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Population genetic structure of *Plasmopara viticola* in the Western Cape Province of South Africa

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SUMMARY

Plasmopara viticola populations in South Africa were studied for two consecutive grape growing seasons, in an organically managed and a conventional fungicide-sprayed vineyard. Three to four samplings in each season were genotyped with four microsatellite markers (GOB, CES, ISA and BER). Population differentiation (*Fst*) between the conventional fungicide-sprayed vineyard and organically managed vineyard was low (0.004 and 0.016) in both growing seasons, suggesting one metapopulation. However, differences in the relative contribution of the predominant and new genotypes to epidemics in the two vineyards suggested that fungicide applications may have selected for reduced pathogen diversity. In both years and vineyards, sexual (oosporic) reproduction and/or migration occurred throughout the year and contributed between 12 and 74% to the epidemic. Hardy–Weinberg analyses suggest that South African *P. viticola* populations are randomly mating. Epidemics in both years and vineyards were dominated by one or two genotypes that each contributed between 14 and 67% to the epidemic through asexual reproduction. The remaining genotypes showed low levels of asexual reproduction, with most genotypes never being able to reproduce asexually. However, for some genotypes asexual reproduction was important, as it enabled survival of the genotypes from one season to the next. In total, ten genotypes were able to survive asexually or vegetatively from one season to the next. The populations were further characterized by the presence of a high frequency of isolates that most likely have elevated ploidy levels.

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INTRODUCTION

Downy mildew, one of the most destructive diseases of grapevines, is caused by the diploid, obligate biotrophic oomycete *Plasmopara viticola* (Berk et Curt.) Berl. et de Toni (Lafon and Clerjeau, 1994; Wong *et al*., 2001). Similar to most other oomycetes, *P. viticola* (kingdom Stramenopila, phylum Oomycota) can reproduce asexually through the production of sporangia that release zoospores, as well as sexually through thick-walled resistant oospores (Förster *et al*., 1990; Harper *et al*., 2005; Lafon and Clerjeau, 1994). As *P. viticola* is heterothallic, sexual reproduction can only take place if both mating types (A1 and A2) are present (Wong *et al*., 2001).

Plasmopara viticola is native to Northern America, from were it was accidentally introduced to Europe and the rest of the world. This introduction in the late 1870s was indirectly due to the devastating root-feeding insect phyloxera (*Daktulosphaira vitifoliae*), which prompted the replacement of old vines with new vines grafted on to phyloxera-resistant rootstocks from North America that contained downy mildew inoculum (Alexopoulos *et al*., 1996; Lafon and Clerjeau, 1994). The introduction of *P. viticola* into Europe most probably consisted of more than one introduction event from North America (Gobbin *et al*., 2006a). In South Africa, downy mildew was officially reported for the first time in 1907 in the Eastern Cape Province, although it was thought to be present in this region prior to the reported date (Dewar, 1907). However, in the largest grape-growing region in South Africa, the Western Cape Province, the pathogen was not reported until 1968 (Marais, 1973). Subsequent to its report in this region, the pathogen was found sporadically in some regions in the Western Cape, whereas in other regions (Stellenbosch, Wellington, Paarl and Robertson) it was more prevalent and was detected in each of five consecutive growth seasons (Marais, 1973). The source population(s) for the introduction into South Africa is unknown.

World-wide all *P. viticola* populations, except the North American populations, are considered as founder populations that were founded through the importation of grapevines. Owing to genetic drift and natural selection, all of these populations

should be characterized by a reduced level of allelic diversity. However, as South African *P. viticola* populations in the Western Cape Province have only been colonizing grapevines for approximately 39 years (Marais, 1973), compared with 125 years in Europe (Gobbin *et al.*, 2006a), there would have been less opportunities for multiple introductions. Therefore, it is hypothesized that the South African *P. viticola* populations in the Western Cape will have a strong signal of genetic drift. This signal is expected to be even stronger in populations that are subjected to fungicide treatment due to genetic drift and selection. Application of fungicides for which resistance have been reported, including carboxylic acid amides (iprovalicarb, benthiavalicarb, dimethomorph and flumorph) phenylamides (metalaxyl) and cyanoacetamide-oximes (cymoxanil) (Gisi *et al.*, 2007; Gullino *et al.*, 1997; Leroux and Clerjeau, 1985), will most likely result in fixation of alleles in clonal populations leading to adaptation by sorting non-randomly among mutations. Therefore, it can be hypothesized that genetic differentiation will be present between clonally reproducing *P. viticola* populations from vineyards that are managed organically vs. vineyards where fungicides have been applied. If populations are randomly mating, the signal of genetic differentiation among populations will be lost due to random sorting of genes among individuals.

The asexual phase (sporangia), also referred to as secondary inoculum, of *P. viticola* has long been viewed as the life-cycle stage that is most important in causing expansion of epidemics. The pathogen has many asexual cycles during the season under favourable environmental conditions. These cycles result in mass production of inoculum over short time spans, as well as long-distance dispersal of sporangia. By contrast, the role of the sexual phase (oospores) has been primarily viewed as only providing the initial primary inoculum of the epidemic at the start of the season (Blaise *et al.*, 1999; Lafon and Clerjeau, 1994). However, *P. viticola* population genetic studies in Europe, mainly using microsatellite markers but also randomly amplified polymorphic DNA (RAPD) markers, have recently challenged these long-standing epidemiological views (Gobbin *et al.*, 2006a,b; Stark-Urnau *et al.*, 2000). These studies have concluded that oosporic infections not only contribute to primary infections, but that they can also contribute significantly (26–78%) to the development of epidemics throughout the growing season (Gessler *et al.*, 2006; Gobbin *et al.*, 2003b, 2005, 2006a; Rumbou and Gessler, 2004). Furthermore, it has been shown that only a few genotypes will significantly contribute, through asexual reproduction, to expansion of the epidemic, with most genotypes not producing a significant number of secondary infections. Currently, it is hypothesized that long-distance migration of asexual spores are limited (Gessler *et al.*, 2006; Gobbin *et al.*, 2003a,b, 2005, 2006a,b; Rumbou and Gessler, 2004, 2006).

Microsatellite markers for genotyping *P. viticola* isolates have been developed independently by two research groups. The first set of markers was developed by Gobbin *et al.* (2003a), and consists of five markers containing a range of 2–101 alleles per locus. These markers have been used to study several European populations (Gobbin *et al.*, 2003a,b, 2005; Rumbou and Gessler, 2004, 2006), one North American population (Eugster *et al.*, 2003; Kennelly *et al.*, 2005, 2006) as well as five Australian populations (Hug *et al.*, 2006). Recently, Delmotte *et al.* (2006a) developed a second set of seven microsatellite markers that contains relatively low levels of polymorphisms, ranging from one to six alleles per locus. These markers have only been used to characterize *P. viticola* populations on a more limited scale in Europe (1008 lesions) and North American (50 lesions) (Delmotte *et al.*, 2006a,b).

In South Africa, the severity of downy mildew epidemics varies between years, due to substantial variation in climatic conditions (Marais, 1973). In most years, epidemics can be readily controlled due to low precipitation levels and high temperatures. However, in years with high rainfall and lower temperatures explosive epidemics occur that are difficult to control. The relative contribution of sexual and asexual reproduction, as well as migration to the development of epidemics in South Africa is currently unknown. This is an important epidemiological aspect of the disease that will influence management strategies.

The primary focus of this study was to investigate the population genetic structure of *P. viticola* to understand better the biology of the disease in the Western Cape Province of South Africa, by drawing comparisons mainly with populations from Europe. Because of different climatic conditions in South Africa and a younger grape industry, it is likely that the population structure of *P. viticola* is significantly different from European populations. The source population(s) from which the South African population was founded, would also have played an important role in the current population structure. The study was conducted in one organically managed and one conventional fungicide-treated vineyard in two consecutive growing seasons in the Western Cape Province, which is the largest grape-growing region in South Africa. The specific aims of the study were (1) to determine whether these populations are randomly mating, and what the relative contribution of sexual and asexual reproduction and/or migration is to the development of epidemics, (2) to determine whether *P. viticola* genotypes can survive asexually or vegetatively from one season to the next, and (3) to determine whether vineyard management, organic vs. conventional fungicide treatment, will influence the *P. viticola* population structure.

RESULTS

Weather conditions and development of epidemics

Downy mildew symptoms appeared at approximately the same time period (beginning to mid October) in the two sampling seasons (2004/05 and 2005/06) after a period of high rainfall (36

and 60 mm/day) in September. In general, the 2004/05 season was more conducive to the development of downy mildew than the 2005/06 season. Within the survey period, there were a total number of 13 rain events in 2004/05 and only seven rain events in the 2005/06 season. The average daily temperatures and relative humidity were 18 °C and 90%, 22.2 °C and 62%, and 24.1°C and 50% at the start (September–October), middle (November–December) and end (January–February) of the 2004/05 season, respectively, whereas temperatures and relative humidity were 18.1°C and 75%, 26.5°C and 50%, and 31.2°C and 36% at the corresponding times in the 2005/06 season.

In the organic vineyard, the initiation of epidemics was slow at the beginning of both seasons but increased in severity as the year progressed (Table 1). In both years, approximately 60–90% of the vines were heavily infected at the end of the season. The epidemic in this vineyard continued into January and February in both years, most likely due to the weekly, alternating application of compost tea and a copper–sulphur mixture that were not effective in stopping expansion of the epidemic.

In the conventional fungicide-managed vineyard, the disease initially progressed at a rapid rate in 2004/05, but was readily controlled with fungicide applications in the middle and at the end of the season. In the 2004/05 season fosetyl-Al and mancozeb, iprovalicarb and propineb, as well as potassium phosphate were applied. Unfortunately, the exact application time of these fungicides is unknown due to a change in ownership of the farm. In the 2005/06 season the epidemic in the fungicide vineyard was initially slow but became more severe during the middle of the season (November) after which the epidemic stopped. The spray programme during this season consisted of four phosphoric acid and four cymoxanil (cyanoacetamide-oximes) sprays. In both seasons no new or living lesions were found from December through to February, in the conventional fungicide vineyard. This is most likely due to the stringent spray programmes that were applied, along with unfavourable environmental conditions for disease development at the end of November through to January.

Table 1 Occurrence of *Plasmopara viticola* genotypes and genotypic diversity of populations collected at different sampling times in two consecutive growing seasons in an organic and conventional fungicide-managed vineyard.

Sample	Sampling date	Lesions analysed*	Lesions by predominant genotype (%)†	Total no. genotypes	New genotypes (%)‡	Genotypes causing > 5 lesions (%)	Lesions by new genotypes (%)§	E_H¶
Organic vineyard								
2004/05	1st—04/10/04	16 (P)	0 (6)	5	100	0	100	—
	2nd—08/12/04	76 (T)	18 (20)	32	88	6	74	0.40
	3rd—08/02/05	113 (P)	14 (12)	53	58	9	30	0.38
	Total**	205	11 (13)	71	—	8	—	0.36
2005/06	1st—06/10/05	11 (T)	18 (0)	8	100	0	100	—
	2nd—14/11/05	98 (T)	44 (7)	27	85	15	48	0.37
	3rd—28/11/05	110 (P)	21 (13)	51	69	6	42	0.39
	4th—30/01/06	97 (P)	2 (16)	49	69	10	44	0.39
	Total	316	22 (10)	106	—	13	—	0.38
Conventional fungicide vineyard								
2004/5	1st—04/10/04	64 (P)	14 (2)	31	100	3	100	0.43
	2nd—26/10/04	67 (T)	28 (22)	23	22	9	12	0.38
	3rd—08/12/04	53 (T)	39 (25)	18	33	28	15	0.38
	Total	184	27 (16)	48	—	10	—	0.41
2005/06	1st—06/10/05	13 (T)	23 (0)	6	100	0	100	—
	2nd—14/11/05	23 (T)	30 (17)	11	73	9	39	0.26
	3rd—28/11/05	121 (T)	67 (2)	17	82	18	28	0.25
	Total	157	40 (6)	30	—	13	—	0.25

*Lesions were collected using either a partial (P) or total (T) sampling strategy. During a total sampling strategy all the lesions were collected, whereas in the partial sampling strategy only two lesions were sampled from every second vine.

†Number of lesions containing the most predominant genotype, followed by the number of lesions caused by the second most dominant genotype in parentheses.

‡Number of genotypes that were not detected in the previous sampling, divided by the total number of genotypes detected for each specific sampling time.

§Number of lesions caused by new genotypes divided by the total number of lesions of each specific sampling time.

¶Shannon's equitability index calculated by H'/H'_{max}, where $H'_{max} = \ln N$ (N = number of individuals in the sample).

**Values represent those from pooled samples.

Microsatellite amplification

All loci had two alleles per locus as in a typical diploid system, except GOB for which a relatively high percentage of the genotypes (41%) contained three alleles. Genotypes with three alleles at GOB occurred in similar frequencies in both the organic and the fungicide-managed vineyards. In isolates where three alleles occurred at GOB, the locus was excluded in allele-based analyses, but retained for genotypic analyses (Tables 1 and 3).

Genetic structure in the organic and conventional fungicide managed vineyards

In total, in the organic vineyard 530 lesions were genotyped in the two seasons, compared with 340 lesions in the fungicide-managed vineyard. The total number of genotypes detected in the organic vineyard was 71 (out of 206 lesions) and 106 (out of 324 lesions) in the 2004/05 and 2005/06 seasons, respectively, compared with a total number of 48 (out of 183 lesions) and 30 (out of 157 lesions) genotypes in the corresponding seasons in the fungicide managed vineyard (Table 1). In 2004/05, half of the lesions from the first sampling in both vineyards could not be amplified. Therefore, these two samples were considered as sampled partially (Table 1).

The diversity of loci and genotypic diversity were similar in the organic and conventional fungicide treated vineyards. The most diverse locus according to the number of alleles and allelic richness after rarefaction and Nei's gene diversity was GOB, followed by CES. Loci ISA and BER were similar in their diversity (Table 2). Genotypic diversity (E_H) was similar for all populations (0.36–0.41) except for low values obtained in 2005/06 in the fungicide-managed vineyard (0.25) (Table 1).

The percentage of lesions caused by the predominant genotype in individual samples ranged from 2 to 44% in the organic vineyard and 14 to 67% in the fungicide-managed vineyard (Table 1). On average across samples within a year, the percentage of lesions caused by the most predominant genotype in the organic vineyard was 11% in 2004/05 and 22% in 2005/06. In the fungicide-managed vineyard, this ranged from 27% in 2004/05 to 40% in 2005/06 (Table 1).

Spatial–temporal analysis of the predominant genotypes in the organically and conventional fungicide-managed vineyards (Table 1) in each of the growing seasons showed that five of the eight genotypes expanded from an initial focus, with the number of lesions increasing over time with limited dispersal. It should be noted that the first infections caused by one of the predominant genotypes (G1) in the organic vineyard in the 2004/05 season were not detected, probably due to the limited nature of the

Table 2 Number of alleles, dominant alleles, private alleles and gene diversity of *Plasmopara viticola* populations sampled during different times in an organic and conventional fungicide-managed vineyard in the 2004/05 and 2005/06 grape-growing seasons.

		Number of alleles (k)				Allelic richness				Dominant allele size*				Private alleles				Gene diversity†			
Sampling		k_{ISA}	k_{GOB}	k_{CES}	k_{BER}	ISA	GOB	CES	BER	ISA	GOB	CES	BER	ISA	GOB	CES	BER	ISA	GOB	CES	BER
Organic vineyard																					
2004/05	1st	2	7	5	2					144.1	369/385.8	171	180.9					0.18	0.84	0.72	0.32
	2nd	2	16	9	2					144.1	385.8	139	180.9		1			0.29	0.84	0.79	0.37
	3rd	3	20	10	2					144.1	385.8	139	180.9	1	1			0.37	0.88	0.78	0.29
	Total	3	22	11	2	2.3	14.4	7.9	2.0	144.1	385.8	139	180.9	1	2			0.32	0.88	0.80	0.34
2005/06	1st	2	6	4	2					144.1	385.8	171	180.9					0.38	0.80	0.65	0.12
	2nd	2	13	8	2					144.1	385.8	139	180.9					0.38	0.88	0.74	0.18
	3rd	2	13	7	2					144.1	385.8	139	180.9					0.43	0.88	0.67	0.21
	4th	2	17	8	2					144.1	385.8	139	180.9		3			0.46	0.87	0.72	0.35
	Total	3	21	11	2	2.0	12.3	6.9	2.0	144.1	385.8	139	180.9		3			0.44	0.88	0.75	0.33
Conventional fungicide vineyard																					
2004/05	1st	2	14	10	2					144.1	369.3	171	180.9					0.26	0.77	0.75	0.23
	2nd	2	16	9	2					144.1	385.8	139	180.9					0.26	0.81	0.77	0.34
	3rd	2	11	4	2					144.1	385.8	139	180.9			1		0.31	0.81	0.69	0.24
	Total	2	17	11	2	2.0	11.0	9.1	2.0	144.1	369.3	139	180.9			1		0.28	0.80	0.79	0.24
2005/06	1st	2	7	5	2					144.1	369/385.8	139/165	180.9					0.28	0.82	0.74	0.38
	2nd	2	12	4	2					144.1	385.8	139	180.9					0.3	0.90	0.64	0.24
	3rd	2	14	8	2					144.1	385.8	139	180.9		1			0.24	0.79	0.81	0.51
	Total	2	18	11	2	2.0	14.0	8.5	2.0	144.1	385.8	165.0	180.9		1			0.28	0.85	0.82	0.38

*Allele sizes were calibrated according to a set of European *Plasmopara viticola* isolates (Gobbin *et al.*, 2003a).

†Nei's gene diversity.

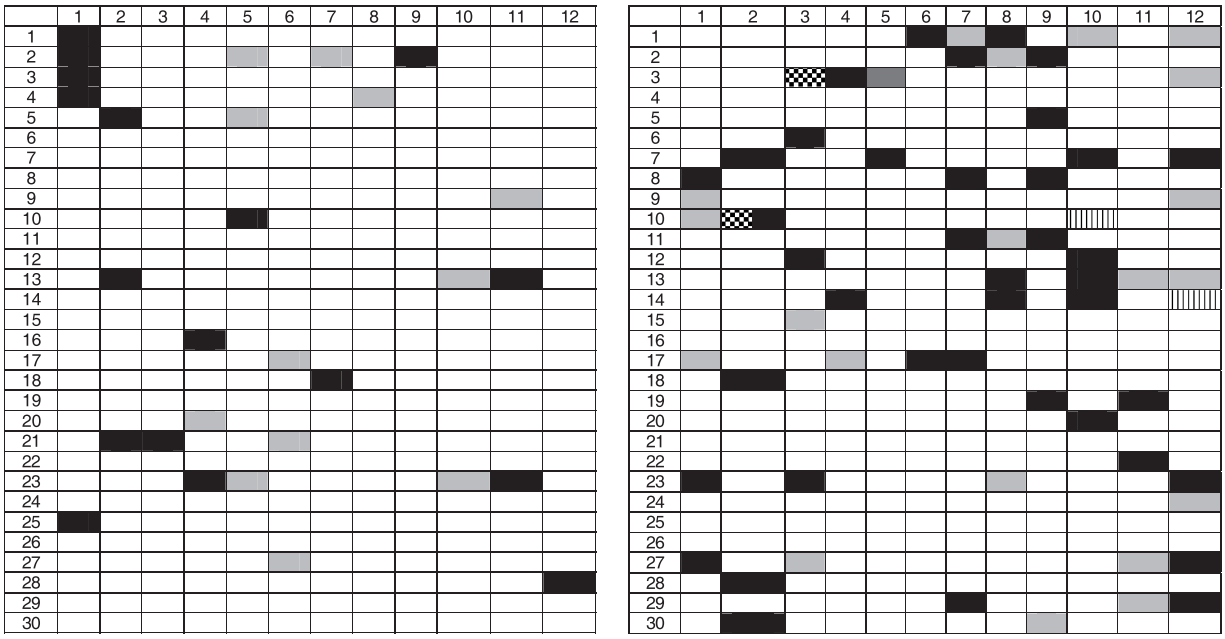

Fig. 1 Schematic representation of the predominant genotype G1 in an organically managed vineyard where *Plasmopara viticola* populations were studied in the (a) 2004/5 and (b) 2005/6 growing seasons. Each square represents one vine, with vines being spaced 2 m within rows and 3 m between rows. The dispersion pattern of the predominant genotype G1 in each growing season is shown at four consecutive samplings times (1st = ▩, 2nd = ■, 3rd = ▭, 4th = ▥).

sampling in that epidemic (Table 1, Fig. 1a). The three genotypes that did not show the aforementioned spatial–temporal pattern included genotype G1 in the organically managed vineyard in the 2005/06 season that initially expanded from a focus, but declined during the epidemic until only two lesions were detected in the fourth sampling (Fig. 1b). Also, the most predominant genotype (G8) in the 2005/06 season in the conventional fungicide-managed vineyard expanded from a focal point and had a random widespread distribution throughout the plot, whereas the second most predominant genotype in this vineyard and season fluctuated over time. Examples of the spatio-temporal spread of three genotypes in the two vineyards and seasons are represented in Figs 1and 2.

New genotypes were detected throughout the year with most genotypes, not being able to cause much more than five lesions in each sampling except for the four predominant genotypes (Table 1, column 4). The percentage of lesions caused by new genotypes was higher in samples from the organic vineyard (30–74%) than in the fungicide-managed vineyard (12–39%) (Table 1). The percentage of new genotypes detected during sampling periods was lowest in the 2nd and 3rd sampling collected in 2004/05 from the conventional fungicide-managed vineyard. The percentage of genotypes causing more than five lesions was highest in the conventional fungicide-managed orchard in the 3rd samplings of both years (Table 1), representing samples taken after five fungicide applications.

Occurrence of genotypes between years and across the two vineyards

Altogether in the two vineyards, ten genotypes were detected in both seasons, whereas five genotypes were shared among the two vineyards (Table 3). A subset of the lesions containing these genotypes, which were representative of the sampling dates and vineyards, was further genotyped using the microsatellite markers of Delmotte *et al.* (2006a). These markers also identified the lesions of each of the genotypes (G1–G10) as being clonal. One of the genotypes (G1) was detected in both growing seasons in the organically managed vineyard, and was also the predominant genotype in both seasons in this vineyard (Table 3).

Although six of the genotypes were detected in the organic vineyard as well as in the conventional fungicide-sprayed vineyard in both seasons, some of these genotypes were predominant in the one vineyard, but had a much lower frequency in the other (Table 3). The predominant genotype G10 in the conventional fungicide-sprayed vineyard in the 2005/06 season was also detected in the organic vineyard, but was present at only low frequencies in this vineyard (Table 3). Similarly, the predominant genotype G1 that was present in the organic vineyard in both seasons was only present at a low frequency in the 2004/05 season and absent in the 2005/06 season in the conventional fungicide-managed vineyard (Table 3). The predominant genotype G8 that was present in the 2004/05 season in the conventional

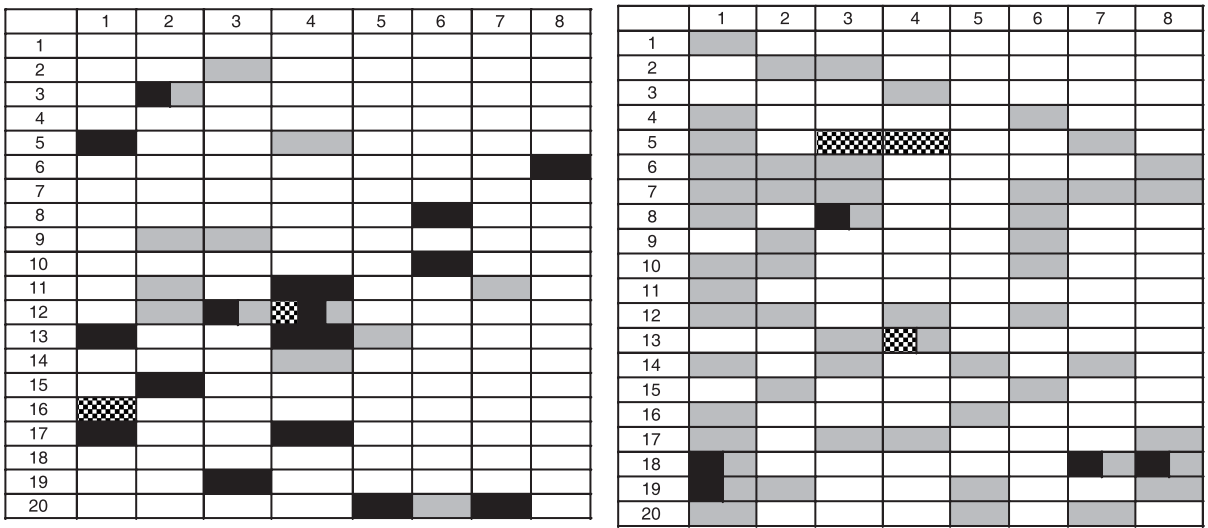

Fig. 2 Schematic representation of the predominant genotypes (a) G8 in the 2004/05 growing season and (b) G10 in the 2005/06 growing season in a fungicide-managed vineyard where *Plasmopara viticola* populations were studied. Each square represents one vine, with vines being spaced 2 m within rows and 3 m between rows. The dispersion pattern of the two predominant *P. viticola* genotypes in each growing season is shown at three consecutive samplings times (1st = , 2nd = , 3rd =).

Table 3 *Plasmopara viticola* genotypes that were able to survive between seasons (2004/05 and 2005/06) in an organic or conventional fungicide-managed vineyard. The number of lesions of each specific genotype at the 1st, 2nd, 3rd or 4th sampling time within each vineyard and year is shown. The predominant genotypes during 2004/05 were G1 and G7, and G1 and G6 during 2005/06 in the organic vineyard, whereas G8 and G10 were the predominant genotypes in the fungicide-managed vineyard during the 2004/05 and 2005/06 seasons, respectively.

	Organic 04/05‡			Organic 05/06				Fungicide 04/05			Fungicide 05/06		
Geno.†	1st	2nd	3rd	1st	2nd	3rd	4th	1st	2nd	3rd	1st	2nd	3rd
G1	0 (0)	14 (18)	16 (14)	2 (18)	43 (44)	22 (20)	2 (2)	1 (2)	5 (8)	3 (6)	0 (0)	0 (0)	0 (0)
G2	0 (0)	2 (3)	10 (9)	1 (9)	2 (2)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
G3*	1 (6)	2 (3)	3 (3)	0 (0)	1 (1)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (8)	0 (0)	0 (0)
G4*	0 (0)	0 (0)	6 (5)	1 (9)	3 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
G5	0 (0)	0 (0)	1 (1)	2 (18)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
G6	0 (0)	3 (4)	3 (3)	0 (0)	6 (6)	12 (12)	15 (15)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
G7	1 (6)	16 (20)	13 (12)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	1 (2)	2 (4)	0 (0)	0 (0)	0 (0)
G8	0 (0)	3 (4)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	2 (3)	19 (28)	20 (38)	1 (8)	0 (0)	0 (0)
G9*	0 (0)	2 (3)	2 (2)	0 (0)	5 (5)	9 (9)	1 (1)	1 (2)	5 (8)	5 (10)	1 (8)	4 (17)	2 (2)
G10	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	3 (3)	1 (2)	0 (0)	2 (4)	4 (30)	8 (34)	80 (67)

†Ten different genotypes (G1 to G10). Genotypes followed by a asterisk contained three alleles at the GOB locus.
‡Values in each column represent the number of lesions with the specific genotype, followed by the percentage of lesions with the specific genotype in brackets.

fungicide-managed vineyard was also present in the organically managed vineyard in both seasons, but remained at low levels (Table 3).

Population subdivision between organic and fungicide-managed vineyards

Gene diversities were similar in both vineyards. Three private alleles were observed in each sampling year from the organic vineyard, compared with one only in each of the sampling years from the conventional fungicide-managed vineyard (Table 2). No significant population differentiation (*Fst*) could be observed between sampling years of the organic vineyard or the fungicide-treated vineyard. However, significant *Fst* values were observed between samples of the organic and fungicide-managed vineyards. This differentiation was mainly due to differences between the 1st sampling in 2004/05 and the 3rd sampling in 2005/06, both from the fungicide-managed vineyard (Table 4).

Table 4 Estimates of pair-wise *Fst* values averaged over four microsatellite loci of 13 sampling times in two vineyards.

	Organic 04/05			Organic 05/06				Fungicide 04/05			Fungicide 05/06		
	1	2	3	1	2	3	4	1	2	3	1	2	3
Organic 04/05	1												
	2	0.00											
	3	0.00	0.00										
Organic 05/06	1	0.00	0.00	0.00									
	2	0.00	0.00	0.00	0.00								
	3	0.00	0.00	0.00	0.00	0.00							
	4	0.00	0.00	0.00	0.00	0.00	0.00						
Fungicide 04/05	1	0.00	0.03	0.05*	0.14**	0.04*	0.04*	0.09**					
	2	0.00	0.02	0.05*	0.12*	0.03	0.03	0.08**	0.00				
	3	0.00	0.00	0.00	0.03	0.00	0.00	0.02	0.00	0.00			
Fungicide 05/06	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.03	0.00		
	2	0.00	0.00	0.02	0.05	0.00	0.00	0.03	0.00	0.00	0.00	0.00	
	3	0.00	0.06	0.09**	0.20**	0.08**	0.08**	0.13**	0.00	0.00	0.03	0.12	0.00

**$P = 0.01$, *$P = 0.05$.

Because of low *Fst* values in pair-wise comparisons of all samples collected in a specific vineyard and sampled in the same growing season (Table 4), samples were pooled into four populations representing the vineyard and growing season. Significant ($P \leq 0.05$) *Fst* values were obtained in all pair-wise comparisons among the four pooled populations, except between populations sampled from the same vineyard in consecutive growing seasons (Table 5). Corresponding *Nm* values ranged from 4.2 to 6.9 in pair-wise comparisons among populations from organic and fungicide-managed vineyards (Table 5).

Random mating

Clone-corrected populations sampled at different sampling times were all analysed for Hardy–Weinberg equilibrium. Hardy–Weinberg expectations were satisfied for all loci except locus BER in all populations and locus ISA in the 2005/06 organic vineyard with a G^2 test (Table 6). For the combined populations, also only locus BER was in significant Hardy–Weinberg disequilibrium (Table 6). Excess heterozygosity (*Fis*) was observed at loci ISA and BER in both populations (Table 6).

Table 5 Estimates of pair-wise *Fst* (below diagonal) and gene flow (*Nm* = above diagonal) values averaged over four microsatellite loci of two vineyards sampled in consecutive years.

	Organic 04/05	Organic 05/06	Fungicide 04/05	Fungicide 05/06
Organic 04/05	x	~	6.5	6.9
Organic 05/06	0.00	x	4.2	4.6
Fungicide 04/05	0.04*	0.06*	x	~
Fungicide 05/06	0.04*	0.05*	0.00	x

*$P \leq 0.05$.

The analysis of molecular variance (AMOVA) showed that 95% of the genetic diversity observed in the South African *P. viticola* population can be attributed to differences within populations (d.f. = 561, MS SS = 1 445 570, $P = 0.062$). None of the variation was due to differences among populations sampled at different times during the growing season (d.f. = 11, SS = 2774, $P = 0.116$), and only 5% of the variation observed was due to differences between vineyards grown either organically or treated with fungicides (d.f. = 1, SS = 35873, $P = 0.137$).

DISCUSSION

The population genetic structure of *P. viticola* in South Africa was investigated by genotyping 521 downy mildew lesions from an organically treated vineyard and 341 lesions from a conventional fungicide-treated vineyard with four microsatellite markers. Similar to populations from Europe (Gobbin *et al.*, 2006a), the South African *P. viticola* populations have high levels of gene diversity, especially for loci GOB and CES, which also have the most alleles and highest allele richness. Furthermore, South African epidemics are also characterized by new infections throughout the growing season, as has been found in Europe (Gobbin *et al.*, 2006a; Rumbou and Gessler, 2004).

This study has revealed negative *Fis* values (–0.05 to –0.47) for three of the four loci analysed. Negative *Fis* values usually indicate an excess of heterozygosity, consistent with outbreeding, although significant migration among populations will also result in a heterozygosity excess. *Fis* values were large and positive (0.56–0.74) in all populations sampled for locus BER only. High *Fis* values indicate a reduction in heterozygosity due to

Table 6 Observed and expected heterozygosity of the different sampling times during the 2004/05 and 2005/06 seasons in an organically managed and conventional fungicide-treated vineyard.

		Heterozygosity (observed/expected)							
		ISA		GOB		CES		BER	
		O/E	*Fis*†	O/E	*Fis*†	O/E	*Fis*†	O/E	*Fis*†
Organic 04/05	1	—		—		—		—	
	2	0.35/0.30		1.00/0.87		0.94/0.81		0.10/0.37**	
	3	0.48/0.37		0.97/0.90		0.98/0.79		0.10/0.30**	
	Total	0.40/0.33	–0.24	0.98/0.89	–0.12	0.97/0.80	–0.21	0.10/0.34**	0.74
Organic 05/06	1	—		—		—		—	
	2	0.52/0.39*		0.92/0.91		1.00/0.76		0.04/0.18**	
	3	0.63/0.44**		0.87/0.91		0.90/0.68		0.03/0.16**	
	4	0.73/0.47**		0.96/0.89		0.93/0.73		0.09/0.33**	
	Total	0.66/0.45**	–0.47	0.91/0.89	–0.05	0.92/0.75	–0.30	0.08/0.33**	0.57
Fungicide 04/05	1	0.31/0.27		0.88/0.80		0.88/0.79		0.04/0.24**	
	2	0.30/0.26		1.00/0.84		1.00/0.82		0.11/0.27*	
	3	0.44/0.36		1.00/0.84		0.94/0.72		0.06/0.24**	
	Total	0.40/0.33	–0.20	0.98/0.89	–0.17	0.97/0.80	–0.18	0.10/0.34**	0.74
Fungicide 05/06	1	—		—		—			
	2	—							
	3	0.28/0.25		0.85/0.82		0.78/0.86		0.24/0.47*	
	Total	0.33/0.28	–0.17	0.90/0.87	–0.08	0.85/0.83	–0.10	0.19/0.38*	0.56

Loci which were in Hardy–Weinberg disequilibrium with a G^2 test and 1000 randomizations. $*P = 0.05$; $**P = 0.01$.

†*Fis* = Inbreeding coefficient as determined in Popgene v 3.2.

—, Populations with insufficient sample size for HWE analyses.

non-random mating within its subpopulation. Positive estimates of *Fis* usually indicate an inbreeding mating system. However, positive *Fis* values may also be recorded in outcrossing populations where there are high frequencies of null alleles at the marker loci used to estimate *Fis*, and where there is strong population substructure. Significant population structure (*Fst*) was observed between organic and fungicide-managed vineyards, but because BER is the only locus with high positive *Fis* values, it suggests that null alleles did indeed cause the large positive *Fis* values at BER. The occurrence of null alleles in *P. viticola* at BER has been suggested previously (Gobbin *et al.*, 2006a). Thus, excluding BER due to the possible occurrence of null alleles at this locus, *P. viticola* populations from South Africa appear to be randomly mating due to negative *Fis* values, Hardy–Weinberg equilibrium of the remaining three loci in most populations and high genotypic diversities.

In South Africa, oosporic (sexual) inoculum and/or migration appear to play a role in the development of epidemics throughout the season, given that new genotypes were identified throughout the growing season. The contribution of new genotypes in each sampling to disease development varied from 12 to 74%, with the contribution being the lowest in the 2004/05 conventional fungicide-managed vineyard. These new infections prevailed up until the end of each growing season, causing between 15 and 44% of the disease incidence. In Europe, Gobbin *et al.* (2005) and Rumbou and Gessler (2004) concluded through their microsatellite studies that new genotypes mostly originated from oosporic infections. Kennelly *et al.* (2005, 2006) have recently also shown the importance of oosporic infections through a field infection study where potted seedlings became infected throughout the season upon placement, at 2–5-day intervals, on naturally infected vineyard soil that was removed from the vineyard and placed in a downy mildew-free region. However, even though populations appeared to be in Hardy–Weinberg equilibrium in South Africa, suggesting random mating, we were unable to differentiate whether the new infections were due to immigrants from neighbouring vineyards or indeed oosporic inoculum. The occurrence of the same genotypes between vineyards and seasons suggests that migration also plays a significant role in establishing new infections.

In our study, the ability of genotypes that contributed to epidemics through asexual reproduction appeared to be genotype-specific. In general, only one or two genotypes dominated epidemics in both vineyards and seasons. Only one predominant genotype contributed significantly (67%) to disease development in only one of the analysed samples. All the predominant genotypes each accounted for a moderate number of lesions (average 6–40%) throughout the growing seasons. The reported frequency of a specific predominant *P. viticola* genotype varies between vineyards and countries (Gobbin *et al.*, 2006a).

However, there is a tendency in isolated mountain populations, Greek island and Australian populations for the predominant genotype to contribute to a significant amount of disease (40–94%) (Gobbin *et al*., 2003b, 2005, 2006a; Hug *et al*., 2006; Rumbou and Gessler, 2006). In these populations, there may be little sexual reproduction, or the majority of isolates in a population could have a low fitness, subjecting them to genetic drift during bottlenecks at the end of the growing season, resulting in only one or two predominant genotypes that drive epidemics.

In both analysed vineyards, only a few genotypes (3–28%) were able to show some low level of asexual reproduction, except for the four predominant genotypes (Table 1, column 4) that showed marked clonal reproduction. Most genotypes were never observed to reproduce asexually. Similar results were found in central Europe, Australia and in Greek populations (Gobbin *et al*., 2005; Hug *et al*., 2006; Rumbou and Gessler, 2006, 2007). The limited number of *P. viticola* genotypes that was able to produce more than five lesions through asexual reproduction is surprising, considering that approximately 2.4×10^5 *P. viticola* sporangia are produced on 1 cm^2 of an artificial infected lesion (Reuveni, 2003). Therefore, only a small number of sporangia ultimately seem to succeed in infecting new host material. The reason for this failure may be linked to lower levels of asexual spore production under field conditions, or a high sensitivity of sporangia to unfavourable environmental conditions such as long-wavelength UV light and high temperatures (Mizubuti *et al*., 2000; Rotem *et al*., 1985).

The presence of only a limited number of genotypes that are able to reproduce asexually could be due to the genetic background of these isolates. This would suggest that the generation through sexual recombination of a genotype that is also able to reproduce asexually might be a rare event in *P. viticola*. Limited evidence for this can be found in a laboratory study by Kast (2004) and Kast *et al*. (2001), which showed that *P. viticola* isolates differ in virulence and that not all field lesions are able to produce asexual spores under laboratory conditions. Similar observations were made in this study (unpublished data). In other oomycetes such as *Phytophthora infestans*, it has indeed been shown that in some sexual crosses the progeny lack pathogenic fitness (Mayton *et al*., 2000). Furthermore, in *P. infestans* and *P. cinnamomi* (soil-borne oomycete), most of the progeny from a sexual cross were found to be significantly less virulent than the parental isolates (Linde *et al*., 2001; Mayton *et al*., 2000). Consequently, Mayton *et al*. (2000) concluded from their study on *P. infestans*, where laboratory and field inoculations were conducted, that most progeny are not as virulent and fit as the parental strains, and that these will most likely not survive as epidemiologically important individuals. This inability of sexual progeny to survive indicates that genetic inbreeding, which is common in founder populations, may also be present in *P. viticola* populations in South Africa. Alternatively, the limited observed asexual reproduction could also be due to a limited number of niches, which when occupied by other genotypes will prevent certain genotypes from spreading clonally.

The survival of *P. viticola* genotypes from one season to the next as asexual spores or as vegetative mycelium has only recently been reported from a study in one Greek island population (Rumbou and Gessler, 2006). In South Africa, ten different genotypes survived between seasons in an asexual or vegetative form. Moreover, in the organically managed vineyard, the predominant genotype G1 in the 2004/05 season was able to survive as asexual spores or as vegetative mycelium to the next season (2005/06) where it also dominated the epidemic. It is interesting to note that the spread of the G1 genotype in the 2005/06 season also followed a step-wise dispersal pattern with only two lesions being detected at the start of the season. It is impossible to determine whether these first infections originated from within the sampled block, or whether the surrounding area could have contributed the inoculum. Survival of genotypes between seasons in South Africa might be due to the mild winters, where vegetative mycelium or sporangia may overwinter in grapevine buds or leaves that often remain hanging on the vines until the next season. Another hypothesis for the apparent survival of isolates between seasons could be that the microsatellite markers of Gobbin *et al*. (2003a) were unable to identify true clonal genotypes in South Africa, due to low levels of polymorphism in two of the tested loci (ISA and BER). However, additional genotyping of these isolates with new markers published by Delmotte *et al*. (2006a) also identified the specific genotypes as clones. Furthermore, considering the polymorphisms found with both microsatellite marker sets, the estimated number of genotypes that could be detected is 3.6×10^5 (number of allele combinations per locus = $n(n + 1)/2$, where n = number of alleles), which is a considerably higher number than what was detected in this study. We therefore conclude that the isolates are clonal.

The majority of previous *P. viticola* population genetic studies have been conducted in vineyards that were not treated with any fungicides. Where populations were sampled from fungicide-treated vineyards, specific comparisons were not made between treated and untreated vineyards (Gobbin *et al*., 2005, 2006a; Rumbou and Gessler, 2006). Therefore, this study is the first study attempting to address the effect of fungicide applications on the population structure of *P. viticola*. Population genetic analyses showed that *P. viticola* populations from organic and fungicide-treated vineyards belong to the same metapopulation because (1) there is low population differentiation (*Fst*) between the conventional fungicide- and organically managed populations (*Fst* = 0.04–0.06), (2) the majority of alleles were shared among populations with only six private alleles in the organic vineyard and two in the fungicide-treated vineyard, and (3) genotypes

were shared between populations. The sharing of genotypes between the fungicide and organic vineyard, which were separated by 7 km, provides direct evidence for effective genotype flow between vineyards. Nonetheless, it is important to note that the predominant genotype in the organic vineyard was not the predominant genotype in the fungicide-treated vineyard, even though it was present at low frequencies at all 2004/05 sampling times in this vineyard. In addition, the predominant genotypes in the fungicide-treated vineyards contributed more to the epidemic than those in the organic vineyard, suggesting selection of specific genotypes. Furthermore, fewer new genotypes could be detected during the 2004/05 season in the fungicide-treated vineyard, and genotypic diversity in the conventional fungicide-treated vineyard decreased dramatically towards the end of the season in 2005/06. Thus, although the population genetic structure of *P. viticola* supports one metapopulation, there are indications that fungicide applications (iprovalicarb, propineb, cymoxanil, mancozeb and phosphoric acid) reduced pathogen diversity. Further investigations are required in which the fitness and fungicide sensitivity of isolates from both vineyards need to be compared to determine if the reduced pathogen diversity was due to a chance effect or selection for fungicide resistance. In a study on another oomycete, Grünwald *et al.* (2006) tested the influence of fluazinam, cymoxanil, dimethomorph, metalaxyl and propamocarb fungicide applications on the genetic diversity of *P. infestans* populations in the Toluca valley. In that study, metalaxyl was the only fungicide that resulted in a reduction in genetic diversity of the pathogen (Grünwald *et al.*, 2006). Comparison between this and the *P. infestans* study is difficult as only cymoxanil is shared between the studies and the effect of fungicide applications on the genetic diversity of *P. infestans* populations (Grünwald *et al.*, 2006) was only studied during one field season.

The South African *P. viticola* populations contained some genotypes (41%) that have three alleles at the GOB locus, similar to what has recently been reported in two Greek island populations (Rumbou and Gessler, 2006). However, unlike the Greek island populations, the South African genotypes did not contain three alleles at other loci such as CES and ISA (Rumbou and Gessler, 2006). The presence of three alleles at the GOB locus in some South African genotypes, as well as in two Greek island genotypes (Rumbou and Gessler, 2006), suggests that there is either a gene duplication at this locus, or that the isolates are either triploid ($3n$) or trisomic ($2n + 1$). The presence of only three alleles in all the genotypes, with no genotypes having four alleles, suggests that the genotypes are trisomic rather than triploid. The presence of *P. viticola* isolates with elevated ploidy levels is not unexpected, as it has also been found in other oomycetes including *P. infestans, P. nicotianae, P. ramorum* and *P. cinnamomi*. In *P. infestans* and *P. nicotianae* the presence of polyploids has been shown through DNA content measurements and cytology (Sansome, 1985; Tooley and Therrien, 1991). The presence of three microsatellite alleles in some *P. cinnamomi* isolates was shown through sexual inheritance studies to be due to partial trisomy in these isolates (Dobrowolski *et al.*, 2002). Furthermore, in *P. infestans* and *P. ramorum* microsatellite analyses have also identified genotypes with three alleles at some loci (Ivors *et al.*, 2006; Lees *et al.*, 2006), suggesting elevated ploidy levels. However, the trisomic nature of these isolates suggested by the authors still needs to be investigated through sexual inheritance studies (Ivors *et al.*, 2006; Lees *et al.*, 2006).

The presence of *P. viticola* isolates with possible elevated ploidy levels is important, as this could potentially not only influence the success with which isolates reproduce sexually, but also their ability to adapt to environmental conditions (Rumbou and Gessler, 2006). In plants, it has been found that cultivars differing in ploidy level are difficult to cross (Sanford, 1983). Furthermore, Dick (1972) hypothesized that in oomycetes the development of polyploidy and possibly aneuploidy would increase heterozygosity, which could compensate for the loss of evolutionary potential often associated with a fixed genotype. It is noteworthy that two of the most virulent and persistent clonal lineages (US-8 and US-11) of *P. infestans* in the USA are most likely aneuploids (trisomic, $2n + 1$) (Fry and Goodwin, 1997; Goodwin *et al.*, 1992), supporting the hypothesis of Rumbou and Gessler (2006) that *P. viticola* isolates with elevated chromosome numbers might have an adaptive advantage. However, although in our study only three of the ten isolates that survived between seasons had three GOB alleles (Table 3), definite conclusions cannot be made since the sample sizes were too small. The generation of oomycete genotypes with elevated chromosome numbers might occur through the production of unreduced gametes, meiotic non-disjunction during sexual reproduction (Dobrowolski *et al.*, 2002; Goodwin *et al.*, 1992) or zoospore fusion (Judelson and Yang, 1998).

The findings from this study, along with those of Rumbou and Gessler (2006, 2007), suggest that the population structure and epidemiology of *P. viticola* in warmer climates might be different than what is found in regions with more favourable environmental conditions for disease development. In regions with warmer climates, such as the Greek islands, South Africa and Australia, populations often experience bottlenecks during unfavourable climatic conditions during the growing season, most likely resulting in a strong selection for genotypes capable of surviving adverse environmental conditions. In Australia the absence of oospores in many vineyards (Killigrew and Sivasithamparam, 2005) suggests that epidemics are dominated by highly adapted asexual clonal lineages that can survive between seasons. More populations need to be analysed from South Africa to support this conclusion. Investigations into the frequency and distribution of genotypes that are able to survive as vegetative mycelium or asexual spores will be particularly important in South Africa, as the downy mildew forecasting models that are used currently

predict the initiation of epidemics based on conditions favourable for oospore germination.

EXPERIMENTAL PROCEDURES

Vineyard sites

Downy mildew lesions were collected from two Red Globe vineyards. One vineyard was organically managed with only a copper–sulphur mixture and compost teas being sprayed on an alternating weekly basis, starting at the end of August until the beginning of January. The conventional fungicide-managed vineyard was sprayed with synthetic downy mildew fungicides including fosetyl-Al and mancozeb, iprovalicarb, propineb and potassium phosphate in the 2004/05 season. Unfortunately, the exact time of fungicide applications in this season is unknown due to a change in farm ownership. In the 2005/06 season in the fungicide vineyard, applications commenced at the end of August with a mancozeb application, followed by four applications of a mixture of phosphoric acid and cymoxanil approximately every 2 weeks, until the end of October. The vineyards are situated in the Northern Paarl area (Western Cape province) and are approximately 7 km apart. The experimental blocks in the organically managed vineyard consisted of 12 rows each containing 30 vines. In the conventional fungicide-managed vineyard, there were eight rows each containing 20 vines. Vine spacing was 2 m within rows and 3 m between rows in both vineyards. Weather data, which included hourly temperature, rainfall, leaf wetness and relative humidity, were recorded with a weather station in the organically grown vineyard to estimate infection periods of the disease.

Sampling

Sampling dates were based on predictions made by the Plant Plus downy mildew forecasting software (Dacom Plant Service, the Netherlands). Downy mildew lesions (known as oil spots) were collected three times during each of the 2004/05 and 2005/06 seasons, with the exception of the organically managed vineyard in which there were four sampling times in the 2005/06 season. Single lesions were sampled by removing a small section of each lesion with a sterilized scissor. The remainder of the lesion was left, allowing the genotype to continue to contribute to the epidemic. The sampled lesions were labelled according to the row, plant and leaf number in order to identify their exact location in the vineyard. The number of lesions collected at each sampling time was determined by the severity of infections, resulting in either a total (T) or partial (P) sampling strategy. A total sampling strategy was followed when the disease incidence was low and all lesions could be collected, whereas a partial sampling strategy was followed at high disease incidences, with only two lesions sampled from every second vine.

DNA isolation

DNA extraction was done using the Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Each sampled lesion was placed in an Eppendorf tube with 0.5-g glass beads (2 mm diameter), and subsequently snap frozen in liquid nitrogen. Lesions were homogenized by shaking the tubes for 10 min at high frequency (30 1/s) using a Mixer Mill Type MM 301 (Retsch Gmbh & Co. KG, Germany), equipped with a 2×12 tube (1.5 mL) adapter set. Immediately after homogenization, extraction buffer (Gobbin *et al.*, 2003a) was added and tubes were placed at 65 °C for 10 min, followed by centrifugation for 5 min at 20 000 ***g*** in order to precipitate cellular debris. The cleared supernatant (350 μL) was mixed with 525 μL of binding buffer AP3/E (Qiagen). The columns were each washed twice with 0.5 mL AW Buffer (Qiagen), followed by an ethanol wash and membrane drying according to the manufacturer's instructions (Qiagen). DNA was eluted twice with 100 μL of sterile distilled water.

Microsatellite amplification

All sampled lesions (Table 1) were genotyped using microsatellite markers (ISA, GOB, BER, CES) developed by Gobbin *et al.* (2003a). PCR amplification of ISA and BER was done in a multiplex reaction, whereas GOB and CES were each amplified in single reactions. PCR reactions consisted of a total volume of 10 μL, containing 5 μL DNA solution (not quantified), 1× Supertherm Gold buffer (Southern Cross Biotechnology Ltd, Cape Town, South Africa), and specific dNTPs, primer, Taq polymerase and $MgCl_2$ concentrations for amplification of each of the different loci. The dNTPs concentration for the ISA and BER multiplex reaction was 0.3 mM, whereas the concentrations for CES and GOB amplifications were 0.4 and 0.3 mM, respectively. The final $MgCl_2$ concentrations for the ISA and BER multiplex reaction was 1.9 mM $MgCl_2$, whereas the concentrations for GOB and CES were 1.9 and 1.8 mM $MgCl_2$, respectively. One unit Taq polymerase (Supertherm Gold, Southern Cross Biotechnology) was used for amplification of GOB, and 0.7 U Taq for CES amplification as well as for the ISA and BER multiplex reaction. Primer concentrations for amplification of the different loci were: BER_f, 0.4 μM; BER_r, 0.4 μM; ISA_f, 0.12 μM; ISA_r, 0.12 μM; CES_f, 0.14 μM; CES_r, 0.14 μM; GOB_f, 0.4 μM; GOB_r, 0.4 μM. Forward primers were all labelled with the following dyes: GOB_f and ISA_f with 6-FAM, CES_f with VIC and BER_f with PET (Applied Biosystems, Foster City, CA). PCR was performed in a Gene Amp PCR system 9700 (Applied Biosystems) under the following conditions: 7 min at 95 °C, 32 cycles of 30 s at 96 °C, 30 s at 60 °C and 50 s at 72 °C, with a final extension of 7 min at 72 °C.

A subset of *P. viticola* oil spots, consisting of 64 lesions that were representative of ten specific genotypes obtained from different sampling times, seasons and vineyards (Table 3), were also genotyped using five (Pv7, P13, Pv14, Pv17 and PV31) of the

seven microsatellite markers developed by Delmotte *et al.* (2006a). These lesions, which included some of the rare genotypes appearing in either the organic or fungicide vineyard, were genotyped with the additional markers in order to provide additional support that the putative clones that survived between seasons were indeed clonal. PCR amplification of the five markers consisted of two multiplex reactions (Pv17 along with Pv31, and Pv7 along with Pv13), whereas Pv14 were amplified in a single reaction. PCR reactions consisted of a total volume of 10 μL, containing 5 μL DNA solution (not quantified), 1× Supertherm Gold buffer, 0.7 U Supertherm Gold Taq, 0.3 mM dNTPs and specific primer and $MgCl_2$ concentrations for amplification of the different loci. The $MgCl_2$ concentration for the multiplex reactions was 2.5 mM, whereas the concentration for Pv14 amplification was 2 mM. Primer concentrations for amplification of both forward and reverse primers of Pv17, Pv31 and Pv13 were 0.1 μM, whereas 0.2 μM was used for Pv14 and 0.5 μM for Pv7 primers. Forward primers were all labelled with the following dyes: $Pv7_f$ and $Pv14_f$ with 6-FAM, $Pv17_f$ with VIC, $Pv13_f$ with PET and $Pv31_f$ with NED (Applied Biosystems). PCR was also performed in a Gene Amp PCR system 9700 (Applied Biosystems) for the multiplex reactions under the following conditions: 2 min at 94 °C, 32 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, with a final extension of 2 min at 72 °C. Amplification conditions for Pv14 were similar as for the multiplex reactions, except that the annealing temperature was 55 °C.

Fragment analysis was performed on an ABI 3100 (Applied Biosystems) genetic analyser. PCR products of GOB (3.5 μL), CES (1.5 μL) and the ISA and BER multiplex (1.0 μL) amplifications were mixed with 8 μL dH_2O. One microlitre of this mixture was added to 0.5 μL 500 LIZ™ size standard and 10 μL HiDi (Applied Biosystems) in the sequencing plate. Fragment analysis of the PCR products of the Delmotte *et al.* (2006a) loci were analysed in a similar manner, except that 1 μL of PCR product was diluted in 6 μL dH_2O. Fragments were visualized with Gene Mapper 3.0™ (Applied Biosystems). For each locus, different fragment lengths were considered as different alleles. Combining the information provided by the scoring of different loci, samples showing identical allele patterns were interpreted as clonal and thus derivatives of the same oosporic infection.

Allele sizes of the South African populations were calibrated to the allele sizes of published European populations (Gobbin *et al.*, 2003a). This was done by analysing PCR fragments from 30 European genotypes (DNA kindly provided by D. Gobbin, Institute of Integrative Biology, Swiss Federal Institute of Technology, Zürich, Switzerland) on the sequencer used in this study.

Data analyses

The relative amount of asexual reproduction during the growing season was determined with the Shannon–Wiener index (H') of genotype richness and evenness in a sample. We specifically used Shannon's equitability index ($E_H = H'/H'_{max} = H'/\ln N$) where N = sample size, which is normalized for sample size to account for populations with different sizes. The E_H of individual populations (samples) as well as for pooled samples was measured from vineyards collected from a particular growing season.

All other analyses were performed with clone-corrected data sets to prevent over-representation of alleles in frequently occurring clones. Population differentiation (*Fst*) (Weir, 1997) between pair-wise comparisons of all populations as well as between pooled samples from vineyards sampled in a particular growing season were conducted in GenALEx 6. Based on low and non-significant *Fst* values among samples collected during the same growing season, the samples were pooled to represent only four populations, namely Organic 04/05, Organic 05/06, Fungicide 04/05 and Fungicide 05/06. These pooled populations were subjected to another clone correction, *Fst* analyses among populations and also gene flow analyses among populations in GenALEx 6 (Peakall and Smouse, 2001). The allele richness of these four pooled populations was determined in FSTAT as a measure of diversity to compare organic vs. fungicide-managed populations. Because larger populations are more likely to include rare alleles, we analysed the allele richness R after rarefaction to a sample size of the populations with the fewest individuals ($N = 23$) with complete allele information at all loci.

The expected heterozygosity (*He*) (Nei, 1973) and the number of alleles and private alleles in individual and pooled populations were determined in POPGENE 3.2 (Yeh and Boyle, 1997). To determine the mating structure and relative contribution of asexual vs. sexual reproduction to the epidemic, departures of Hardy–Weinberg equilibrium were tested in POPGENE 3.2 by testing the significance of association between genotypes at loci in each population with a log-likelihood ratio G^2 statistic where probability values are obtained with a weighted randomization test. The excess heterozygosity within populations (*Fst*) (Weir and Cockerham, 1984) was tested with a permutation test (1000 permutations) where alleles were permuted among individuals within the clone-corrected populations.

A hierarchical analyses of molecular variance (AMOVA) was performed in GenALEx 6 (Peakall and Smouse, 2001) to partition the genetic variation. One thousand permutations were performed in the analysis. Hierarchies analysed were within the 13 samples (including different sampling times), among the 13 samples collected throughout the growing season in the two years from the organically and fungicide-treated orchards, and between pooled populations from the organically grown (seven pooled samples) and fungicide-treated vineyards (six pooled samples).

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