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Population Biology

e-Xtra*

Population Genetic Structure and Cryptic Species of *Plasmopara viticola* in Australia

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ABSTRACT

Downy mildew of grape caused by *Plasmopara viticola* is a global pathogen of economic importance to commercial viticulture. In contrast to populations in the northern hemisphere, few studies have investigated the population biology, genetic diversity, and origin of the pathogen in Australian production systems. DNA was extracted from 381 *P. viticola* samples from *Vitis vinifera* and alternate hosts collected via fresh and herbarium leaves from populations within Australia and Whatman FTA cards from North America, Brazil, and Uruguay. A total of 32 DNA samples were provided from a French population. The populations were genotyped using 16 polymorphic microsatellite markers. Representative samples from within Australia, Brazil, and Uruguay were also genotyped to determine which of the cryptic species (clades) within the *P. viticola* species complex were present. Our findings suggest the Australian and South American populations of *P. viticola* are more closely related to the European population than the North American population, the reported source of origin of the pathogen. The Western Australian population had similarities to the South Australian population, and the tight clustering of samples suggests a single introduction into Western Australia. *P. viticola* clade *aestivalis* was the only clade detected in Australian and South American populations. Analysis of the Western Australian population suggests that it is reproducing clonally, but additional research is required to determine the mechanism as to how this is occurring.

Keywords: downy mildew, grape, oomycetes, *Plasmopara viticola*, population genetics

Grapevine downy mildew caused by the oomycete *Plasmopara viticola* (Berk & MA Curtis) Berl & De Toni is a global pathogen recorded in >90 countries worldwide (CABI 2018; Emmett et al. 1992b). Endemic to eastern North America, it is believed that *P. viticola* was imported to Europe with native American *Vitis* species used as rootstock to prevent infection from the insect grape phylloxera, which at the time, was causing significant losses for European viticulture (Fontaine et al. 2013; Gessler et al. 2011; Gobbin et al. 2006; Rossi et al. 2013; Rouxel et al. 2014; Viennot-Bourgin 1981). After its introduction to France in 1878, it spread rapidly eastward into major grape production regions of Europe and over the Mediterranean Sea to Algeria by 1881 (Fontaine et al. 2013; Galet 1977; Gessler et al. 2011; Rossi et al. 2013). The rapid movement was believed to have been aided by human-mediated transfer of grapevine material throughout Europe (Fontaine et al. 2013; Gobbin et al. 2006). Additional human-aided global spread followed with detections in Brazil in 1893, South Africa in 1907, and New Zealand in 1926 (Koopman et al. 2007; Viennot-Bourgin 1981; Woodfin 1926).

The first Australian report of *P. viticola* was its occurrence in several commercial vineyards in Rutherglen, Victoria in 1917 (de Castella and Brittlebank 1917). After this detection, downy mildew spread rapidly within grape-growing regions of eastern Australia, being recorded in New South Wales in 1918, South Australia and Queensland in 1920 and 1921, and Tasmania in 1959 (Emmett et al. 1992a; Laffer 1918; Osborn and Samuel 1922; Quinn 1924). The rapid spread of the disease across eastern Australia resulted from a combination of wind and human-aided dispersal, despite climatic conditions being considered less conducive for disease development than they are in Europe (Osborn and Samuel 1922).

It was thought that natural isolation from other grape-growing regions (McLean et al. 1984), an absence of suitable spring and summer rainfall (Emmett et al. 1992b), and restrictions in movement of host material and machinery from other states and territories (McKirdy et al. 1999) would prevent the establishment of *P. viticola* in the state of Western Australia. However, in 1997, a detection occurred in Kalumburu in the north of the state on a small number of vines, which were subsequently destroyed (Riley 1998). This is a very remote area, accessible only by unsealed roads and separated by desert; it is ~2,200 km from commercial viticulture in the southwest of Western Australia. In October 1998, another detection occurred in the commercial production area of the Swan Valley in southwest Western Australia, and it was soon determined that eradication was not possible (McKirdy et al. 1999). *P. viticola* has since been found in all growing regions of Western Australia, and it has caused economic impact (Taylor and Cook 2018; Williams et al. 2007). The source of *P. viticola* in Western Australia remains unknown as well as whether the 1997 and 1998 detections were related.

Five cryptic species or clades within the *P. viticola* species complex have been identified with specificity to different *Vitis* species and related hosts (Rouxel et al. 2013, 2014). These cryptic species were delineated through a combination of genetic analysis using multiple genes, sporangial morphology, and virulence exhibited in cross-inoculated experiments of different host plants (Rouxel et al. 2013). Because the type specimen of *P. viticola* has not yet been examined and greater morphological analysis is required, a provisional nomenclature system of clades is used in place of forma specialis (Rouxel et al. 2014). A survey of eastern North America highlighted the presence and distribution of all *P. viticola* clades throughout its center of origin on native and

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introduced *Vitis* hosts (Rouxel et al. 2014), several of which are known to occur in Australia.

The development of *P. viticola*-specific microsatellite markers has enabled a greater understanding of the genetic diversity in populations and subsequent epidemic spread of the pathogen in a number of different production systems (Delmotte et al. 2006; Gobbin et al. 2003, 2005, 2006; Koopman et al. 2007; Kosev et al. 2015; Li et al. 2016; Rouxel et al. 2012). The genetic diversity in the Australian grape downy mildew population has only been evaluated in a small number of growing regions with a limited number of isolates (Hug 2005; Williams 2005) using only four microsatellite markers developed by Gobbin et al. (2003).

Our work focuses on populations in Western Australia to determine the likely origin and population structure relative to Australian and global populations. We sampled >400 strains, including herbarium specimens, to test several hypotheses. We genotyped strains using microsatellites. We asked a series of questions to characterize the evolutionary history of downy mildew in Australia. Did populations in Western Australia emerge from Australian populations? If not, were they introduced from Europe or North America? Were there multiple introductions into Western Australia? What cryptic species (clades) are present in Australia? Is the population in Western Australia randomly mating? The answers to these questions will provide insights into how a grape pathogen established itself in Western Australia, a viticulture production area previously considered climatically unfavorable for establishment, and they will provide insights to avoid additional introductions of pathogens in agricultural and natural ecosystems.

MATERIALS AND METHODS

***P. viticola* samples.** A total of 413 samples were collected as part of this study from Australia, the United States, France, Brazil, and Uruguay (Fig. 1). Samples were collected predominantly from *Vitis vinifera* but also included alternate hosts (Table 1).

Collection strategy. *Australia.* The presence of *P. viticola* in Australian grape-growing regions is sporadic, with incidence and severity being restricted by the timely application of fungicides and climatic conditions (Wicks and Hall 1990; Wicks et al. 1991), making structured sampling regimes problematic. Therefore, to obtain a meaningful number of samples for analysis, vineyard owners and consultants across Australia were sent sample packs containing sealable bags and a return envelope from 2014 to 2016, and they were asked to return infected leaves if present. Fresh samples were also collected opportunistically by the authors. Global positioning system (GPS) coordinates of the samples were also requested for data analysis purposes. A 1-cm^2 section was removed from individual oil spot lesions for DNA extraction. Herbarium specimens of *P. viticola* from leaves of dried host plants from the Western Australian Herbarium and Agricultural Scientific Collections Trust in New South Wales were also included in the study.

North America and South America. P. viticola DNA from infected leaves from a range of *Vitis* host plants were collected during 2016 and 2017 by transfer to Whatman FTA micro cards (GE Healthcare Life Sciences). Leaves with individual active oil spots were collected and kept overnight in humid zip-lock bags for sporulation. Using the direct leaf press method outlined in Whatman FTA Protocol BD05, each individual oil spot was transferred to an individual card. GPS coordinates and host cultivar were recorded on the card.

France. DNA samples from the Bordeaux region in France were supplied by Delmas et al. (2017).

DNA extraction. Extractions from fresh leaf tissue were performed according to the Powerplant Pro DNA Isolation Kit protocol (Mo Bio Laboratories). A total of 60 to 80 µl of DNA was eluted at the final step. For herbarium samples, the same protocol was used, except that the samples were ground using a micropestle with the addition of silica beads at the lysis stage. The homogenate was maintained at 55°C overnight before additional steps. Only 50 µl of the elution solution was used, and this was heated to 37°C before use.

DNA extractions from FTA cards followed the protocols described by Ndunguru et al. (2005) and Owor et al. (2007) for viral DNA. Initially, three discs were punched from the cards using a 2-mm Harris Uni-Core and washed with 300 µl of Tris-HCl buffer (10 mM Tris-HCl, pH 8) for 5 min before removing the buffer. The discs were then sequentially washed for 5 min with 300 µl of 70% ethanol and two washes with the FTA Purification Reagent (GE Healthcare Life Sciences). After drying for 2 h in a new 1.5-ml Eppendorf, 20 µl of Tris-HCl buffer (10 mM Tris-HCl, pH 8) was added, and the solution was maintained overnight at 4°C.

DNA sequencing and analysis. For determination of cryptic *P. viticola* species, representative samples from each state in Australia and the international sources were selected for sequencing of the partial internal transcribed spacer (ITS) region with primer pair ITS1-O/ITS2 designed by Rouxel et al. (2013). PCR

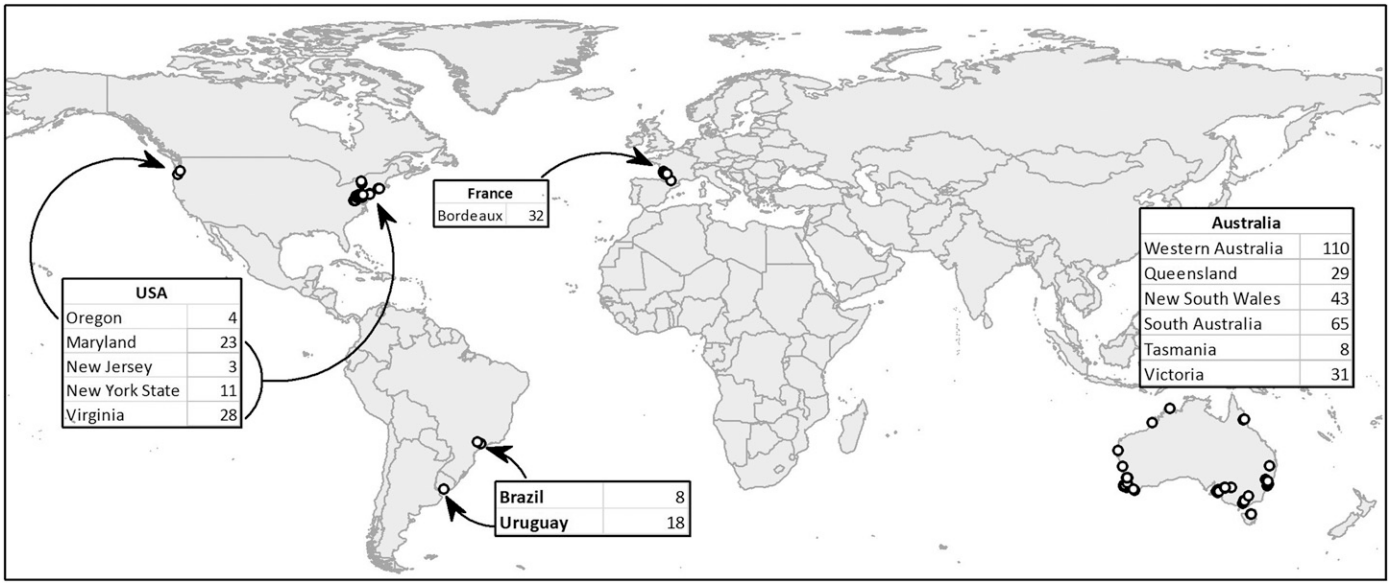

Fig. 1. Global sample of *Plasmopara viticola* isolates indicating the number of samples from each state within Australia and the United States. Isolates from Brazil, Uruguay, and France came from a single state. Circles indicate the locations of isolate collection.

amplifications were carried out with a final volume of 25 µl containing 5 µl of 5× colorless buffer (Promega), 2.5 µl of $MgCl_2$ solution (25 mM), 1 µl of bovine serum antibody, 1.5 of µl dNTP (10 mM; Promega), 0.5 µl of unlabeled forward and reverse primers (10 µM), 0.125 U of GoTaq Hot Start polymerase (5 µ/µl; Promega), and 2 µl of 1:10 diluted DNA. PCR reactions were performed using a BioRad T100 Thermocycler with the following conditions: 95°C for 4 min and 40 cycles at 95°C for 40 s, 58°C for 45 s, and 72°C for 90s with final extension of 72°C for 10 min.

The PCR and sequencing products were cleaned using Sephadex G-50 spin columns (Sigma Aldrich) and sent to the Australian Genome Research Facility (http://www.agrf.org.au/) for sequencing as described by Taylor et al. (2018). Representative sequences of the five known clades within the *P. viticola* species complex were used as a backbone to identify isolates recovered in this study. Sequences were aligned using the MAFTT algorithm plugin in the Geneious v9 software (https://www.geneious.com/) (Kearse et al. 2012). Bayesian estimation of phylogeny was conducted using the MrBayes plugin in Geneious using the GTR+G substitution model >1,000,000 generations; trees were saved each 1,000 generations, and burn in was set at 200,000 generations well after likelihood values had converged to stationary, leaving 800 trees from which the consensus trees and posterior probabilities were calculated.

Microsatellite multiplexing. A total of 16 microsatellite primers were selected to be used for population analysis based on the evaluation of Australian *P. viticola* samples by Taylor et al. (2018) (Supplementary Table S1). The 16 microsatellites were combined into three multiplex reactions based on previously published allele sizes (Delmotte et al. 2006; Gobbin et al. 2003; Rouxel et al. 2012) using Multiplex Manager v1.2 (Holleley and Geerts 2009). Each forward primer was assigned one of four fluorescent dyes (VIC, 6FAM, PET, or NED; Applied Biosystems), and pig tails (GTTT) were added to the 5′ end of the reverse primer to reduce stutter peaks (Brownstein et al. 1996). Multiplex PCRs were performed with the Qiagen Mutiplex PCR kit using the microsatellite cycling protocol with a BioRad T100 Thermocycler and an annealing temperature of 57°C for 35 cycles. Initially, 25-µl reactions were conducted, but these were later reduced to 10 µl. The PCR products were diluted 1:100 in molecular-grade ultrapure H_2O, and 2 µl of the diluted product was added to 15 µl of HiDi Formamide (Applied Biosystems) containing GeneScan 600 LIZ size standard v2.0 (Applied Biosystems) with a ratio of 15 µl of standard to 1,500 µl HiDi Formamide. Samples were run using the GeneMapper50_POP7 module and G5 filter set on an ABI PRISM 3730XL automated DNA sequencer (Perkin-Elmer Applied Biosystems). DNA from a single Western Australia isolate was included in all submitted plates as a reference isolate to ensure PCR reproducibility. Allele sizes were scored using GeneMarker software (SoftGenetics).

Microsatellite data analysis. Microsatellite allele sizes for each isolate were entered into GenAlEx 6.5 data format (Peakall and Smouse 2012). The formatted data were uploaded into the R package poppr 2.5 for population analysis (Kamvar et al. 2014, 2015). Raw data were initially analyzed to investigate whether asexual reproduction was occurring and therefore, its influence on the determination of mating structure within populations. Clonality was established in a number of populations (Supplementary Table S2), and as a result, the data were clone corrected to remove duplicated genotypes as were loci with 5% or more missing data. A sample hierarchy of country and state from where the isolate was collected (country/state) was used for clone correction. Clone-corrected data prevent bias within the statistical analysis by collapsing the samples into one observation per multilocus genotype and are recommended for populations having both sexual and asexual modes of reproduction (Grünwald et al. 2017). A genotype accumulation curve was created to ensure that the number of loci used was sufficient to capture most of the genetic diversity within each of the populations. For comparison purposes, American states were subsequently pooled into a single population named North America, and the Brazil and Uruguay samples were pooled to form a South American population.

Population summaries based on microsatellite data were calculated in poppr by calculating the Shannon–Wiener index (Shannon 2001), Stoddart and Taylor's index (Stoddart and Taylor 1988), Nei's gene diversity (Nei 1978), evenness, and the expected number of multilocus genotypes from populations with a minimum of 10 samples. In populations where all unique multilocus genotypes are equally distributed, Stoddart and Taylor's index will equal the number of isolates, expected multilocus genotypes will be equal to 10, and Nei's diversity and evenness will equal 1. For inference of mode of reproduction, clonal or sexual, within all populations, the index of association and the standardized index of association were calculated. An index of association value of zero

TABLE 1. Summary of global samples of *Plasmopara viticola* isolates indicating number and year of sampling, host species, sample type, and collector[a]

Country and state	Year	No. of samples	Host species	Sample type	Collector(s)
Australia					
WA	1997, 1999, 2009, 2014, 2015, 2016, 2017	110	*Vitis vinifera*	Fresh, herbarium	Various
QLD	2015, 2017	29	*V. vinifera*, *Vitis labrusca*	Fresh	Stephen Tancred
NSW	1976, 2015, 2016	43	*V. vinifera*, *Cissus hypoglauca*	Fresh, herbarium	Various
SA	2013, 2015, 2016, 2017	65	*V. vinifera*	Fresh	Various
TAS	2015, 2017	8	*V. vinifera*	Fresh	Dennis Patten
VIC	2015, 2016	31	*V. vinifera*	Fresh	Various
North America					
Virginia	2016	28	*V. vinifera*, *Vitis aestivalis*, interspecific *V. vinifera* hybrid	FTA card	Andrew Taylor
Maryland	2016	23	*V. vinifera*, interspecific *V. vinifera* hybrid	FTA card	Andrew Taylor
New York	2016	11	*V. vinifera*, *Vitis riparia*	FTA card	Andrew Taylor
New Jersey	2016	3	*V. vinifera*, interspecific *V. vinifera* hybrid	FTA card	Andrew Taylor
Oregon	2016, 2017	4	*Parthenocissus tricuspidata*	FTA card	Andrew Taylor
Brazil					
São Paulo	2016	8	*V. vinifera*, *V. labrusca*	FTA card	Ricardo Feliciano dos Santos
Uruguay	2017	18	*V. vinifera*	FTA card	Eduardo Abreo
France					
Bordeaux	2012	32	*V. vinifera*	DNA	Delmas et al. (2017)

[a] Year of isolate collection is considered the year of harvest to differentiate southern hemisphere production systems, which run over calendar years. NSW, New South Wales; QLD, Queensland; SA, South Australia; TAS, Tasmania; VIC, Victoria; WA, Western Australia.

indicates linkage equilibrium and is used to infer a sexually recombining population. If it is significantly greater than zero, linkage disequilibrium exists, and the population is considered clonal or of mixed mode. Statistical significance for linkage disequilibrium between the microsatellite loci of all populations was calculated using 999 permutations. Using the R package pegas, all loci for each population were tested to determine whether they were at Hardy–Weinberg equilibrium (HWE). Both the χ^2 and the exact test based on Monte Carlo permutations (999) were calculated with the null hypothesis that all alleles were randomly joining to create genotypes and met HWE assumptions.

To determine the extent of genetic similarity between populations of different countries and states within countries, an analysis of molecular variance (AMOVA) was conducted (Excoffier et al. 1992) on the microsatellite data. A total of 1,000 permutations were performed to determine whether significant differences occurred at each level of the hierarchy. A minimum spanning network (MSN) was developed in the poppr package to differentiate individual genotypes and populations using Bruvo's distance (Bruvo et al. 2004). A discriminant analysis of principal components (DAPC) (Jombart et al. 2010) was performed to determine clustering between the populations used in this study. Because no known reference population was included in the study, the DAPC used sequential *K*-means clustering to determine the optimal number of groups based on comparisons of the Bayesian information criterion (BIC) for different group numbers.

RESULTS

Cryptic species (clades) within *P. viticola*. We determined which clades of *P. viticola* exist in Australia sensu Rouxel et al. (2014). Sequences were successfully obtained from the partial ITS region from 19 samples collected during this study from both fresh leaves and FTA cards. Three of the five clades as outlined by Rouxel et al. (2014) were detected from the 19 samples sequenced (Fig. 2). All samples from each state of Australia, Brazil, and Uruguay from both *V. vinifera* and *Vitis labrusca* host species aligned with the *P. viticola* clade *aestivalis*. A single isolate from a *Vitis riparia* host in New York, United States aligned with *P. viticola* clade *riparia*. A *P. viticola* isolate from a *Parthenocissus tricuspidata* host plant from Oregon, United States aligned closely with the *P. viticola* clade *quinquefolia* sequence acquired from GenBank from Rouxel et al. (2014). Sequence data and collection information for the 19 samples have been deposited in GenBank (Supplementary Table S3). We thus accept the hypothesis that the clade *aestivalis* exists in Australia.

Fig. 2. Representative sequences of the five known clades within the *Plasmopara viticola* species complex were used as a backbone to identify isolates recovered in this study. The posterior probability is shown at the nodes. *Plasmopara euphrasiae* is included as an outgroup. Samples KF652198, JF897779, JF897782, JF897781, and JF897780 were used as "known" samples reported from Rouxel et al. (2013, 2014). BRA, Brazil; NSW, New South Wales; NY, New York; OR, Oregon; QLD, Queensland; SA, South Australia; TAS, Tasmania; URY, Uruguay; VI, Virginia; VIC, Victoria; WA, Western Australia.

Allelic diversity and clonality. Of the 16 microsatellites, the loci BER and PV74 had >5% missing, and these genotypes were removed from additional analysis. Despite seeming to indicate polymorphism, the loci GOB and CES, which contained compound repeats, made accurately determining allele calls difficult, and they were also removed from further analysis. Genotype accumulation curves were used to determine how the number of loci used in a study affects the number of genotypes observed. Ideally, they show a point of leveling where the addition of more loci does not result in more genotypes, indicating that a sufficient number of loci were used in the study. Genotypic accumulation curves conducted on all populations indicated that the 12 remaining loci were sufficient to describe the amount of genetic diversity in each population (Supplementary Fig. S1). The loci PV103 and PV146 were polymorphic across the entire dataset but had a number of populations where only a single allele was present in the population. Clone correction of the data reduced the total number of samples from 413 to 321, and the Western Australian samples reduced the most: from 110 to 27 (Tables 1 and 2).

Genotypic diversity, AMOVA, index of association, and HWE. Of the total of 321 clone-corrected isolates, there were 318 distinct multilocus genotypes between all populations in the study, indicating a high level of genetic diversity in all populations (Table 2). With only eight isolates, the Tasmanian population was relatively small and could not be compared meaningfully with the other populations within the study. All remaining populations, except for Western Australia and New South Wales, had expected multilocus genotypes equal to the sample size of 10 and Stoddart and Taylor's index values equal to the number of isolates, indicating unique genotypes within all populations (Table 2). On a global scale, the North American population had the highest genotypic diversity as indicated by the Shannon–Weiner index (4.23). The South Australian population had the second highest genotypic diversity, and it was highest among all Australian states based on the same indices. Nei's gene diversity ranged from 0.62 for the North American population to 0.26 for the Western Australian population. The AMOVA showed there was significant differentiation between Australian states (degrees of freedom = 9, sum of squared differences = 5.75, $P = 0$) (Supplementary Table S4).

Despite the diversity indices indicating that all populations in this study are genetically diverse, the index of association provided evidence of linkage disequilibrium within the Western Australian, Queensland, New South Wales, and South American populations (Table 2). The associations of the loci within these populations were all significantly different from 0 ($P < 0.01$) (Table 2), rejecting the hypothesis of linkage equilibrium and indicating that random mating or sexual reproduction is not occurring within these populations. Within all populations, there were a number of loci with significant departures from HWE (Supplementary Fig. S2). For the populations from Western Australia, Queensland, North America, and South America, there were more loci deviating from HWE (seven loci) than those in HWE. The HWE results for Western Australia and Queensland suggested that these populations were not randomly mating. A greater proportion of loci were within HWE than in departure for the France, South Australia, and Victoria populations, and therefore, they met the assumptions of random mating.

MSN. The Western Australian multilocus genotypes cluster tightly together within the MSN, indicating a close genetic

relationship despite the diversity within the population (Fig. 3). This clustering supports a scenario of a single introduction into Western Australia potentially from either South Australia or France, with subsequent clonal divergence in Western Australia. The close proximity of a number of multilocus genotypes from South Australia and France suggested that these populations were most closely related to the Western Australian population. The range of multilocus genotypes within the eastern Australian populations, South America, and France is more diverse and has various unique groupings. The multilocus genotypes from North America seemed to be the least genetically linked to all other populations analyzed in this study, forming a large distinct grouping of multilocus genotypes.

DAPC. A DAPC analysis provided additional support for the clustering observed in the MSN. We first performed *K*-means clustering using 40 principal component axes to determine how many groups to use and their membership. After four groups, there did not seem to be an appreciable decrease in our optimality criterion (BIC) by adding more groups (Supplementary Fig. S3). We performed DAPC using 40 principal component axes and 4 discriminant analysis axes with the sample divided into two through four groups and plotted the results in ggplot2 (Wickham 2009). For $K = 2$, the North American population is a distinct cluster from the remaining populations in this study (Fig. 4). At $K = 3$, the Western Australian and South Australian populations are predominantly differentiated from the remaining populations (Fig. 4). This analysis supports a likely scenario of one introduction into Western Australia, most likely following a stepping stone model from other regions in Australia and with a potential origin from France.

DISCUSSION

Viticulture in Australia has been historically linked to Europe, with multiple introductions of grapevine planting material occurring during the 18th and 19th centuries (Beeston 2001; Kerridge and Antcliff 1999), after which *P. viticola* was first identified. Our findings indicate that the populations of *P. viticola* within Australia and Europe are genetically similar. Analysis of the microsatellite markers from all populations in this study except those from North America could not be clearly differentiated, suggesting that the spread of the pathogen to Australia and South America most likely occurred from European production systems, where *V. vinifera* species were historically cultivated, rather than North American populations. It is unclear whether the introduction of *P. viticola* into Australia occurred directly from Europe or from a secondary population, such as South Africa or Brazil, because vine material is known to have been collected from there on passages to Australia (Beeston 2001; Kerridge and Antcliff 1999). The number of Brazilian samples in this study was too small to make clear inference of their relationship to the Australian population. With only a single population of *P. viticola* from Europe included, we were unable to determine whether the populations in Australia clustered more closely to the western or eastern European cluster of *P. viticola* as identified by Fontaine et al. (2013).

The North American population exhibited the highest level of genetic diversity among the populations of *P. viticola* in this study. This is not surprising given that *P. viticola* is believed to have originated in eastern North America, and five clades of the disease are found to exist there (Rouxel et al. 2014). *P. viticola* was first described in North America in 1834 compared with 1878 for France (Kassemeyer et al. 2015). A genetic bottleneck or introductions from a single-source population were proposed for the weak structure of the *P. viticola* population in Europe (Fontaine et al. 2013). Our study has found that the population genetic diversity among the French samples was similar to the South American populations and a number of the states in Australia. The Western Australian population had the lowest genetic diversity of all the populations studied. It is likely an additional example of a demographic bottleneck given that the pathogen was only detected in 1997 or 1998 (McKirdy et al. 1999; Riley 1998), and quarantine requirements still restrict any potential for additional introductions of new genotypes. In an earlier study using microsatellite primers, Hug (2005) found that Western Australia had low genetic diversity when comparing *P. viticola* populations from different states of Australia.

Despite the lower genotypic diversity within the European and Australian populations of *P. viticola*, the diversity represented within the populations differs enough to warrant a need to test newly bred resistant cultivars of *V. vinifera* in multiple locations to account for this diversity. A number of countries, including France and Australia, have breeding programs to introduce and release *V. vinifera* cultivars with known resistance to *P. viticola* (Dry et al. 2017; Venuti et al. 2013; Weidemann-Merdinoglu et al. 2017). Breakdown of host resistance has already been observed from a *P. viticola* isolate of European origin (Peressotti et al. 2010).

An aim of this research was to determine the most likely source of the Western Australian population of *P. viticola*. The AMOVA suggests that there are significant differences between the populations within different states of Australia, although it does seem from the DAPC and MSN data that there are similarities

TABLE 2. Genotypic diversity statistics of the clone-corrected data within all populations examined in this study

Population[a]	*N*[b]	MLG[c]	eMLG[d]	SE[e]	*H*[f]	*G*[g]	λ[h]	E.5[i]	*H*exp[j]	rbarD[k]	p.rD[l]
AU_Western Australia	27	26	9.87	0.33	3.24	25.14	0.96	0.98	0.26	0.14	0.01
AU_South Australia	63	63	10	1.38E-06	4.14	63	0.98	1	0.44	0.02	0.12
AU_Queensland	26	26	10	1.09E-06	3.26	26	0.96	1	0.45	0.15	0.01
AU_New South Wales	39	38	9.94	0.24	3.63	37.10	0.97	0.99	0.39	0.07	0.01
AU_Victoria	31	31	10	1.78E-06	3.43	31	0.97	1	0.42	–0.00	0.70
AU_Tasmania	8	8	8	0	2.08	8	0.88	1	0.39	0.12	0.02
N_America	69	69	10	5.43E-06	4.23	69	0.99	1	0.62	0.01	0.14
S_America	26	26	10	1.09E-06	3.26	26	0.96	1	0.49	0.09	0.01
FR_Bordeaux	32	32	10	0	3.47	32	0.97	1	0.51	0.02	0.11
Total	321	318	9.99	0.05	5.76	315.11	1.0	0.99	0.58	0.16	0.01

[a] AU, Australia; FR, France; N_America, North America; S_America, South America.
[b] Number of isolates in the population.
[c] Multilocus genotypes.
[d] Expected number of MLG at the smallest. samples size >10.
[e] Standard error based on the eMLG.
[f] Shannon-Wiener index of MLG.
[g] Stoddard and Taylor's index of MLG diversity.
[h] Simpson's index.
[i] Evenness.
[j] Nei's unbiased gene diversity.
[k] Standardized index of association.
[l] Significance of rbarD ($P < 0.01$).

between the Western Australian population and the South Australian population when up to four population groups are assigned. The tight clustering of Western Australian genotypes with a few South Australian genotypes in the MSN indicated that there might have been one introduction into Western Australia. The inclusion of herbarium samples highlights that no major population changes have occurred within the Western Australian population since the 1997 invasion. Trace back of the host planting material of the 1997 detection in northern Western Australian did not define a conclusive source, but there was a possibility that vines were introduced from the Northern Territory (Riley 1998). Until isolates of *P. viticola* from the Northern Territory are analyzed, we cannot conclusively determine whether the source of the Western Australian population of *P. viticola* is from South Australia or the Northern Territory or whether there is a relationship between all three populations. However, the potential of a natural pathway does exist in northern Australia, because several native *Vitaceae* species, including *Ampelocissus* and *Cissus* species, are reported to be susceptible to *P. viticola* infection (Emmett et al. 1992b).

Sequence analysis on representative samples taken from all states in Australia and those from populations in Brazil and Uruguay revealed all isolates to be *P. viticola* clade *aestivalis*. This clade is likely to have originated from colonization of a *Vitis aestivalis* host and undergone host range expansion to *V. labrusca* and the European grapevine species *V. vinifera* (Rouxel et al. 2013). The representative samples in this study came from both *V. labrusca* and *V. vinifera* hosts. *P. viticola* clade *aestivalis* is the predominate clade infecting *V. vinifera* cultivars in North America (Rouxel et al. 2014), and therefore, it is not surprising that this clade is the most widely spread in global *P. viticola* populations. Representative isolates of *P. viticola* clade *riparia* and *P. viticola* clade *quinquefolia* in this study were isolated from *V. riparia* and *Parthenocissus tricuspidata* hosts, respectively, confirming the findings of Rouxel et al. (2013, 2014). The discovery of clades within *P. viticola* with different host ranges may represent new biosecurity threats to the native *Vitaceae* species present within Australia, some of which have conservation status owing to restricted distribution (https://florabase.dpaw.wa.gov.au/).

The grape downy mildew lifecycle is dimorphic, consisting of both sexual and asexual reproductive stages (Rossi et al. 2008), and therefore, it is not unusual to observe clones within population studies. After clone correcting the data, the index of association

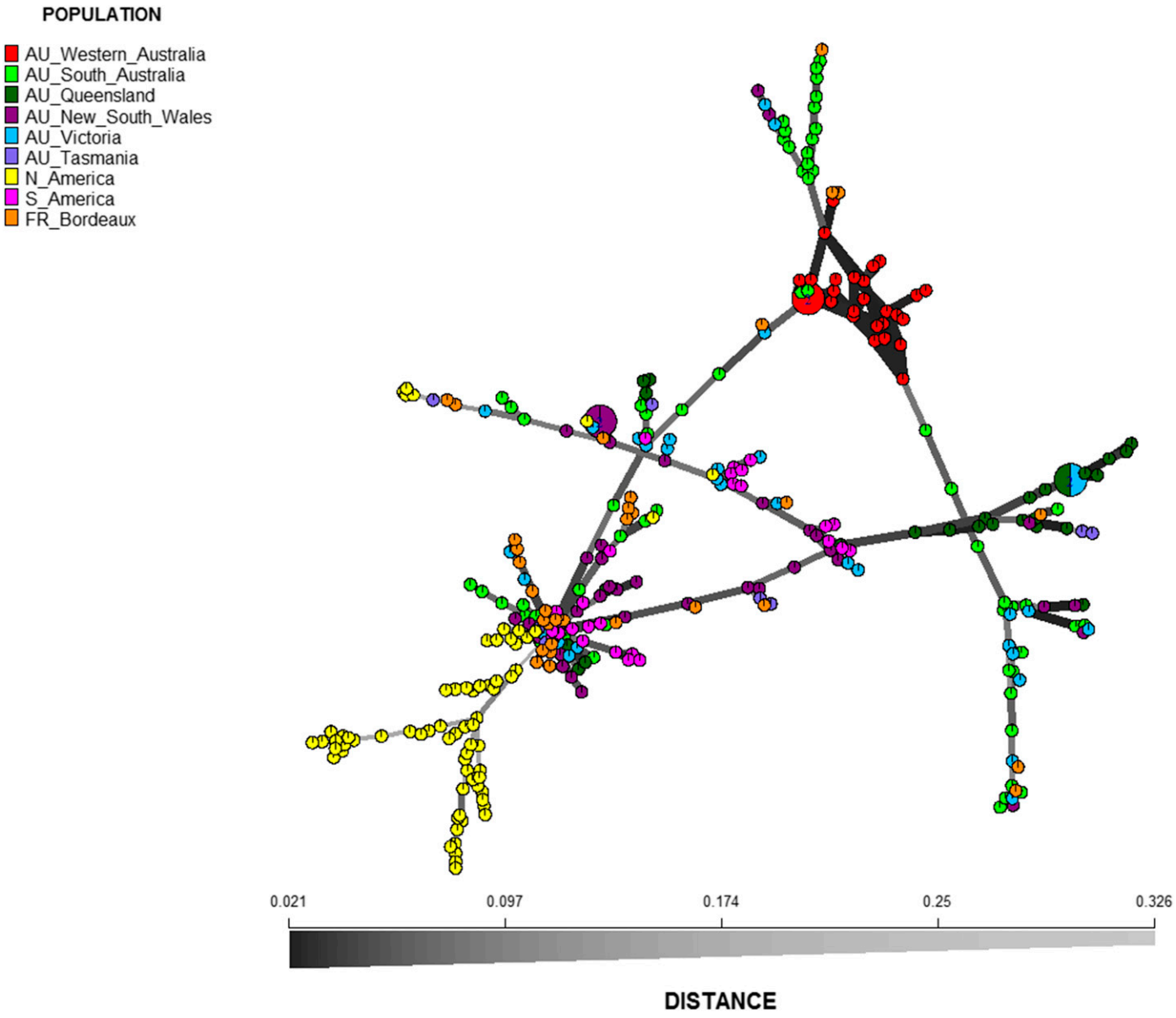

Fig. 3. Minimum spanning network of all isolates of *Plasmopara viticola* after clone correction based on Bruvo's distance. Each node represents a multilocus genotype, with nodes of the same color indicating the same population with a country/state hierarchy. The greater the genetic distance, the thinner and lighter the color of the line. The larger the node size, the greater the number of samples with the same microsatellite profile. AU, Australia; FR, France; N, North America; S, South America.

analysis of the Western Australian, New South Wales, Queensland, and South American populations within our study seems to show reproduction either asexually or via cloning. This is interesting, because the isolates for several of these populations, particularly Western Australian, were collected over several seasons. Studies in Greece and South Africa have also detected the presence of the same genotype over successive seasons (Koopman et al. 2007; Rumbou and Gessler 2006). It has been proposed that *P. viticola* can survive in an asexual form within grapevine material in areas that experience mild winters, including Greece, South Africa, and Australia (Killigrew 2006; Koopman et al. 2007; Rumbou and Gessler 2006). It has long been thought that the pathogen may be able to overwinter within dormant buds similar to grape powdery mildew, *Erysiphe necator* (Killigrew 2006; Rumbou and Gessler 2006), but an investigation suggests that this is not occurring in Western Australia (Taylor 2018). The sheer distances between isolates collected within mature vineyards in Western Australia, >250 km, indicate that natural spread between regions is unlikely to occur in a single season.

Findings in this study raise questions about the understanding of the mating system and its plasticity within *P. viticola*. European and American populations of *P. viticola* are strictly heterothallic, with two distinct mating types being required for the formation of oospores (Scherer and Gisi 2006; Wong et al. 2001). Our study indicates that random mating is still occurring within French and North American populations, where optimal climatic conditions are prevalent. The prevailing Mediterranean climate experienced in Western Australian is only sporadically favorable to the development of *P. viticola*, leading to regular extinction and recolonization events (Hug 2005; McLean et al. 1984; Weltzien 1981). Similar conditions occur within growing regions of Greece and South Africa (Koopman et al. 2007; Rumbou and Gessler 2006). Surveys of the majority of the viticulture regions within Western Australian have detected *P. viticola* oospores (Taylor and Wicks 2017). The presence of oospores in a clonal population may be evidence of *P. viticola* secondary homothallism or selfing as a mechanism to survive periods of unsuitable climatic conditions. This would not be unusual for an oomycete pathogen in Western Australian. For example, *Phytophthora cinnamomi* has been reported to survive the dry hot summers by producing selfed, sexual oospores in a number of hosts, despite the presence of both mating types (Crone et al. 2013). The breakdown of mating type regulation may result as an evolutionary advantage for heterothallic oomycetes to, on occasion, form oospores for resisting harsh environments (Judelson 2009). Scherer and Gisi (2006) observed *P. viticola* oospore formation without crossing of isolates and suggested that both mating types may be present within the same lesion. Rather, the observation of sexual oospores without mating type crosses could be evidence of the ability of *P. viticola* to self. The formation of selfed oospores would explain the rapid and long-distance spread of the disease and its ability to survive after extinction events in Western Australia. Additional investigations are warranted to understand the survival mechanism and reproduction of *P. viticola* under suboptimal environments.

The successful use of the FTA cards in storage and amplification of *P. viticola* DNA during this study will aid in the understanding of the population structure of the disease globally. Less is known about populations of *P. viticola* than other economically damaging oomycete pathogens, such as *Phytophthora infestans*. This lack of knowledge is in some part because of *P. viticola* being a biotroph, requiring plant material to be collected, stored appropriately, and in some instances, mailed to different research laboratories to obtain results. This creates issues in regard to samples being perishable or quarantine regulations with the movement of live plant material. The FTA cards, with viability reported to last over a decade (Smith and Burgoyne 2004), remove some of the problems encountered in working with *P. viticola* populations.

Fontaine et al. (2013) discussed the requirement to understand the incursion pathway of *P. viticola* into New World viticulture. This study has highlighted the movement of the pathogen to two New World viticulture regions in Australia and South America and in doing so, has increased the knowledge surrounding the population structure of *P. viticola* globally. The addition of South Africa to *P. viticola* population studies is required to completely understand any linkages with Australian populations given the historical movement of grape material between the two countries. The discovery of clonal or selfing populations of *P. viticola* with the presence of oospores presents potential capacity for long-distance movement of the pathogen previously not considered possible.

Fig. 4. Discriminant analysis of principal components clustering of *Plasmopara viticola* isolates with two to four populations (*K*). Bars of the same color represent the likelihood of the same genetic cluster based on microsatellite data analysis. Bars of mixed colors are admixed individuals/isolates. Fr, France; NAm, North America; NSW, New South Wales; QLD, Queensland; SA, South Australia; SAm, South America; T, Tasmania; VIC, Victoria; WA, Western Australia.

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