Population genetic analyses of *Phytophthora cinnamomi* reveals three lineages and movement between natural vegetation and avocado orchards in South Africa

Juanita Engelbrecht[1*], Tuan A. Duong[1] Trudy Paap[1], Joseph M. Hubert[1], Juanita J. Hanneman[1] and Noëlani van den Berg[1]

[1]Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI) , University of Pretoria, Pretoria 0002, South Africa.

Corresponding author: Juanita Engelbrecht, Email: Juanita.engelbrecht@fabi.up.ac.za

Abstract

Phytophthora cinnamomi is the causal agent of root rot, canker and dieback of thousands of plant species around the globe. This oomycete not only causes severe economic losses to forestry and agricultural industries, but also threatens the health of various plants in natural ecosystems. In this study, 380 isolates of *P. cinnamomi* from four avocado production areas and two regions of natural vegetation in South Africa were investigated using 15 microsatellite markers. These populations were found to have a low level of genetic diversity and consisted of isolates from three lineages. Shared genotypes were detected between isolates from avocado orchards and natural vegetation, indicating the movement of isolates between these areas. The population from the Western Cape natural vegetation had the highest genotypic diversity and unique alleles, indicating this could be the point of introduction of *P. cinnamomi* to South Africa. Index of association analysis suggested that five out of six populations were under linkage disequilibrium suggesting a clonal mode of reproduction whereas genotypes sampled from a recently established avocado orchard in the Western Cape were derived from a randomly recombined population. This study provided novel insights on the genetic diversity and spread of *P. cinnamomi* in South Africa. It also reported on the predominance of triploidy in natural occurring populations and provided evidence for recombination of *P. cinnamomi* for the first time. The presence of two dominant genotypes in all avocado production areas in South Africa highlight the importance of considering them in disease management and resistance breeding programmes.

Keywords: oomycetes, Phytophthora cinnamomi, population biology

Introduction

Phytophthora cinnamomi Rands is a soilborne oomycete pathogen that is among the 100 worst invasive species in the world (Lowe *et al.* 2000). This pathogen was first described in Sumatra in 1922 (Rands 1922) and since then it has been reported from numerous countries on all continents except for Antarctica. It is thought to be indigenous to Papua New Guinea where the highest genetic diversity has thus far been reported (Linde *et al.* 1999, Old *et al.* 1988, Old *et al.* 1984). However, alternate centres of origin have also been suggested, including other parts of East and Southern Asia such as Indonesia, Malaysia and Taiwan (Arentz 2017, Arentz and Simpson 1986, Ko *et al.* 1978, Shepherd 1975). In a recent study Shakya *et al* 2021 conducted a global study of *P. cinnamomi* using genotyping-by-sequencing of isolates from various countries and also suggested Taiwan as the candidate centre of origin as it contained the highest genotypic diversity and the most private alleles.

Phytophthora cinnamomi is heterothallic with two mating types (designated as A1 and A2), with the presence of both mating types required for sexual reproduction. Although both mating types have been found in close proximity in the natural vegetation of South Africa and in avocado orchards in Australia, evidence of sexual reproduction in nature has never been reported (Oh *et al.* 2011, Old *et al.* 1988, Old *et al.* 1984). Asexual reproduction, through the formation of sporangia that release motile zoospores (Hardham 2005, Linde *et al.* 1997), seems to be the predominant mode of spread of *P. cinnamomi* in agricultural crops and natural vegetation. Jung *et al.* (2014) proposed that only the A1 mating type is of ancient origin from Taiwan and that the A2 mating type may have emerged more recently (around 19th century). In a recent study, Arentz (2017) proposed that the A2 mating type was derived from the A1 mating type, and used this to explain why no evidence of sexual reproduction has been found for *P. cinnamomi*.

In the genus *Phytophthora, P. cinnamomi* has the largest host range, estimated to be more than 5000 plant species (Hardham and Blackman 2018). Additionally, it is also the most widely distributed *Phytophthora* species across the world affecting several economic and ecologically important plants (Zentmyer 1980). Its global distribution has been attributed to the movement of infected soil and plant material in the horticultural trade between continents over the last 100+ years (Arentz 2017, Linde *et al.* 1999). Despite its relevance and widespread occurrence, *P. cinnamomi* still remains understudied when compared to other notable *Phytophthora* spp. such as *Phytophthora infestans* and *Phytophthora sojae*.

A number of studies have investigated the genetic variability of *P. cinnamomi* using various markers including isozymes, RAPD, AFLP, microsatellites, mitochondrial haplotypes, and direct gene sequencing (Chang *et al.* 1996, Dobrowolski *et al.* 2003, Duan *et al.* 2008, Linde *et al.* 1997, Linde *et al.* 1999, Martin and Coffey 2012, Old *et al.* 1988, Old *et al.* 1984, Oudemans and Coffey 1991, Pagliaccia *et al.* 2013). All of these studies found that *P. cinnamomi* had a low genetic variability within and among geographically defined populations. Isolates of the A1 mating type displayed a higher genetic diversity when compared to the A2 mating type isolates (Arentz 2017, Dobrowolski *et al.* 2002). The invasion of *P. cinnamomi* globally has mainly been achieved by the clonal spread of the A2 mating type, as indicated by the dominance of this mating type in various studies (Arentz 2017, Kamoun *et al.* 2015). It has also been shown that the A2 mating type is more aggressive than the A1 mating type (Arentz 2017, Jung *et al.* 2013, Kamoun *et al.* 2015, Zentmyer 1980, Zentmyer 1988). The A2 mating type is also the dominant type isolated from forestry and agricultural tree crops in South Africa (von Broembsen 1984). Between 1991 and 1993, Linde *et al.* (1997) collected 49 isolates from South Africa, of which only 10 were of mating type A1 and these were all from the Western Cape province. In addition to the A1 mating type, isolates of the A2 mating type have also been found in the natural vegetation of the Western Cape region of South Africa (Linde *et al.* 1997, Oh *et al.* 2011).

After almost 100 years since *P. cinnamomi* was first reported in South Africa (Doidge and Bottomley 1931), it remains one of the major pathogens affecting fruit trees and forestry species as well as natural vegetation. In the fruit tree sector, avocado is severely affected, resulting in significant economic losses annually. Despite this, little is known regarding the source of *P. cinnamomi* introduction and its genetic diversity in avocado production areas. Thus, the aims of this study were to investigate the genetic diversity and population biology of *P. cinnamomi* from different avocado orchards across South Africa, and to compare it to populations obtained from natural vegetation in order to investigate the possible movement of *P. cinnamomi* between these two types of environments.

Materials and Methods

Collection of isolates, DNA extraction and confirmation of identity

Avocado roots and soil samples surrounding avocado trees were collected from Limpopo (8 orchards), Mpumalanga (7 orchards), KwaZulu-Natal (2 orchards) and the Western Cape province (2 orchards) of South Africa between 2012 – 2017. Orchard blocks sampled were

selected at random, with 10 avocado trees sampled per block. Isolations from root material were made by embedding surface sterilized root pieces (1.5% Bleach for 30 s, 70% Ethanol for 30 s, rinsed in water and blotted dry) in *Phytophthora* selective NARPH media (20% clarified V8 juice agar containing 50 mg/L nystatin, 200 mg/L ampicillin, 10 mg/L rifampicin, 20 mg/L PCNB, 50 mg/L hymexazol, and 20 g/L agar). Isolations from soil samples were made by baiting with avocado leaves followed by plating the pieces of infected leaves onto NARPH agar plates. Isolates from natural vegetation were obtained between 2016 – 2018 by baiting rhizosphere soil samples (from *Leucadendron argenteum*, *Orthamnus zeyheri*, *Podocarpus latifolius*, *Ficus macrophylla* and *Protea* spp.) with leaves/petals of three plant species (*Rosa* sp., *Hedera helix* and *Quercus ilex*) and plating the baited leaf pieces on NARPH or PARPH media (20% clarified V8 juice agar containing 5 mg/L pimaricin, 200 mg/L ampicillin, 10 mg/L rifampicin, 20 mg/L PCNB, and 50 mg/L hymexazol, and 20 g/L agar). For KwaZulu-Natal samples were mostly collected from the Pietermaritzburg National botanical garden and for the Western Cape the Garden route botanical garden and Kirstenbosch National botanical garden were sampled. Upon plating the roots or infected leaf tissues onto NARPH/PARPH media, the plates were incubated for up to one week at room temperature and regularly inspected for mycelial growth. Once observed, cultures that displayed the characteristically coralloid-type mycelia with hyphal swelling resembling that of *P. cinnamomi* were transferred onto half strength potato dextrose agar (½ PDA) plates (19.5 g/L PDA powder, Merck, South Africa, and 7 g/L agar) and single hyphal tip isolations were made and transferred onto fresh ½ PDA plates.

Single hyphal tip cultures were used to inoculate malt-yeast extract broth (2% Difco malt extract, 0.5% Difco yeast extract (DB, France)) and grown for four days at 25°C, 200 rpm. Mycelial mass was harvested, freeze dried and stored at -20°C until further use. Genomic DNA was extracted from freeze dried mycelia using the PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, USA) as described by Engelbrecht *et al.* (2017). Identity of isolates was confirmed by PCR using species specific primers that amplify the *Ypt1* gene (Ycin3: 5’ GTCCTATTCGCCTGTTGGAA 3’ and Ycin4R: 5’ GGTTTTCTCTACATAACCATCCTATAA 3’) (Schena *et al.* 2008) or by amplification and sequencing of ITS regions using a combination of ITS1 or ITS6 or DC6 and ITS4 primers (Cooke *et al.* 2000).

SSR genotyping and confirmation of allele size

Fifteen out of the 16 Simple Sequence Repeat (SSR) markers previously developed by Engelbrecht *et al.* (2017) were used in the genotyping of isolates. The remaining marker (Pc_SSR17) resulted in the amplification of two variable microsatellite regions and thus was not included in this study. PCR genotyping was carried out in two multiplex PCRs using Platinum® Multiplex PCR master mix (Applied Biosystems, CA, USA) as previously described (Engelbrecht *et al.* 2017). Fragment analysis was conducted using an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems) with GeneScan 500 LIZ as the Size Standard (Applied Biosystems). Allele sizes were determined using the software GeneMapper v4.0 (Applied Biosystems). Since isolates of *P. cinnamomi* have been shown to be triploid (Engelbrecht *et al.* 2021), trisomy analysis was conducted using the trisomy function in GeneMapper which takes peak area into account for allele assignment. The presence of three alleles at one or more SSR markers and/or the apparent unequal peak areas when two alleles were present was used as evidence for triploidy. Once a given isolate was confirmed to be triploid, all markers from the isolate were carefully examined and scored so that they all had three alleles. Samples showing no evidence of trisomy across all markers were considered to be diploid.

All observed alleles were confirmed by Sanger sequencing. To do this, isolates representing distinct allele sizes were selected and PCRs were conducted for each of the markers using non-labelled primers as previously described (Engelbrecht *et al.* 2017). PCR products of homozygous alleles were sequenced directly whereas PCR products of heterozygous alleles were cloned using CloneJET PCR cloning kit (Thermo Scientific, USA), followed by colony PCR amplification and sequencing of the resulting clones using the same SSR primers as used in the initial PCR amplifications. Multiple clones were sequenced until all the expected alleles were recovered and confirmed. Sequences obtained with forward and reverse primers were assembled and the exact allele size was determined from the sequence data. The allele sizes determined by sequencing was used to correct the sizes obtained from GeneScan analysis for all isolates prior to being used for data analyses, as described in Engelbrecht *et al.* (2017).

Population genetic analysis

The R package *poppr* (Kamvar *et al.* 2014) was used to calculate basic population statistics, including (i) Stoddart and Taylor's index, $G = 1/\Sigma_i p^2_i$, where p_i is the observed frequency of i^{th} genotype (Stoddart and Taylor 1988) and (ii) evenness, $E_5 = (1/\lambda) - 1/e^{H'} - 1$, where λ is

Simpson's index and H′ is Shannon-Wiener's index (Grünwald *et al.* 2003, Ludwig and Reynolds 1988). E_5 is a preferred index of evenness because it is less dependent on the number of genotypes in a sample (Grünwald *et al.* 2003).

Bruvo distances were calculated using the R package POLYSAT v1.3.3 (Bruvo *et al.* 2004, Clark and Jasieniuk 2011) as it supports the analysis of datasets with different ploidy levels (Kamvar *et al.* 2014). A Neighbour joining tree was generated from the calculated Bruvo distance matrix using the nj function as part of ape package v5.0 (Paradis and Schliep 2019). To investigate the possible evolutionary relationships among multilocus genotypes (MLGs) a Minimum Spanning Network (MSN) was constructed using Bruvo's distance using the *poppr* package (Kamvar *et al.* 2014).

Test for signature of sexual reproduction in populations was conducted using index of association statistics. The observed rBarD value for each population was calculated and compared to that obtained from a 999-replicate randomized simulation. The calculations were made on the clone corrected dataset using the ia function as part of the *poppr* package (Kamvar *et al.* 2014).

Results

SSR genotyping of *P. cinnamomi* isolates

A total of 380 *P. cinnamomi* isolates were included in this study. Of these, 211 isolates were genotyped previously (Engelbrecht *et al.* 2017) and 169 were genotyped in the current study. The 380 isolates were divided into six populations according to their geographic origin and sampling source (avocado orchards or natural vegetation). Four populations were obtained from avocado orchards in four different provinces in South Africa including 121 isolates from Mpumalanga, 90 from Limpopo, 34 from KwaZulu-Natal and 40 from Western Cape, hereafter referred to as Mpumalanga-Avo, Limpopo-Avo, KwaZulu-Natal-Avo and Western Cape-Avo, respectively. Two populations were obtained from natural vegetation including 37 from KwaZulu-Natal and 58 from Western Cape, hereafter referred to as KwaZulu-Natal-NV and Western Cape-NV, respectively.

All 15 primer pairs successfully amplified the targeted SSR regions in all isolates. GeneScan data analysis indicated that 311 isolates showed three alleles in at least one of the 15 markers and these included isolates from all six populations. A total of 38 alleles were detected with 15 markers, with the number of alleles ranging from 2 to 4 alleles (average 2.53) per locus (Table 1). A total of 69 isolates consistently displayed only two alleles across all of the markers

Table 1. Allele frequency of *Phytophthora cinnamomi* populations from Mpumalanga-Avo, Limpopo-Avo, KwaZulu-Natal-Avo, KwaZulu-Natal-NV, Western Cape-Avo, and Western Cape-NV.

Locus	Allele size	Mpumalanga-Avo	Limpopo-Avo	KwaZulu-Natal-Avo	KwaZulu-Natal-NV	Western Cape-Avo	Western Cape-NV
Pc-SSR2	**266**	0.579	0.617	0.529	0.514	0.500	0.867
	269	0.421	0.383	0.471	0.486	0.500	0.133
Pc-SSR3	**377**	0.079	0.117	0.015	0.014	-	0.220
	380	0.921	0.883	0.985	0.986	1.000	0.617
	389	-	-	-	-	-	0.163
Pc-SSR4	**173**	0.921	0.883	0.985	0.986	1.000	0.783
	176	0.079	0.117	0.015	0.014	-	0.217
Pc-SSR7	**262**	0.921	0.878	0.985	0.986	1.000	0.443
	267	-	-	-	-	-	0.020
	268	-	-	-	-	-	0.250
	270	0.079	0.122	0.015	0.014	-	0.287
Pc-SSR8	**211**	0.004	-	-	-	-	0.153
	217	0.426	0.383	0.485	0.486	0.500	0.127
	220	0.570	0.617	0.515	0.514	0.500	0.720
Pc-SSR9	**350**	0.079	0.117	0.015	0.014	-	0.208
	353	0.921	0.883	0.985	0.986	1.000	0.792
Pc-SSR12	**211**	0.576	0.607	0.515	0.500	0.500	0.727
	214	0.424	0.393	0.485	0.500	0.500	0.273
Pc-SSR13	**437**	0.469	0.450	0.495	0.495	0.500	0.265
	443	0.482	0.472	0.495	0.495	0.500	0.432
	452	-	-	-	-	-	0.156
	458	0.049	0.078	0.010	0.009	-	0.146

Locus	Allele size	Mpumalanga-Avo	Limpopo-Avo	KwaZulu-Natal-Avo	KwaZulu-Natal-NV	Western Cape-Avo	Western Cape-NV
Pc-SSR14	**212**	0.492	0.500	0.485	0.486	0.500	0.653
	215	0.508	0.500	0.515	0.514	0.500	0.347
Pc-SSR15	**320**	0.067	0.118	0.015	-	-	0.341
	326	0.933	0.882	0.985	1.000	1.000	0.659
Pc-SSR16	**339**	0.508	0.511	0.500	0.500	0.500	0.653
	343	0.492	0.489	0.500	0.500	0.500	0.347
Pc-SSR19	**171**	0.421	0.394	0.485	0.486	0.500	0.283
	174	0.579	0.606	0.515	0.514	0.500	0.717
Pc-SSR20	**316**	0.579	0.617	0.515	0.514	0.500	0.873
	319	0.421	0.383	0.485	0.486	0.500	0.127
Pc-SSR22	**253**	0.326	0.275	0.343	0.324	0.342	0.083
	256	0.360	0.354	0.299	0.338	0.317	0.299
	262	0.314	0.371	0.358	0.338	0.342	0.618
Pc-SSR23	**333**	0.076	0.067	0.010	0.009	-	0.297
	336	0.452	0.456	0.495	0.495	0.488	0.277
	339	0.472	0.478	0.495	0.495	0.513	0.427

which included 11 isolates from Limpopo-Avo, 28 from Mpumalanga-Avo, two from Western Cape-Avo, four from KwaZulu-Natal-Avo and 24 from Western Cape-NV. Trisomy analysis with manual verification for all the markers indicated that only 23 isolates from the Western Cape-NV showed no evidence of trisomy for all the markers tested (i.e. did not have a 2:1 or 1:2 peak ratio at loci with two alleles) and therefore they were considered as diploid.

In total, 92 MLGs were detected among the 380 isolates, 69 of which were represented by only a single isolate. Mpumalanga-Avo had 45 MLGs, Limpopo-Avo 30 MLGs, Western Cape-Avo 5 MLGs, KwaZulu-Natal-Avo 13 MLGs, Western Cape-NV had 20 MLGs and KwaZulu-Natal-NV had 6 MLGs. There were 14 MLGs that were shared between at least two populations. Two dominant genotypes were present that accounted for 55% of all the isolates, each including isolates from all six populations. These two genotypes accounted for 48% of Mpumalanga-Avo, 58% of Limpopo-Avo, 59% of KwaZulu-Natal-Avo, 93% of Western Cape-Avo, 89% of KwaZulu-Natal-NV and 16% of Western Cape-NV isolates. Shannon-Wiener's index (H) was similar for Limpopo-Avo, Mpumalanga-Avo, KwaZulu-Natal-Avo and Western Cape-NV whereas Western Cape-Avo and KwaZulu-Natal-NV had slightly lower values (Table 2). The Stoddart and Taylor's index (G) for Western Cape-Avo and KwaZulu-Natal-NV were also much lower when compared to the other populations (Table 2). The unbiased gene diversity (Hexp) was similar between Limpopo-Avo, Mpumalanga-Avo and Western Cape-NV but different from Western Cape-Avo, KwaZulu-Natal-Avo and KwaZulu-Natal-NV which had similar values (Table 2).

Test for random mating

The index of association test rejected the null hypothesis of random mating ($P < 0.001$) in five populations including Mpumalanga-Avo, Limpopo-Avo, KwaZulu-Natal-Avo, KwaZulu-Natal-NV and Western Cape-NV, suggesting a clonal mode of reproduction in these populations. In the case of the avocado orchard population from Western Cape-Avo, the observed rBarD value was $-1.46e^{-16}$ and a *P*-value of 0.53 was obtained, supporting the hypothesis of random association between alleles at different loci as a result of outcrossing, indicating evidence of sexual reproduction in this population. The observed I_A, rBarD and *P*-value for all populations are presented in Table 2 and Fig. 1.

Table 2. Genotypic diversity statistics for *Phytophthora cinnamomi* populations from South Africa.

Population	N	MLG	eMLG	H	G	lambda	E.5	Hexp	Ia *	rbarD *	P.rD *
Mpumalanga - Avo	121	45	17	2.73	5.25	0.81	0.64	0.45	7.96e+00	0.624	< 0.001
Limpopo - Avo	90	30	15	2.35	4.47	0.78	0.37	0.40	7.77e+00	0.61	< 0.001
KwaZulu-Natal - Avo	34	13	13	2.05	5.25	0.81	0.63	0.33	7.56e+00	0.587	< 0.001
Western Cape - Avo	40	5	6	0.72	1.52	0.343	0.50	0.32	-1.11e-16	-1.46e-16	0.53
Western Cape - NV	58	20	14	2.47	8.00	0.875	0.64	0.45	7.10e+00	0.533	< 0.001
KwaZulu-Natal - NV	37	6	6	1.04	2.16	0.538	0.64	0.33	7.69e+00	0.7	< 0.001
Total	380	92	15	2.87	6.22	0.84	0.31	0.40	7.80e+00	0.596	< 0.001

N = Number of individuals observed, **MLG** = Number of multilocus genotypes observed, **eMLG** = The number of expected MLG at the smallest sample size ≥ 10 based on rarefaction, **H** = Shannon-Wiener Index of MLG diversity, **G** = Stoddart and Taylor's Index of MLG diversity, **Lambda** = Simpson's Index, **E.5** = Evenness (E_5), **Hexp** = Nei's unbiased gene diversity, **Ia** = The index of association I_A[6], **rbarD** = The standardized index of association $\bar{r}_d$, **p.rD** = *P*-value for $\bar{r}_d$, * = Values were calculated using clone corrected data.

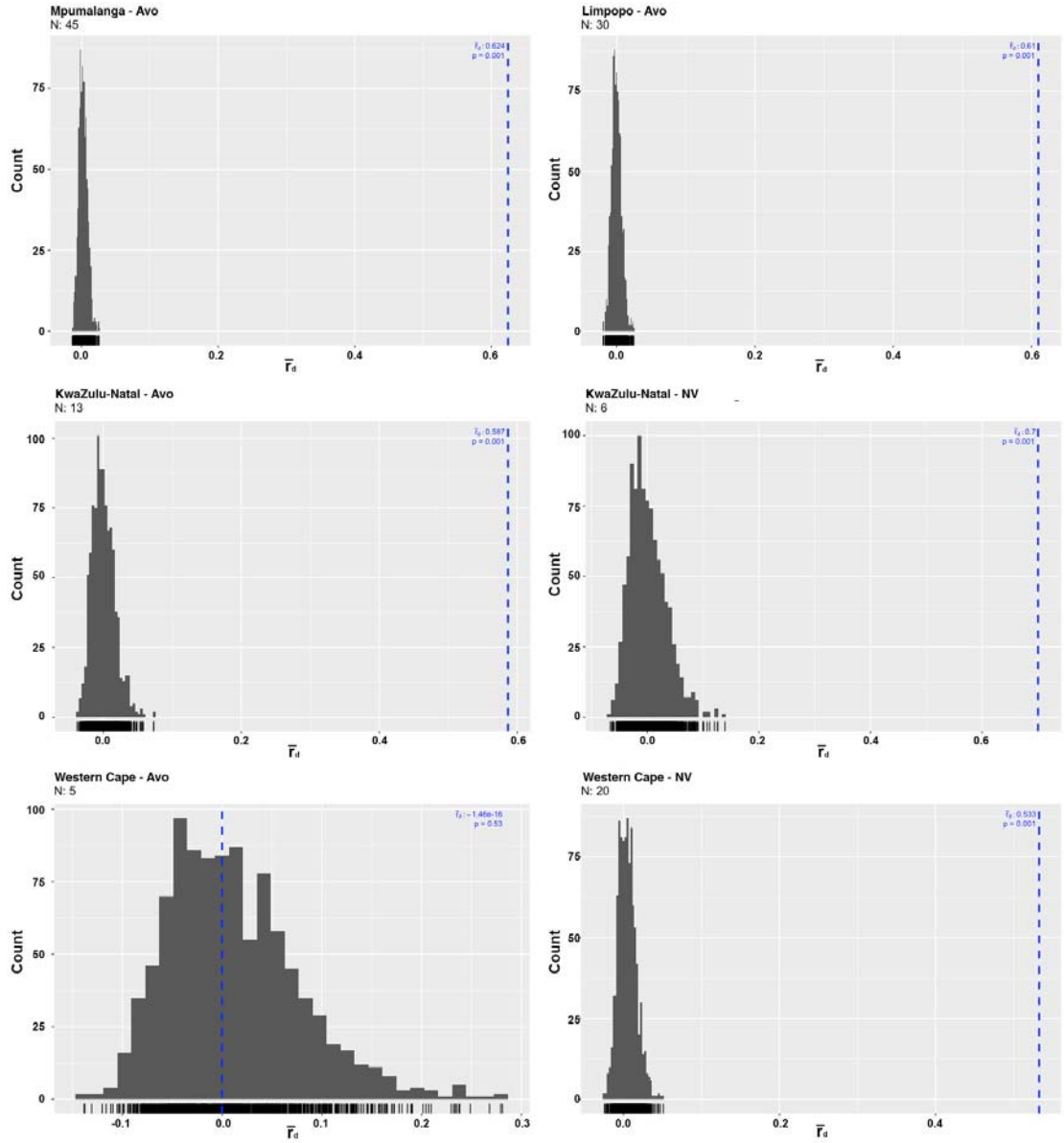

Figure 1. Histograms constructed from rbarD values obtained from 999 time randomized datasets of Mpumalanga - Avo (A), Limpopo - Avo (B), KwaZulu-Natal - Avo (C), KwaZulu-Natal - NV(D), Western Cape - Avo (E) and Western Cape - NV (F). Vertical lines indicate the observed rbarD for each population using clone corrected data.

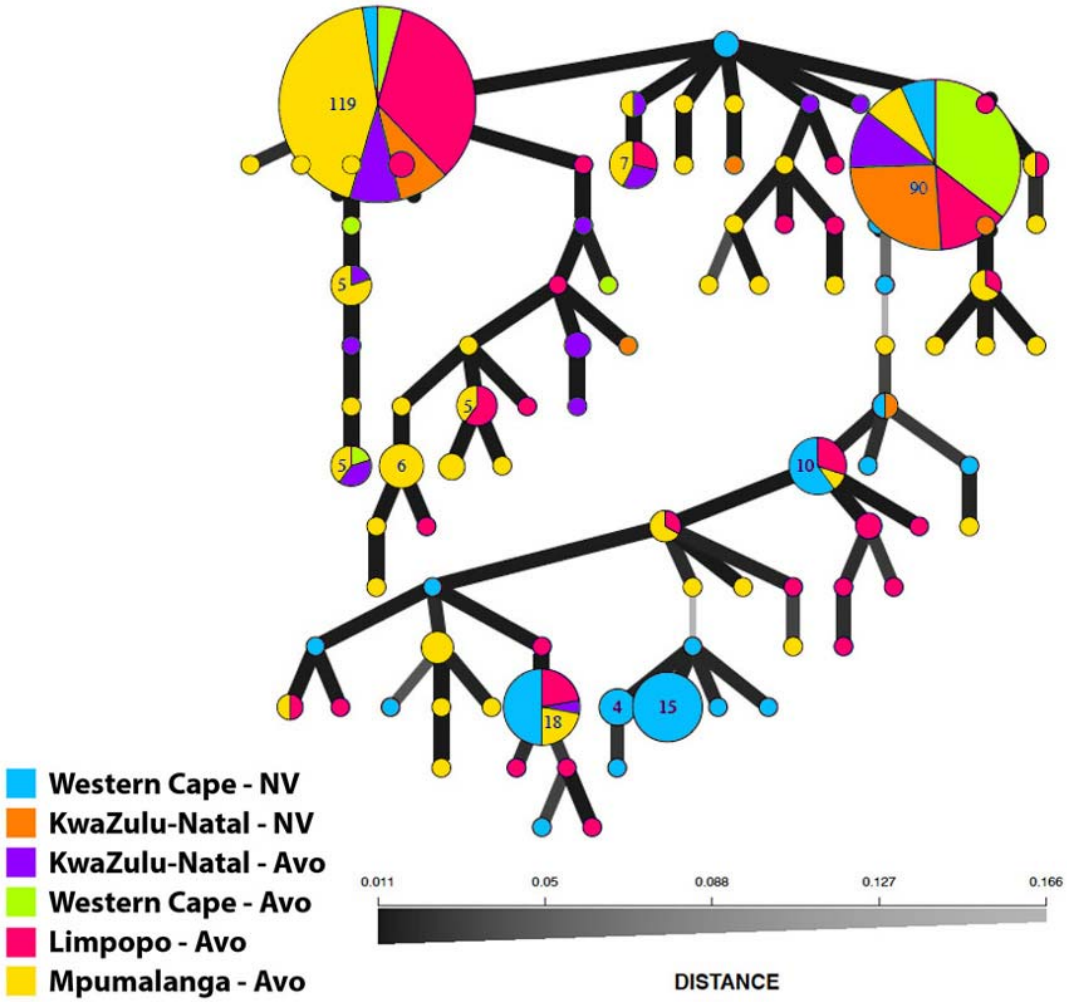

Figure 2. Minimum spanning network constructed from Bruvo's distances. The size of the nodes is proportional to the number of isolates. The thickness and colour gradient of the connecting lines represent the Bruvo's genetic distance between nodes where thicker and darker lines denote smaller genetic distance between genotypes and thinner and lighter lines denote greater genetic distance between genotypes.

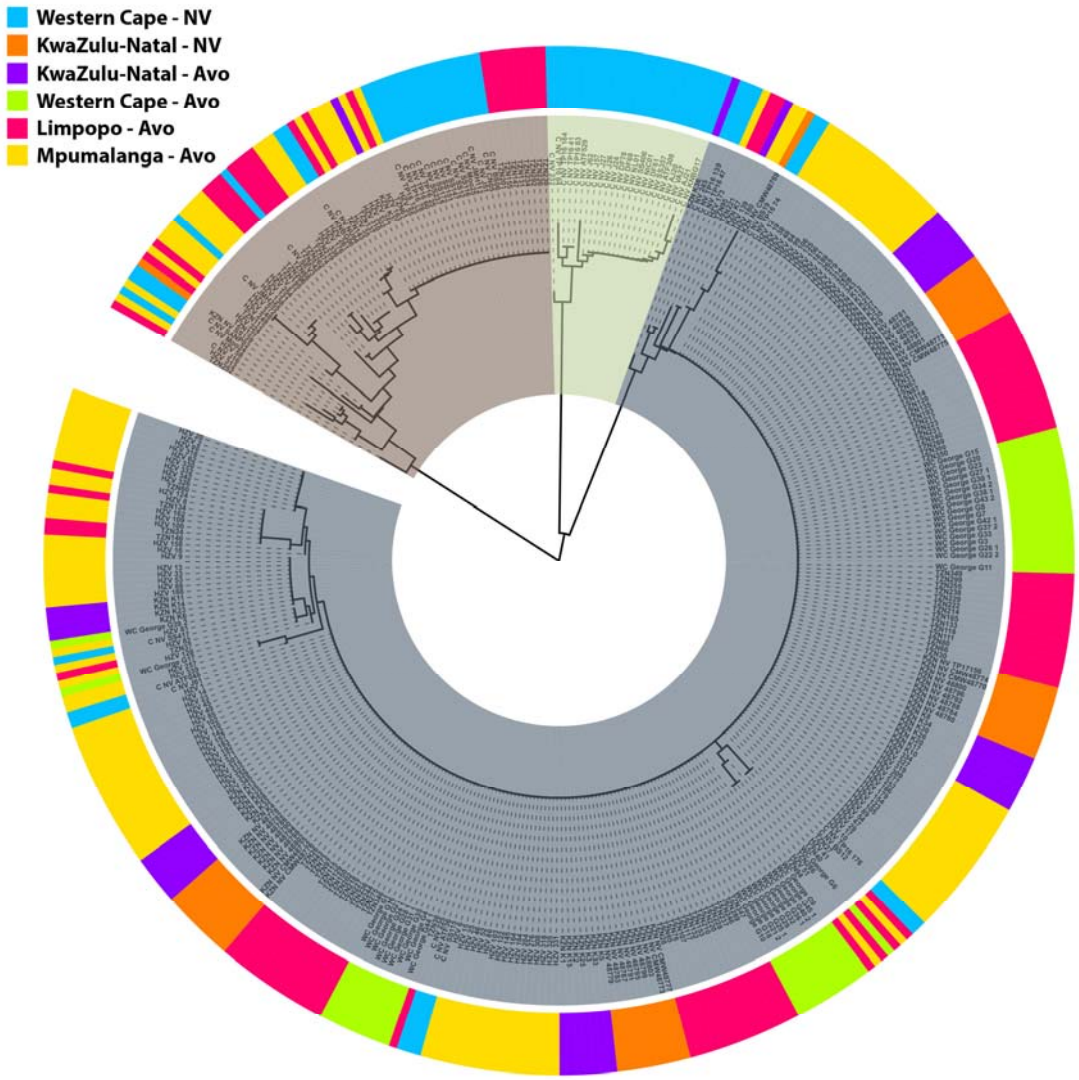

Figure 3. Neighbour joining tree of *Phytophthora cinnamomi* isolates constructed from Bruvo's distance calculated from SSR data obtained using 15 SSR markers. The inner ring indicates the three major clades identified. The outer ring indicates localities of isolates: Western Cape - NV - blue, Western Cape - Avo - green, KwaZulu-Natal - Avo - purple, KwaZulu-Natal - NV - orange, Limpopo - Avo - dark pink and Mpumalanga -Avo - yellow.

Population structure of *P. cinnamomi*

The MSN constructed using Bruvo's distance revealed three main clusters (Fig. 2). One cluster consisted only of isolates collected from the natural vegetation of the Western Cape (Fig. 2) while the other two clusters consisted of isolates from five (all except for Western Cape-Avo) and all six populations, respectively. Isolates from the Western Cape-NV were present in all

three clusters. The two dominant MLGs resided in the same cluster (Fig. 2). The Neighbor joining tree constructed using Bruvo's distance revealed similar results as the MSN where three lineages, corresponding to the three clusters as identified from MSN analysis, were identified (Fig. 3).

Discussion

In this study, 380 isolates of *P. cinnamomi* belonging to six populations from four provinces in South Africa were analysed using 15 SSR markers. A total of 92 MLGs were detected which resided in three major lineages. Two lineages consisted of isolates from all provinces while one lineage was only represented by isolates from the natural vegetation of the Western Cape. Evidence of sexual reproduction in a population of *P. cinnamomi* was also detected for the first time and this was from avocado orchards in the Western Cape.

Around 94% of isolates (357 out of 380) considered in this study were shown to be triploid. These isolates either had three different alleles per locus at certain SSR markers or had support from trisomy analysis to have a 2:1 or 1:2 peak ratio at markers with two alleles. The same pattern was detected in Engelbrecht *et al.* (2017) where several markers showed three alleles per locus, however, in that study it was interpreted as evidence for aneuploidy. Recently, the genome sequence of a South African *P. cinnamomi* isolate (GKB4) has been generated and ploidy analysis indicated that this isolate was triploid at the genome level (Engelbrecht *et al.* 2021). In addition, data analysis of two previously sequenced *P. cinnamomi* isolates from New Zealand and Australia also showed evidence of genome-wide triploidy (Engelbrecht *et al*. 2021). The genome-wide triploidy has also been reported in other *Phytophthora* spp. using flow cytometry and SNP data (Catal *et al.* 2010, Hamed and Gisi 2013, Knaus *et al.* 2020, Li *et al.* 2017). In *P. infestans* it has been shown that progenies from sexually reproducing populations are diploid, whereas isolates from asexual lineages were found to be triploid (Knaus *et al.* 2020, Li *et al.* 2017). From this result, it is highly likely that most triploid isolates of *P. cinnamomi* from South Africa (except for those in the Western Cape avocado orchard as discussed below) are also the result of asexual reproduction. This explains the clonality and limited diversity observed in these populations.

Despite using 15 SSR markers on a large set of isolates, we found a low level of genetic diversity for *P. cinnamomi* in South Africa. This finding is similar to studies conducted by Linde *et al.* (1997 & 1999) using RFLP and isozymes markers. In their studies, they also reported a low level of genetic diversity and concluded that sexual reproduction occurs rarely,

if at all, in South Africa. Similar to their findings which reported an average of 1.43 alleles per locus, the current study found an average of 2.53 alleles per locus using SSR markers, which is expected to offer better resolution than traditional markers. The low number of alleles per locus coupled with limited genetic diversity suggests that *P. cinnamomi* was likely introduced into South Africa, rejecting the hypothesis of von Broembsen & Kruger (1985) which proposed that *P. cinnamomi* could be native to South Africa.

Two dominant genotypes which accounted for 55% of the isolates were detected in this study and included isolates from all six populations. Additionally, 14 other genotypes were shared between at least two populations. The presence of multiple shared MLGs suggests that there is frequent movement of genotypes between populations. The presence of two dominant genotypes represented by a high proportion of avocado isolates supports a predominant clonal mode of reproduction and also suggests that their spread is most probably linked to the transport of avocado planting material in the country.

The index of association analysis supported the hypothesis of random association between alleles at different loci suggesting evidence of sexual recombination in the Western Cape avocado orchard. To the best of our knowledge, this is the first time that evidence of sexual reproduction is reported for a population of *P. cinnamomi* in nature. This result was also unexpected because it is well known that the A2 mating type of *P. cinnamomi* is more invasive and widely distributed, causing the majority of infections (Arentz 2017, Kamoun *et al.* 2015), which is also true for *P. cinnamomi* in avocado orchards in South Africa. Additionally, all isolates from this orchard were shown to be triploid in our SSR analysis. It is, however, noteworthy to mention that the Western Cape avocado orchard was only established nine years ago and prior to its establishment this area was considered natural vegetation. Thus, it is possible that the random combinations of alleles detected in this population was the result of past recombination events that occurred prior to the establishment of avocado orchards. Triploidisation as a mechanism of adaptation has been reported in *Phytophthora capsici* (Hu *et al.* 2020). Thus, it is possible that sexually-derived genotypes from the Western Cape orchards could have become triploid after being associated with avocado, however, this needs to be further investigated. It is also possible that the observed genotypes from Western Cape avocado orchard were derived from parasexual cycles followed by mitotic recombination which has been shown to be a mode of rapid genome evolution in *Phytophthora ramorum* (Dale *et al.* 2019) and this also warrants further investigation.

The evidence in support of recombination in *P. cinnamomi* in this study was surprising given previous studies from around the world reported a clonal mode of reproduction, despite both mating types having been found in close proximity (Oh *et al.* 2011, Old *et al.* 1988, Old *et al.* 1984). Additionally, A1 and A2 mating types have been shown to be divergent lineages, suggesting these mating types may represent two clonal lineages that are not sexually recombining (Brasier 1975, Dobrowolski *et al.* 2003). However, the evidence of recombination detected in this study challenges this hypothesis and warrants further investigation. In the context of South Africa, the presence of both mating types in the country together with evidence for recombination could result in the emergence of highly virulent and adapted lineages. This potentially poses a great threat to plantation forests, fruit tree orchards and natural vegetation.

In contrast to the avocado orchard in the Western Cape, no evidence of sexual recombination was detected within the population from the natural vegetation in the same area, although both A1 and A2 mating types have been shown to be present (Linde *et al.* 1997). Interestingly, the 23 isolates that were shown to be diploid were all from the Western Cape natural vegetation, which could suggest the presence of possible sexual reproduction in this area. It is possible that sexual reproduction could have occurred in this population but at a very low frequency and therefore remained undetected in this study. A follow-up study focusing on this population with more intensive sampling may provide more clarity on this issue.

The source of introduction for *P. cinnamomi* in South Africa still remains unknown after almost 100 years since it was first detected. From the results of this study, it is plausible that the Western Cape province of South Africa was the point of introduction of *P. cinnamomi* into the country given the following reasons; firstly, despite the rather limited sample size, the population from the natural vegetation of the Western Cape is the only population with private alleles that were not found in any of the other populations. Secondly, isolates from this population were represented in all three lineages identified in this study. Lastly, of the three lineages identified, one lineages consisted of only isolates from the Western Cape natural vegetation. If this hypothesis holds true, it is highly probable that *P. cinnamomi* was introduced by the early colonisers of Africa, similar to what has been reported for *Armillaria mellea,* another tree pathogen of significant concern in South Africa (Coetzee *et al.* 2001). The lack of sufficient markers and coordinated efforts has resulted in a limited understanding of the genetic diversity and genotypic profiles of *P. cinnamomi* around the globe. The SSR markers from our previous study (Engelbrecht *et al.* 2017) and the knowledge gained from their

application on the South African *P. cinnamomi* populations in the present study could serve as a foundation for future global research on *P. cinnamomi* population genetics, leading to a better understanding of its origin, movement and invasion patterns.

References

Arentz, F (2017). *Phytophthora cinnamomi* A1: An ancient resident of New Guinea and Australia of Gondwanan origin? *Forest Pathology* **47**(4): e12342.

Arentz, F and J Simpson (1986). Distribution of *Phytophthora cinnamomi* in Papua New Guinea and notes on its origin. *Transactions of the British Mycological Society* **87**(2): 289-295.

Brasier, C (1975). Stimulation of sex organ formation in *Phytophthora* by antagonistic species of *Trichoderma*: II. Ecological implications. *New Phytologist* **74**(2): 195-198.

Bruvo, R, NK Michiels, TG D'souza and H Schulenburg (2004). A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Molecular Ecology* **13**(7): 2101-2106.

Catal, M, L King, P Tumbalam, P Wiriyajitsomboon, WW Kirk and GC Adams (2010). Heterokaryotic nuclear conditions and a heterogeneous nuclear population are observed by flow cytometry in *Phytophthora infestans*. *Cytometry Part* **77A**(8): 769-775.

Chang, T, W Yang and W Wang (1996). Use of random amplified polymorphic DNA markers for the detection of genetic variation in *Phytophthora cinnamomi* in Taiwan. *Botanical Bulletin of Academia Sinica* **37**: 165-171.

Clark, LV and M Jasieniuk (2011). POLYSAT: an R package for polyploid microsatellite analysis. *Molecular Ecology Resources* **11**(3): 562-566.

Coetzee, MP, BD Wingfield, TC Harrington, J Steimel, TA Coutinho and MJ Wingfield (2001). The root rot fungus *Armillaria mellea* introduced into South Africa by early Dutch settlers. *Molecular Ecology* **10**(2): 387-396.

Cooke, DEL, A Drenth, JM Duncan, G Wagels and CM Brasier (2000). A molecular phylogeny of *Phytophthora* and related Oomycetes. *Fungal Genetics and Biology* **30**(1): 17-32.

Dale, AL, N Feau, SE Everhart, B Dhillon, B Wong, J Sheppard, GJ Bilodeau, A Brar, JF Tabima and D Shen (2019). Mitotic recombination and rapid genome evolution in the invasive forest pathogen *Phytophthora ramorum*. *MBio* **10**(2): e02452-02418.

Dobrowolski, MP, I Tommerup, HD Blakeman and PA O'Brien (2002). Non-Mendelian inheritance revealed in a genetic analysis of sexual progeny of *Phytophthora cinnamomi* with microsatellite markers. *Fungal Genetics and Biology* **35**(3): 197-212.

Dobrowolski, MP, IC Tommerup, BL Shearer and PA O'Brien (2003). Three clonal lineages of *Phytophthora cinnamomi* in Australia revealed by microsatellites. *Phytopathology* **93**(6): 695-704.

Doidge, EM and AM Bottomley (1931). A revised list of plant diseases in South Africa. *Memoirs of the Botanical Survey of South Africa* **11**: 1-78.

Duan, C-H, M Riley and S Jeffers (2008). Characterization of *Phytophthora cinnamomi* populations from ornamental plants in South Carolina, USA. *Archives of Phytopathology and Plant Protection* **41**(1): 14-30.

Engelbrecht, J, TA Duong, SA Prabhu, M Seedat and N van den Berg (2021). Genome of the destructive oomycete *Phytophthora cinnamomi* provides insights into its pathogenicity and adaptive potential. *BMC Genomics* **22**(1): 302.

Engelbrecht, J, TA Duong and N van den Berg (2017). New microsatellite markers for population studies of *Phytophthora cinnamomi,* an important global pathogen. *Scientific Reports* **7**(1): 17631.

Grünwald, NJ, SB Goodwin, MG Milgroom and WE Fry (2003). Analysis of genotypic diversity data for populations of microorganisms. *Phytopathology* **93**(6): 738-746.

Hamed, B and U Gisi (2013). Generation of pathogenic F1 progeny from crosses of *Phytophthora infestans* isolates differing in ploidy. *Plant Pathology* **62**(3): 708-718.

Hardham, AR (2005). *Phytophthora cinnamomi*. *Molecular Plant Pathology* **6**(6): 589-604.

Hardham, AR and LM Blackman (2018). *Phytophthora cinnamomi*. *Molecular Plant Pathology* **19**(2): 260-285.

Hu, J, S Shrestha, Y Zhou, J Mudge, X Liu and K Lamour (2020). Dynamic Extreme Aneuploidy (DEA) in the vegetable pathogen *Phytophthora capsici* and the potential for rapid asexual evolution. *PLoS One* **15**(1): e0227250.

Jung, T, T Chang, A Pérez-Sierra, K Hsueh, C Fu, P Abad-Campos and M Horta Jung (2014). Diversity and impact of *Phytophthora* spp. in natural ecosystems of Taiwan. Poster presented at IUFRO Working Party 7.02.09, "*Phytophthora* in Forests & Natural Ecosystems:. Esquel, Chubut, Patagonia, Argentina, 10-14 November 2014.

Jung, T, I Colquhoun and G Hardy (2013). New insights into the survival strategy of the invasive soilborne pathogen *Phytophthora cinnamomi* in different natural ecosystems in Western Australia. *Forest Pathology* **43**(4): 266-288.

Kamoun, S, O Furzer, JD Jones, HS Judelson, GS Ali, RJ Dalio, SG Roy, L Schena, A Zambounis and F Panabières (2015). The top 10 oomycete pathogens in molecular plant pathology. *Molecular Plant Pathology* **16**(4): 413-434.

Kamvar, ZN, JF Tabima and NJ Grünwald (2014). *Poppr* : an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* **2**: e281.

Knaus, BJ, JF Tabima, SK Shakya, HS Judelson and NJ Grünwald (2020). Genome-wide increased copy number is associated with emergence of dominant clones of the irish potato famine pathogen *Phytophthora infestans*. *MBio* **11**(3): e00326-00320.

Ko, W, H Chang and H Su (1978). Isolates of *Phytophthora cinnamomi* from Taiwan as evidence for an Asian origin of the species. *Transactions of the British Mycological Society* **71**(3): 496-499.

Li, Y, H Shen, Q Zhou, K Qian, T van der Lee and S Huang (2017). Changing ploidy as a strategy: The Irish potato famine pathogen shifts ploidy in relation to its sexuality. *Molecular Plant-Microbe Interactions* **30**(1): 45-52.

Linde, C, A Drenth, GHJ Kemp, MJ Wingfield and SL Von Broembsen (1997). Population structure of *Phytophthora cinnamomi* in South Africa. *Phytopathology* **87**(8): 822-827.

Linde, C, A Drenth and MJ Wingfield (1999). Gene and genotypic diversity of *Phytophthora cinnamomi* in South Africa and Australia revealed by DNA polymorphisms. *European Journal of Plant Pathology* **105**(7): 667-680.

Lowe, S, M Browne, S Boudjelas and M De Poorter (2000). 100 of the world's worst invasive alien species: a selection from the global invasive species database, Invasive Species Specialist Group Auckland.

Ludwig, JA and JF Reynolds (1988). Statistical ecology: a primer in methods and computing, John Wiley & Sons.

Martin, FN and MD Coffey (2012). Mitochondrial haplotype analysis for differentiation of isolates of *Phytophthora cinnamomi*. *Phytopathology* **102**(2): 229-239.

Oh, E, B Wingfield, M Wingfield and J Roux (2011). First report of *Phytophthora cinnamomi* associated with stem cankers of *Quercus cerris* in South Africa. *New Disease Reports* **24**.

Old, K, M Dudzinski and J Bell (1988). Isozyme variability in field populations of *Phytophthora cinnamomi* in Australia. *Australian Journal of Botany* **36**(3): 355-360.

Old, K, G Moran and J Bell (1984). Isozyme variability among isolates of *Phytophthora cinnamomi* from Australia and Papua New Guinea. *Canadian Journal of Botany* **62**(10): 2016-2022.

Oudemans, P and MD Coffey (1991). Isozyme comparison within and among worldwide sources of three morphologically distinct species of *Phytophthora*. *Mycological Research* **95**(1): 19-30.

Pagliaccia, D, E Pond, B McKee and G Douhan (2013). Population genetic structure of *Phytophthora cinnamomi* associated with avocado in California and the discovery of a potentially recent introduction of a new clonal lineage. *Phytopathology* **103**(1): 91-97.

Paradis, E and K Schliep (2019). ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* **35**(3): 526-528.

Rands, RD (1922). Stripe canker of cinnamon caused by *Phytophthora cinnamomi* . *Mededelingen van het Instituut voor Plantenziekten* **54**.

Schena, L, J Duncan and D Cooke (2008). Development and application of a PCR-based 'molecular tool box' for the identification of *Phytophthora* species damaging forests and natural ecosystems. *Plant Pathology* **57**(1): 64-75.

Shakya SK, NJ Grünwald, VJ Fieland, BJ Knaus, JE Weiland, C Maia, A Drenth, D Guest, ECY Liew, C Crane, TT Chang, CH Fu, N Minh Chi, P Quang Thu, B Scanu, ES von Stowasser, A Durán, M Horta Jung, & TJung. (2021). Phylogeography of the wide-host range panglobal plant pathogen *Phytophthora cinnamomi*. *Molecular Ecology* **30**, 5164-5178.

Shepherd, C (1975). *Phytophthora cinnamomi* - an ancient immigrant to Australia. *Search* **6**: 484-490.

Stoddart, JA and JF Taylor (1988). Genotypic diversity: estimation and prediction in samples. *Genetics* **118**(4): 705-711.

von Broembsen, SL (1984). Occurrence of *Phytophthora cinnamomi* on indigenous and exotic hosts in South Africa, with special reference to the South-Western Cape Province. *Phytophylactica* **16**(3): 221-226.

Von Broembsen, SL and FJ Kruger (1985). *Phytophthora cinnamomi* associated with mortality of native vegetation in South Africa. *Plant Disease* **69**: 715-717.

Zentmyer, GA (1980). *Phytophthora cinnamomi* and the diseases it causes. St Paul, Minn, APS Press.

Zentmyer, GA (1988). Origin and distribution of four species of *Phytophthora*. *Transactions of the British Mycological Society* **91**(3): 367-378.