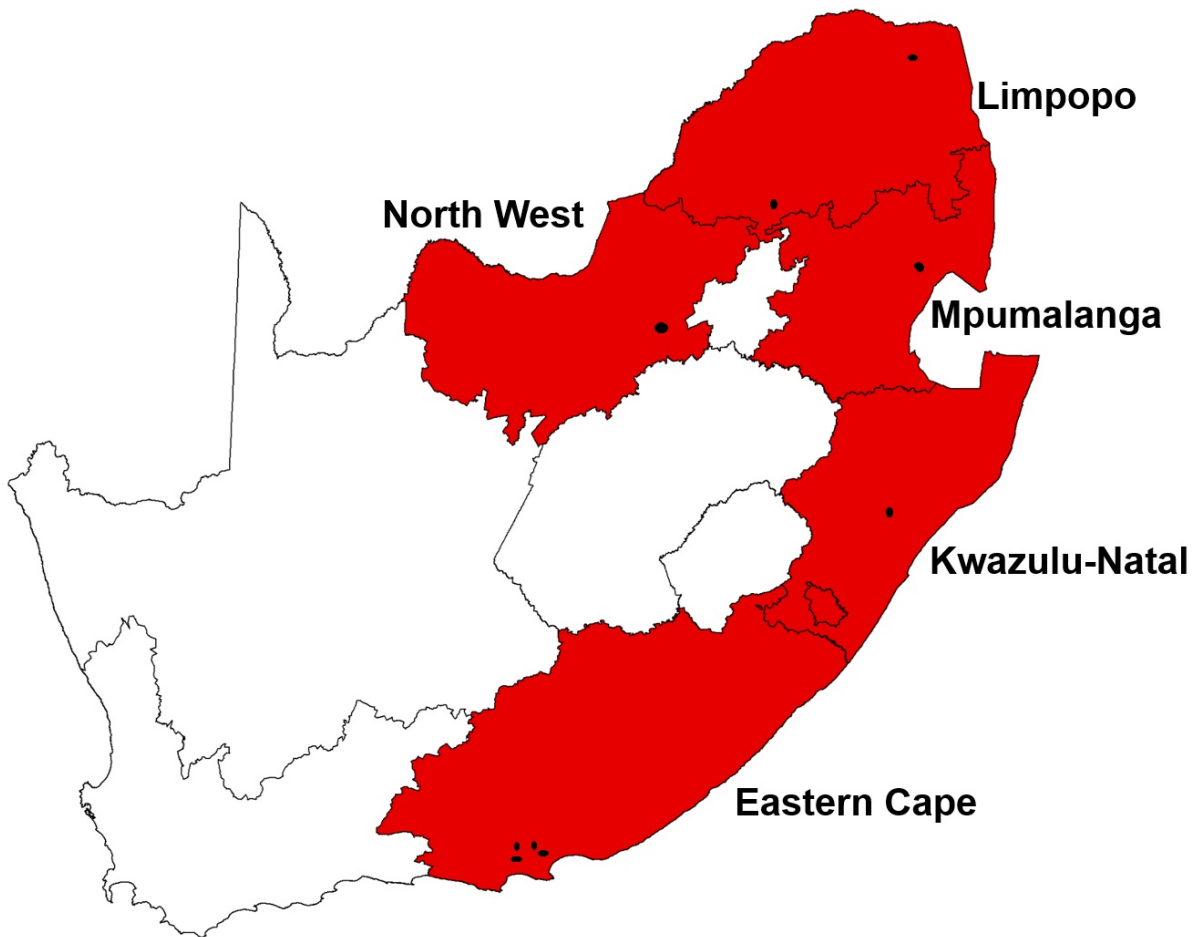
Figure 1. Five provinces (North West, Limpopo, Mpumalanga, KwaZulu-Natal and Eastern Cape) in South Africa where *Phyllosticta citricarpa* populations were sampled from *Citrus* spp. orchards. The populations were used in studies to determine if *P. citricarpa* populations differed in the provinces and if *Citrus* spp. had an effect on population structure.
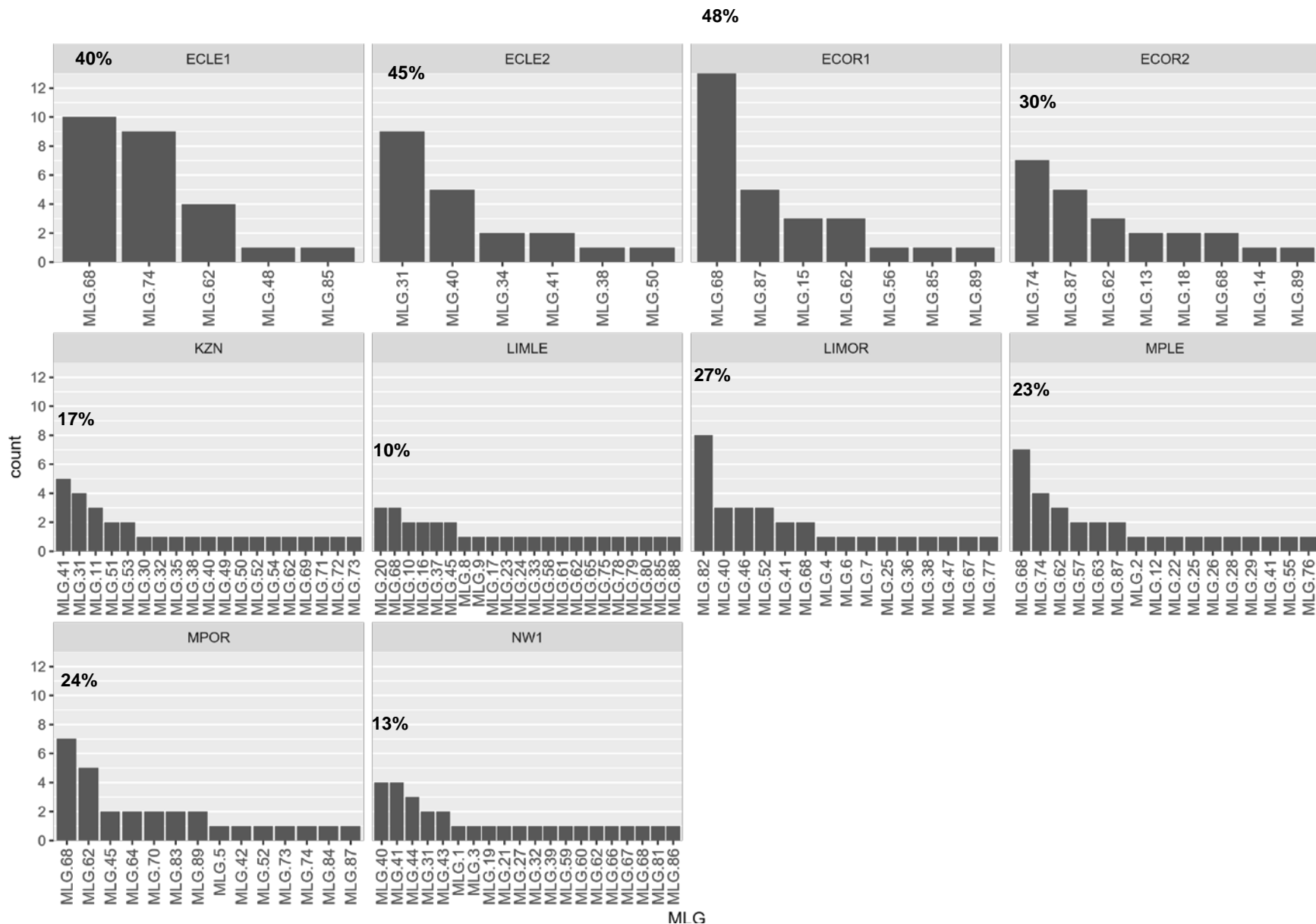
Fig 2. Occurrence of *Phyllosticta citricarpa* multilocus genotypes (MLGs) in ten South African orchards. Above the histogram bars of the dominant MLGs, the percentage is indicated that the dominant MLG represented of the orchard's total population.
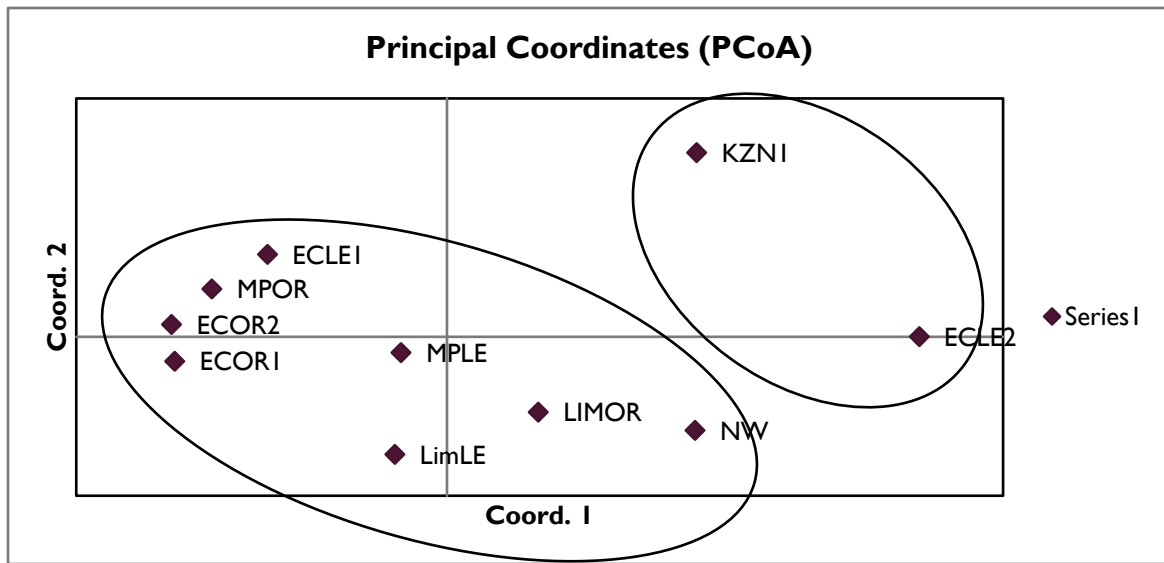
Figure 3. Principal coordinate analysis (PCoA) for ten *Phyllosticta citricarpa* populations collected in five provinces in South Africa, including the North West (NW), Mpumalanga (MPLE, MPOR), Eastern Cape (ECOR1, ECOR2, ECLE2 and ECLE1) and KwaZulu-Natal (KZN). Populations sampled from lemon orchards included ECLE1, ECLE2, LIMLE, MPLE and NW. The KZN population was obtained from a grapefruit orchard. The remaining populations were obtained from orange orchards. The axes explained 82.19% variation for Coordinate 1 and 8.66% for Coordinate 2.
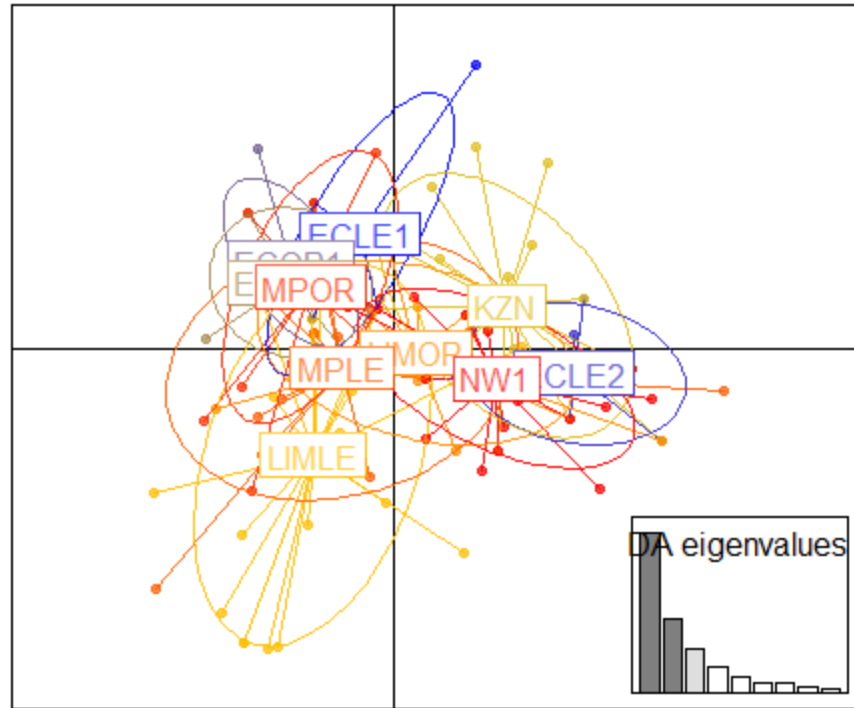
Figure 4. A discriminant analysis of principal components (DAPC) of *Phyllosticta citricarpa* populations (clone corrected) sampled from five provinces in South Africa, including the North West (NW), Mpumalanga (MPLE, MPOR), Eastern Cape (ECOR1, ECOR2, ECLE2 and ECLE1) and KwaZulu-Natal (KZN). Populations sampled from lemon orchards included ECLE1, ECLE2, LIMLE, MPLE and NW. The KZN population was obtained from a grapefruit orchard. The remaining populations were obtained from orange orchards. Populations are indicated by different colours. The number of axes retained for the principal component analysis was 17 and 3 for the discriminant analysis. The eigenvalues chosen represented more than 80% of the total variation.

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CHAPTER 5

Conclusion

Citrus black spot is a fungal disease caused by *P. citricarpa* that influences global citrus production and trade. The epidemiology of *P. citricarpa* has been studied in many countries where the disease is present, but limited information is available on the pathogen's population genetic structure. Markers available to conduct population genetic studies have identified very low or no polymorphisms in *P. citricarpa* populations (Wang et al*.,* 2016). The reproductive system of *P. citricarpa* was only recently resolved, when the mating type locus containing the MAT *1-1-1* or MAT *1-2-1* genes were identified (Wang et al., 2016, Amorim et al., 2017) and when the mating of opposite mating type isolates was achieved under artificial culture conditions (Tran et al., 2017). The aims of this dissertation were to develop more informative markers to determine the distribution of genetic variation in global and South African *P. citricarpa* populations, to investigate the reproductive mode of the pathogen on an international and national scale and to determine the effect of distance (orchard scale), season (temporal - orchard and regional scale) and *Citrus* spp. on the population structure of *P. citricarpa*.

The findings of Chapter 2 were based on the results and analyses of genotyping data of *P. citricarpa* populations from South Africa, USA, Australia, China and Brazil, using seven published (Wang et al., 2016) and eight newly developed polymorphic simple sequence repeats (SSR) (Chapter 2). The study showed that populations differed in their connectivity and differentiation from each other. Limited connectivity was found between the Chinese populations and the populations from the other countries. There was, however, high levels of connectivity between South Africa, Australia and Brazil, as well as between South Africa, Australia and the USA. These findings are most likely due to exchanges of plant material and the associated *P. citricarpa* genotypes dating back to the establishment of the citrus industries in these countries. The Chinese and Australian populations had the highest level of genetic diversities, which correlates with the origin of the *Citrus* host and the first description of CBS in Australia (Benson, 1895). This finding is consistent with a co-evolutionary relationship between the pathogen on its wild hosts. The populations from Brazil, USA and South Africa exhibited characteristics of founder populations, which correlates with the known history of CBS in these countries. Therefore, the source of the South African population could be from the Far East or Australia. Australia or South Africa was identified as a likely source of the Brazilian population. Australia or South Africa can also be the source of the USA population. Migration has thus played an important role in

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determining the population structure of *P. citricarpa* in several countries. Both mating types were found in the populations from South Africa, Australia, China and Brazil. The USA populations, however, contained one mating type only. Linkage disequilibrium analyses indicated the occurrence of sexual reproduction and that asexual reproduction may be important in the pathogen's life cycle. The reproductive structure of the pathogen and presence or absence of both mating types will thus be very important in determining the population's structure and epidemiology of the disease in different countries.

The study conducted in Chapter 2 did not provide a complete picture of the introduction pathways. Therefore, additional populations from other countries in Asia and from New Caledonia and New Guinea should be analysed to determine whether the Oceanian countries, China or another country in Asia was the source population from which dispersal of the pathogen to other continents took place. The data from Chapter 2 provides strong evidence that asexual reproduction is common in all *P. citricarpa* populations world-wide.

To further investigate the population structure of *P. citricarpa* in South Africa at the orchard spatial (distance) and temporal (seasonal) scales, as well as the reproductive system, a detailed study in two orchards was conducted (Chapter 3). Populations were sampled according to a distance based structure over two seasons (2012 and 2013), from two lemon orchards differing in climate (Mpumalanga province - sub-tropical and North West province - semi-arid). The populations were genotyped using 15 SSR markers. Spatial analyses at the orchard scale indicated that subpopulations that were separated by shorter distances (within 200 m), were typically not significantly genetically differentiated, while those separated by longer distances were sometimes significantly differentiated. Temporal analyses of the North West orchard showed that seasonal populations were not significantly genetically differentiated. In contrast, seasonal populations from the Mpumalanga orchard were significantly differentiated, most likely due to higher rainfall and disease pressure in the Mpumalanga orchard. Mating type ratios in both orchards did not deviate significantly from a 1:1 ratio. Linkage disequilibrium analyses again indicated that *P. citricarpa* reproduces sexually and asexually and supported the finding of Chapter 2 that pycnidiospores are important in the epidemiology of CBS in South African orchards. Clonal reproduction was also identified as being important in the two lemon orchards, and was evidenced by low genotype evenness values and dominance by one or two multilocus genotypes. This is in contrast with previous studies from South Africa that indicated that pycnidiospores have a minor role in the epidemiology of CBS. The previous studies, however, failed to distinguish between the heterothallic *P. citricarpa* and the homothallic endophyte *P.*

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capitalensis which draws a lot of the older epidemiological research into question and may have given rise to an over estimation of the role of ascospores in the epidemiology of CBS.

The role of pycnidiospores and ascospores in South Africa should be further investigated by conventional inoculation (fruit and twigs) and leaf removal studies in citrus orchards. New ascospore trap data should be generated involving the specific differentiation of *P. citricarpa* from *P. capitalensis* ascospores. These studies should be conducted in orange orchards, since the relative importance of ascospore and pycnidiospore inoculum sources might differ between citrus types. This was one of the aims of Chapter 4.

The effect of *Citrus* spp. on population structure in South Africa, and whether *P. citricarpa* populations differ among the five provinces where CBS occurs, were investigated along with the reproductive system in Chapter 4. Ten populations from five provinces were genotyped. To study the effect of *Citrus* spp., nine of these populations (obtained from oranges and lemons) were analysed. Analyses of the provincial population structure indicated that the KwaZulu-Natal and Limpopo populations had the highest genetic diversities, while the Eastern Cape had the lowest. This correlates with the historical records of the time period that the disease has been established in the different provinces. Results indicated that there was most likely two separate introductions of the pathogen into South Africa. Populations from the different provinces were not significantly genetically differentiated. *Citrus* spp., specifically oranges and lemons, did not have an effect *on P. citricarpa* population structure. Further studies should be conducted to determine whether KwaZulu-Natal or Limpopo was the first place of introduction of CBS, and how CBS spread within South Africa. Future studies should investigate whether other citrus types such as grapefruit and mandarins have an effect on the population structure of *P. citricarpa.* It is important to note that the one grapefruit population from KwaZulu-Natal was not significantly differentiated from a lemon orchard in the Eastern Cape (Chapter 4).

Linkage disequilibrium analyses and mating type ratios further supported the findings of Chapter 2 and 3 in that *P. citricarpa* reproduces sexually and asexually and that pycnidiospores are more important in the epidemiology of CBS in South African orchards than previously reported. The linkage disequilibrium analyses indicated that asexual reproduction was not more pronounced in lemon populations than orange populations. The extent of clonal reproduction were comparable between lemon and orange orchards (Chapter4).

This was the first study using a population genetics approach to better understand the biology and epidemiology of CBS at a global, regional and orchard scale. The study provides insight into CBS introduction pathways. Although China is considered to be the centre of origin of the host and the pathogen, no connectivity could be identified between the western and eastern

countries. Both mating types were present in all the countries, except for the USA and the three European countries. The study indicated that the contribution of asexual spores to disease development (fruit symptoms) in South African orchards is more important than previously understood and that clonal survival of the pathogen is possible in South Africa. The thesis has shed new light on the population structure of *P. citricarpa* globally and in South Africa. Important evolutionary forces that affect the population structure of *P. citricarpa* at the global scale include the reproductive system of the pathogen and migration. Altogether this thesis has contributed towards a better understanding of CBS epidemiology, which can be used to improve disease management.

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