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Cryptic species and population genetic structure of *Plasmopara viticola* in São Paulo State, Brazil

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Downy mildew (*Plasmopara viticola*) is one of the most important diseases in grape-growing areas worldwide, including Brazil. To examine pathogen population biology and structure, *P. viticola* was sampled during the 2015/16 growing season from 516 lesions on nine grape cultivars in 11 locations in subtropical areas of São Paulo State, Brazil. For identification of cryptic species, a subsample of 130 isolates was subjected to cleaved amplified polymorphic sequence (CAPS) analysis, and for 91 of these isolates the ITS1 region was sequenced. These analyses suggest that the population of *P. viticola* in São Paulo State consists of a single cryptic species, *P. viticola* clade *aestivalis*. Seven microsatellite markers were used to determine the genetic structure of all 516 *P. viticola* isolates, identifying 23 alleles and 55 multilocus genotypes (MLGs). Among these MLGs, 34.5% were clonal and represented 93% of the isolates sampled. Four dominant genotypes were present in at least five different locations, corresponding to 65.7% of the isolates sampled. Genotypic diversity ($\hat{G}$ = 0.21–0.89) and clonal fraction (0.58–0.96) varied among locations (populations). Most populations showed significant deviation from Hardy–Weinberg expectations; in addition, excess of heterozygosity was verified for many loci. However, principal coordinate analysis revealed no clusters among locations and no significant isolation by distance was found, suggesting high levels of migration. The results indicate that downy mildew epidemics result from multiple clonal infections caused by a few genotypes of *P. viticola*, and reproduction of *P. viticola* in São Paulo State is predominantly asexual.

Keywords: downy mildew, epidemiology, genotypic diversity, microsatellite markers, *Vitis* spp.

Introduction

Grape downy mildew (caused by the oomycete *Plasmopara viticola*) is an economically important disease affecting viticulture worldwide, including grape-growing areas in Brazil. The pathogen is native to North America and was introduced into the grape-growing areas of Europe in the late 1870s. The first reports of the disease in Brazil date from the end of the 19th century. It is assumed that *P. viticola* was introduced on grape nursery stock imported from North America (Souza, 1996).

In Brazil, *Vitis labrusca* (fox grape) is the main grape species commercially cultivated, with *V. vinifera* (common grape) being a distant second. Indeed, almost 80% of Brazilian production is from *V. labrusca* and interspecific hybrids, such as Niagara Rosada, Niagara Branca, Isabel, Bordô, Concord, Moscato Embrapa, BRS Lorena and Seyval (Camargo, 2014). In the state of São Paulo, Niagara Rosada (a cultivar of *V. labrusca*) is the most common cultivar and represents 49% of grape production (Oliveira *et al.*, 2008). Grapes may be harvested from December to June in hot temperate regions (east-central São Paulo), while in rainy tropical regions (northwestern São Paulo), harvest extends from June to November (Conceição *et al.*, 2012).

In temperate grape-producing regions, *P. viticola* oospores overwinter in leaf litter on the vineyard floor. Oospores germinate during spring, producing a sporangium that releases zoospores that cause primary infections. Thus, oospores represent the main source of inoculum for primary infections in temperate climates (Lafon & Clerjeau, 1988). In some regions, oospores can also contribute to epidemic progress later in the growing season because they may continue to germinate and cause infections (via sporangia and zoospores) as the season progresses (Rumbou & Gessler, 2004). In contrast, in tropical and subtropical climates, due to the variable periods of harvest and/or lack of severe winters, green tissue is available during most of the year, potentially enabling asexual survival (Gobbin *et al.*, 2006; Rumbou & Gessler, 2006; Koopman *et al.*, 2007; Angelotti *et al.*, 2012). Furthermore, *P. viticola* is heterothallic and the two mating types (P1 and P2) must be present to enable sexual reproduction (Wong *et al.*, 2001). In Brazil, it is unknown whether both mating types co-occur and whether oospores are formed. Because of the climatic conditions and the availability of green tissue year-round,

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it is possible that asexual structures represent a common mechanism of survival in São Paulo.

North America is the centre of origin of *P. viticola*, as well as of many species of *Vitis* (Gessler *et al.*, 2011). The occurrence of several cryptic species of *P. viticola* has been reported recently from North America. Specifically, three cryptic species were identified on cultivated grapes: *P. viticola* clade *riparia* (infects interspecific hybrids), *P. viticola* clade *vinifera* (infects *V. vinifera* cultivars and hybrids), and *P. viticola* clade *aestivalis* (infects *V. vinifera*, *V. labrusca*, and hybrids; Rouxel *et al.*, 2013, 2014). The occurrence of cryptic species of *P. viticola* in Brazil has not been investigated previously.

The genetic structure of *P. viticola* populations has been studied in grape-producing areas around the world, particularly in Europe (Rumbou & Gessler, 2006; Rouxel *et al.*, 2012), North America (Rouxel *et al.*, 2012), South Africa (Koopman *et al.*, 2007), China (Li *et al.*, 2016) and Australia (Hug, 2005). These studies revealed that populations of the pathogen typically have high genetic variability as a consequence of recombination by sexual reproduction (Gobbin *et al.*, 2006; Li *et al.*, 2016). However, in unfavourable climatic conditions for production of oospores, reduced genetic diversity would be expected. In such situations, downy mildew epidemics may occur mainly by multiple clonal infections caused by a few genotypes (Rumbou & Gessler, 2004, 2006). In South America, and specifically Brazil, no studies regarding the population structure and presence of cryptic species of *P. viticola* have been reported. Hence, the purpose of this study was to fill this knowledge gap by shedding light on pathogen diversity and – indirectly – on mode of reproduction and sources of initial inoculum for downy mildew epidemics in the state of São Paulo, Brazil.

Material and methods

Sampling and DNA extraction

Grape leaves with signs and symptoms of downy mildew were sampled during the 2015/16 growing season from 11 locations in São Paulo, including nine cultivars and 26 different vineyards (Table 1; Fig. S1). Atibaia, Jundiai, Indaiatuba and Porto Feliz are traditional regions of grape-growing in São Paulo (since the 19th century), whereas in the other regions commercial grape production has been practised for only a few decades or less. Except for Piracicaba, where samples were collected in an experimental vineyard operated by the University of São Paulo, all other leaf samples were obtained from commercial family farms. Sampling occurred between November and December 2015, except for Votuporanga, where samples were collected in March 2016.

In total, 680 *P. viticola*-infected leaves were sampled and kept in separate bags, one leaf per bag. On each leaf, a single, distinct lesion with a fresh, sporulating colony was excised using an 8-mm cork borer. Each leaf disk was stored in a microcentrifuge tube with 0.4 g $CaCl_2$ covered with a layer of sterile cotton. Tubes with leaf disks were maintained at 4 °C until DNA extraction using a modified cetyl trimethyl-ammonium bromide (CTAB) extraction procedure (Lo Piccolo *et al.*, 2012). DNA samples were stored at −20 °C.

Table 1 Locations in São Paulo State (Brazil), grape cultivars and number of vineyards from which *Plasmopara viticola*-infected leaves were collected.

Location	Cultivar	Grape species	No. vineyards sampled
Atibaia	Niagara Branca	*Vitis labrusca*	1
	Niagara Rosada	*V. labrusca*	1
Indaiatuba	IAC Juliana	Interspecific hybrid	1
	Niagara Rosada	*V. labrusca*	3
Jarinu	Niagara Rosada	*V. labrusca*	2
Jundiaí	Niagara Rosada	*V. labrusca*	2
	Moscato Embrapa	Interspecific hybrid	1
	Isabel	*V. labrusca*	1
Leme	Niagara Rosada	*V. labrusca*	1
Louveira	Niagara Rosada	*V. labrusca*	2
Mogi Mirim	Isabel	*V. labrusca*	1
Piracicaba	Niagara Rosada	*V. labrusca*	1
Porto Feliz	Niagara Rosada	*V. labrusca*	3
	Niagara Branca	*V. labrusca*	1
	Italia	*Vitis vinifera*	1
	Centennial Seedless	*V. vinifera*	1
Santo Antonio de Posse	Patricia	Interspecific hybrid	1
	Thompson Seedless	*V. vinifera*	1
Votuporanga	Moscato Embrapa	Interspecific hybrid	1
Total	9	3	26

Identification of cryptic species

PCR was performed to amplify the internal transcribed spacer 1 (ITS1) region and the *β-tubulin* gene using primer pairs ITS1-O/ITS2 and PVc389-F3/PVc389-R4, respectively. The reaction used Illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare) with 0.5 μL each of forward and reverse primers (10 mM), 1 μL of DNA template, and double-distilled H_2O (ddH_2O) for a final volume of 25 μL. PCR was performed with an initial denaturing temperature of 95 °C for 4 min; 40 cycles of 95 °C for 40 s, 58 °C for 45 s, 72 °C for 90 s; and a final extension step at 72 °C for 10 min (Rouxel *et al.*, 2012).

Cleaved amplified polymorphic sequence (CAPS) analysis of PCR amplicons was conducted to identify the cryptic species of *P. viticola* (Rouxel *et al.*, 2014). ITS1 and *β-tubulin* amplicons were digested with *Ase*I and *Hpy*CH4V enzymes, respectively, in a mixture containing 0.3 μL restriction enzyme, 6.7 μL PCR amplicon, 1 μL 10× buffer and 2 μL ddH_2O. The CAPS reaction was performed in a thermocycler at 37 °C for 11 h followed by 65 °C for 25 min. A total of 130 *P. viticola* DNA samples (referred to as isolates henceforth), comprising five isolates from each of the 26 vineyard × cultivar × location combinations (Table 1), was analysed. Restriction fragments were visualized on 1.2% agarose gels. In addition, 91 *P. viticola* isolates comprising subsamples of three to five isolates from each combination of vineyard × cultivar × location were used for ITS1 sequencing to validate the results of CAPS analysis. The ITS1 PCR product was purified using the QIAquick PCR Purification kit (QIAGEN) and submitted to the Georgia Genomics and Bioinformatics Core (Athens, GA, USA) for Sanger sequencing.

The ITS1 sequences of the 91 isolates were aligned and compared with the sequences of the five *P. viticola* cryptic species obtained from GenBank, using GENEIOUS v. 8.1.8 (Kearse *et al.*, 2012). Phylogenetic relationships among isolates were analysed using the Bayesian inference method. The best-fit model of nucleotide substitution was determined with MRMODELTEST v. 2.3 (Nylander, 2004) based on Akaike's information criterion (AIC). Bayesian inference was performed by Markov chain Monte Carlo (MCMC) algorithm using MRBAYES v. 3.1.1 (Ronquist & Huelsenbeck, 2003). Four MCMC chains were carried out simultaneously with 10 million generations, sampling trees every 1000 generations. The first 25% of trees were discarded as burn-in. TREEVIEW (Page, 1996) was used to visualize and edit the trees.

Genotyping with SSR markers

All 680 isolates were genotyped using seven *P. viticola*-specific simple sequence repeat (SSR) markers previously developed by Rouxel *et al.* (2012) (Table 2). These markers have proved useful for analyses of *P. viticola* populations from various parts of the world including Europe, North America and China (Rouxel *et al.*, 2012; Li *et al.*, 2016). Forward primers of each primer pair were labelled with different fluorescent markers, FAM, VIC, NED and PET, for multiplex PCRs. Depending on the number of loci amplified in each multiplex reaction, each reaction contained 1 μL of DNA template, 5 μL of 2× Type-it Multiplex PCR mix (QIAGEN), 0.4 μL of forward and reverse primer mixture (10 μM), and RNAse-free water added to a final volume of 10 μL. PCR started at 94 °C for 4 min; followed by 38 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 35 s; and final extension at 72 °C for 5 min. PCR products were diluted 15-fold in distilled deionized water, and 1 μL of the diluted product was added to a mixture of 9.9 μL of Hi-Di formamide (Life Technologies) and 0.1 μL of GeneScan-500 LIZ (Applied Biosystems) in 96-well plates. Samples were denatured at 95 °C for 5 min in a thermocycler and submitted to the Georgia Genomics and Bioinformatics Core for fragment analysis.

Measures of genotypic diversity and identification of multilocus genotypes

Alleles at each locus were called using GENEIOUS v. 8.1.8. The numbers of alleles and private alleles were estimated by GENALEX v. 6.503 (Peakall & Smouse, 2012). Because sample sizes were different among populations (locations), allelic richness was assessed by HP-RARE (Kalinowski, 2005) to adjust allele number among populations (Milgroom, 2015). Genotypic diversity ($\hat{G}$) of each population was calculated using MULTILOCUS v. 1.3b (Agapow & Burt, 2001). Unique multilocus genotypes (MLGs) and clonal populations were identified using MLGSIM 2.0 (Stenberg *et al.*, 2003) from which the P_{sex} value was calculated to test whether an MLG observed more than once in a population originated from sexual reproduction. The *P* values for estimating the significance of the P_{sex} value was estimated by 1000 permutations. Significant P_{sex} values indicate that the repeated MLG is more probably the result of clonal reproduction, whereas nonsignificant P_{sex} values indicate the repeated MLG is probably derived from sexual reproduction. A clone-corrected (CC) dataset composed of only one representative of each MLG in each population (excluding those with nonsignificant P_{sex} values) was assembled for further analysis.

Table 2 Characteristics of seven microsatellite markers specific for *Plasmopara viticola* (Rouxel *et al.*, 2012).

Locus	Primer sequences (5′–3′)	Repeat motif	Size range of alleles (bp)
Pv61	TCTTCAGGTAGATGCGACCA GGTGACTCCTCGGACGAATA	$(CA)_9$	181–187
Pv91	ACCAGCCTTTGCGAAGATAA TGAAAGTTACGTGTCGCACC	$(TG)_6$	142–146
Pv101	AACACGGCGCCAAAGTATTA GGGCATTAACGTGCAAATTC	$(CTT)_6$	263–266
Pv83	TGCAGCATTGTTTCATCCAT ACACGGTACTTTGCGTTCCT	$(TG)_6$	238–242
Pv88	AATACCAAAAATGGCCGTCA ACTCTCTTGCCAGCACCATC	$(GT)_6$	202–208
Pv93	TAGCACCGGACTAGGCGTAT TGTACCCTGTTGCCCTCTTC	$(GT)_6$	147–151
Pv103	TGACCTACCACCCATTTACCA ACGGTCAGGTCAAAAGCAGT	$(TG)_6$	277–299

Analyses of random mating and population structure

Hardy–Weinberg equilibrium (HWE) was tested for each population and locus of the CC dataset using GENEPOP v. 4.2 (Rousset, 2008). Observed and expected heterozygosity were estimated by GENALEX v. 6.503. Tests for conformity with HWE expectations were conducted by the Markov chain method with 1000 dememorization and 100 batches of 1000 interactions per batch under the null hypothesis of random mating.

F-statistics for the CC dataset were also estimated using GENALEX v. 6.503. F_{IS} and F_{ST} denote heterozygosity reduction caused by the pathogen breeding system and by population subdivision, respectively (Balloux *et al.*, 2003; Milgroom, 2015). Pairwise F_{ST} values were calculated by analysis of molecular variance (AMOVA) among populations using CC data. *P*-values were determined based on 1000 permutations. AMOVA was performed for CC data using GENALEX v. 6.503.

In order to visualize the population structure of *P. viticola*, principal coordinates analysis (PCoA) of pairwise genetic distances was conducted for the 11 populations with CC data using GENALEX v. 6.503.

Analyses of spatial genetic structure

Isolation by distance (IBD) for the CC dataset was calculated based on the Mantel test using GENALEX v. 6.503. IBD among populations was evaluated by plotting pairwise values of the genetic distance, $F_{ST}/(1-F_{ST})$, against $\log_{10}$ of geographic distance.

Results

Identification of cryptic species

CAPS analysis and ITS1 sequencing showed consistent results for all *P. viticola* isolates tested. CAPS analysis of the ITS1 region of the 133 *P. viticola* isolates yielded similar band sizes (*c.* 300 bp) as the reference isolate of *P. viticola* clade *aestivalis*. The same result was observed for the *β-tubulin* gene, for which all isolates had the same band size (*c.* 700 bp) after CAPS analysis. Similarly, all 91 *P. viticola* isolates sequenced for the ITS1

region (Table S1) were identical with *P. viticola* clade *aestivalis*. In the Bayesian inference phylogenetic tree, all *P. viticola* isolates subjected to the analysis were grouped into a single clade with bootstrap support value >78% (data not shown). Therefore, both CAPS analysis and ITS1 sequencing indicated that *P. viticola* samples collected in São Paulo belonged to a single cryptic species, *P. viticola* clade *aestivalis*.

Allele frequency and genetic diversity

A total of 516 (out of 680) isolates were successfully genotyped with the seven SSR markers used in the analysis. Based on the number of alleles found within the 516 *P. viticola* isolates, the theoretical number of possible genotypes (Gobbin *et al.*, 2003) that could be resolved with these SSR markers was 4×10^5.

The number of alleles (k) at each locus varied from 2 to 6. The most diverse locus was Pv61 ($k = 6$) followed by Pv93 ($k = 4$). Allelic richness varied from 1.43 (Mogi Mirim) to 2.35 (Atibaia). Among all locations, Atibaia (a traditional area of grape production in São Paulo) had the highest genotypic diversity ($\hat{G} = 0.89$) whereas Piracicaba (where the experimental vineyard was established in 2011) had the lowest ($\hat{G} = 0.21$). Conversely, the highest clonal fraction was observed in Piracicaba (0.96) while the lowest was found in Atibaia (0.58). In addition, only five private alleles were observed in two locations, three in Atibaia and two in Porto Feliz; no private alleles were found in the other populations (Table 3).

Fifty-five MLGs were observed among the 516 *P. viticola* isolates, 19 of which were repeated MLGs that probably resulted from clonal reproduction. However, 13 repeated MLGs had a high probability to have resulted from sexual reproduction (Table 4, assigned as 'ns'). Among the clonal population, four dominant MLGs (14, 41, 32 and 13) represented 65.7% of the total isolates analysed. MLG 14 consisted of the highest number of isolates ($n = 159$) and was collected in 10 of the 11 sampling locations. The other dominant MLGs, 41, 32 and 13, were identified in at least five different locations. Even clonal MLGs with a lower number of isolates, such as MLG 9, 30 and 31, were also found across locations, indicating a high level of migration. Indaiatuba (another traditional area of grape-growing) was the population with the highest number of different MLGs, whereas Piracicaba had the lowest (Table 4).

Population genetic structure

Deviation from HWE was observed for most loci, especially locus Pv61, for which eight of 11 populations significantly deviated from HWE (Table 5). Monomorphic loci were observed in many populations, especially with loci Pv88 and Pv83. Disregarding monomorphic loci found in some populations, Pv101 and Pv103 were completely in accordance with HWE expectations. Negative values for the inbreeding coefficient ($F_{IS} < -0.185$) were observed for three loci (Pv61, Pv101 and Pv93), indicating excess of heterozygosity. However, loci Pv88, Pv83 and Pv91 were fixed for homozygosity, expressed by high positive values of F_{IS} (>0.562). Pv103 also had a positive value, but was closest to zero ($F_{IS} = 0.054$; Table 5).

Pairwise F_{ST} values ranged from 0 to a maximum of 0.471 (Table 6). Significant F_{ST} values ($P < 0.05$) were obtained in 63.6% of the pairwise population comparisons, although most of these yielded low to moderate F_{ST} values. F_{ST} values greater than 0.250 were observed in 25.3% of pairs, indicating greater genetic differentiation. Most of these high values of pairwise F_{ST} were observed between Piracicaba or Jarinu with other locations. Piracicaba and Jarinu had higher values of clonal

Table 3 Measures of genotypic diversity of *Plasmopara viticola* populations in São Paulo State, Brazil.

Location	No. isolates (n)	No. of alleles at microsatellite locus							No. MLG (g)	Clonal fraction[a]	$\hat{G}$[b]	Private alleles	Allelic richness[c]
		Pv61	Pv101	Pv88	Pv103	Pv93	Pv83	Pv91					
Atibaia	38	5	2	2	2	2	3	2	16	0.58	0.89	3	2.35
Indaiatuba	68	5	2	2	2	3	1	2	18	0.74	0.78	0	2.15
Jarinu	40	2	2	1	2	2	1	1	5	0.88	0.51	0	1.57
Jundiai	77	4	2	2	2	2	2	2	15	0.81	0.74	0	2.14
Leme	23	4	2	1	2	2	1	1	5	0.78	0.52	0	1.81
Louveira	41	3	2	1	2	2	1	1	7	0.83	0.65	0	1.64
Mogi Mirim	20	2	2	1	1	1	1	2	8	0.60	0.75	0	1.43
Piracicaba	70	2	2	1	2	3	2	1	3	0.96	0.21	0	1.85
Porto Feliz	78	4	2	1	3	3	1	2	14	0.82	0.84	2	2.16
Santo Antonio de Posse	40	4	2	1	2	2	1	2	10	0.75	0.76	0	1.97
Votuporanga	21	3	2	1	2	2	1	2	7	0.67	0.60	0	1.86
Total	516	6	2	3	3	4	3	2	55	0.89	0.84	5	—

[a]Clonal fraction = $1-(g/n)$.
[b]Genotypic diversity.
[c]Rarefied allelic richness.

Table 4 Composition of multilocus genotypes (MLGs) of *Plasmopara viticola* assigned by location in São Paulo State, Brazil.

Location from sampled isolates	Total no. of isolates	No. MLGs	MLG code and number of isolates in each MLG[a]
Atibaia	38	16	8^1, 9^1, $\underline{10}^1$, 14^3, $\underline{17}^1$, $\underline{22}^1$, 30^{10}, 31^3, 32^2, $\underline{33}^{1,ns}$, $\underline{39}^{1,ns}$, $\underline{39}^{1,ns}$, 40^4,41^6, $\underline{46}^1$, $\underline{47}^1$
Indaiatuba	68	18	$\underline{2}^1$, 3^3, $\underline{4}^1$, 6^2, 7^1, 8^1, 9^{13}, $\underline{11}^1$, 13^2, 14^{18}, $\underline{15}^1$, 23^3, $\underline{26}^1$, 30^1, 32^3, 34^1, 41^{14}, $\underline{43}^{1,ns}$
Jarinu	40	5	7^1, 8^1, 9^3, 13^8, 14^{27}
Jundiai	77	15	14^7, $\underline{20}^{1,ns}$, $\underline{20}^{1,ns}$, 23^2, $\underline{24}^1$, $\underline{28}^1$, 30^1, 31^4, 32^{10}, $\underline{33}^{1,ns}$, 32^1, $\underline{35}^1$, 40^2, 41^{37}, 42^7
Leme	23	5	3^3, 9^5, 13^{10}, 14^4, 29^1
Louveira	41	7	$\underline{1}^1$, $\underline{5}^1$, 7^1, 8^4, 9^3, 13^{11}, 14^{20}
Mogi Mirim	20	8	$\underline{18}^1$, 19^1, 23^1, 29^1, 30^3, 31^8, 40^1, 41^4
Piracicaba	70	3	$\underline{12}^1$, 14^{62}, 16^7
Porto Feliz	78	14	9^1, 13^6, 14^{14}, $\underline{25}^1$, 31^1, 32^{21}, 34^4, $\underline{36}^1$, 37^6, $\underline{38}^1$, 41^{18}, $\underline{43}^{1,ns}$, $\underline{44}^1$, $\underline{45}^1$
SA de Posse	40	10	13^2, 14^2, $\underline{27}^1$, 30^1, 31^2, 32^8, 34^1, $\underline{39}^{1,ns}$, 40^4, 41^{18}
Votuporanga	21	7	9^2, 14^2, 19^{13}, $\underline{20}^{1,ns}$, $\underline{21}^{1,ns}$, $\underline{21}^{1,ns}$, $\underline{21}^{1,ns}$

Superscript numbers indicate the number of isolates in each MLG. The superscript 'ns' indicates nonsignificant P_{sex} value, calculated using MLGSIM 2.0 (Stenberg *et al.*, 2003); this suggests the MLG is more probably derived from sexual reproduction and so should be considered as a different MLG. Underlining denotes unique (non-repeated) MLGs.

[a]Each number indicates an MLG.

Table 5 Expected (first number) and observed (second number) heterozygosity and inbreeding coefficient (F_{IS}) for each microsatellite locus for *Plasmopara viticola* populations in São Paulo State, Brazil.

Location	Pv61	Pv101	Pv88	Pv103	Pv93	Pv83	Pv91	Average all loci
Atibaia	0.74/0.94***	0.40/0.44	0.06/0.06[a]	0.22/0.13	0.22/0.25	0.17/0.13	0.49/0.25	0.33/0.31
Indaiatuba	0.75/0.94***	0.38/0.50	0.10/0.00**	0.49/0.33	0.48/0.72	0.00/0.00[a]	0.31/0.06**	0.36/0.37
Jarinu	0.50/1.00	0.32/0.40	0.00/0.00[a]	0.38/0.50	0.50/1.00	0.00/0.00[a]	0.00/0.00[a]	0.24/0.41
Jundiai	0.54/0.80**	0.32/0.40	0.12/0.00**	0.24/0.14	0.23/0.27	0.12/0.13	0.48/0.13**	0.30/0.27
Leme	0.66/1.00*	0.38/0.50	0.00/0.00[a]	0.50/0.50	0.48/0.80	0.00/0.00[a]	0.00/0.00[a]	0.29/0.40
Louveira	0.56/1.00*	0.38/0.50	0.00/0.00[a]	0.38/0.50	0.50/1.00*	0.00/0.00[a]	0.00/0.00[a]	0.26/0.43
Mogi Mirim	0.43/0.63	0.30/0.38	0.00/0.00[a]	0.00/0.00[a]	0.00/0.00[a]	0.00/0.00[a]	0.50/0.43	0.18/0.20
Piracicaba	0.50/1.00	0.50/1.00	0.00/0.00[a]	0.50/1.00	0.61/1.00	0.44/0.00	0.00/0.00[a]	0.36/0.57
Porto Feliz	0.67/1.00***	0.46/0.57	0.00/0.00[a]	0.59/0.21**	0.47/0.50*	0.00/0.00[a]	0.49/0.14**	0.38/0.35
SA de Posse	0.66/1.00***	0.38/0.50	0.00/0.00[a]	0.38/0.10*	0.26/0.3	0.00/0.00[a]	0.48/0.20	0.31/0.30
Votuporanga	0.45/0.29**	0.13/0.14[a]	0.00/0.00[a]	0.24/0.29	0.46/0.71	0.00/0.00[a]	0.34/0.14	0.23/0.22
F_{IS}	−0.484	−0.352	0.784	0.054	−0.558	0.652	0.562	−0.185

[a]Monomorphic locus for the respective population.

Asterisks indicate the population deviates from Hardy–Weinberg equilibrium at the following significance levels: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

Table 6 Estimates of pairwise F_{ST} values[a] averaged over seven microsatellite loci for *Plasmopara viticola* populations sampled in São Paulo State, Brazil.

Location	Piracicaba	Atibaia	Votuporanga	Louveira	Jundiai	Leme	Mogi Mirim	Jarinu	SA de Posse	Porto Feliz	Indaiatuba
Piracicaba	0.000										
Atibaia	0.264*	0.000									
Votuporanga	0.336*	0.053	0.000								
Louveira	0.161*	0.254*	0.262*	0.000							
Jundiaí	0.349*	0.000	0.083*	0.334*	0.000						
Leme	0.076	0.124*	0.126*	0.000	0.203*	0.000					
Mogi Mirim	0.471*	0.017	0.117*	0.449*	0.000	0.291*	0.000				
Jarinu	0.164	0.251*	0.262*	0.000	0.331*	0.000	0.468*	0.000			
SA de Posse	0.284*	0.000	0.053	0.237*	0.000	0.102*	0.027	0.238*	0.000		
Porto Feliz	0.249*	0.025	0.101*	0.203*	0.019	0.095*	0.092*	0.200*	0.000	0.000	
Indaiatuba	0.106*	0.067*	0.062*	0.022	0.122*	0.000	0.178*	0.013	0.041	0.055*	0.000

[a]F_{ST} values represent the reduction in heterozygosity caused by population subdivision. Estimates using clone-corrected dataset.

Significant pairwise F_{ST} values ($*P \leq 0.05$) are based on P values determined by 1000 permutations of the data.

Table 7 Analysis of molecular variance (AMOVA) for *Plasmopara viticola* populations in São Paulo State, Brazil.

Source	df	SS	MS	Estimated variance	Molecular variance (%)
Among populations	10	42.668	4.267	0.155	11.3
Among individuals within populations	97	126.587	1.305	0.092	6.7
Within individuals	108	121.000	1.120	1.120	82.0
Total	215	290.255	—	1.367	100.0

df, degrees of freedom; SS, sum of squares; MS, mean squares.

fraction (>0.88) and the lowest values of genotypic diversity ($\hat{G} < 0.51$), which may explain the high values of pairwise F_{ST} between these populations and populations from other locations.

The AMOVA showed that 82.0% of the molecular variance of *P. viticola* in São Paulo was due to differences within individuals. Differences among individuals within populations and among populations contributed only 6.7% and 11.3% of genetic variance, respectively (Table 7).

The first two principal components of PCoA captured 63.8% of the variance among populations (48.1% for coordinate 1 and 15.7% for coordinate 2; Fig. 1). No obvious population clusters were identified among the sampled locations, indicating the *P. viticola* populations in São Paulo belonged to a genetically similar group.

The Mantel test yielded no significant correlations between genetic distance and geographic distance ($r^2 = 0.0018$, $P = 0.600$), i.e. no IBD. This indicates high levels of gene flow or migration among populations in different locations in São Paulo.

Discussion

To the authors' knowledge this is the first study to investigate the occurrence of cryptic species and the

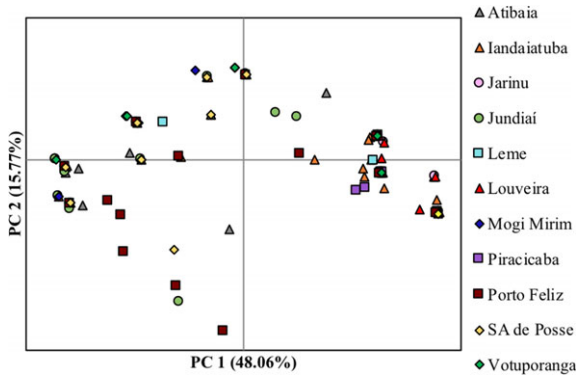

Figure 1 Principal coordinates analysis based on genetic distance matrix of clone-corrected dataset of *Plasmopara viticola* isolates ($n = 516$) from 11 locations in São Paulo State, Brazil.

population structure of *P. viticola* in Brazil. Regardless of the cultivar, *P. viticola* clade *aestivalis* was the only cryptic species found to be infecting grapevine in São Paulo State. Previous studies in North America revealed that *P. viticola* is a complex of five cryptic species, each associated with different wild or cultivated grape species (Rouxel *et al.*, 2014). In North America, clade *aestivalis* is predominant in vineyards cultivated with *V. vinifera*, whereas on interspecific hybrids, clade *riparia* is predominant. On *V. labrusca*, the proportion of clade *aestivalis* (45.7%) and clade *vinifera* (54.3%) was similar, although all isolates sampled from cultivar Niagara belonged to clade *vinifera* (Rouxel *et al.*, 2014). Such a variability of cryptic species was not found in vineyards sampled in São Paulo, where a single cryptic species represented by clade *aestivalis* was identified. This may indicate a genetic bottleneck where only one of the cryptic species was introduced into São Paulo.

Low levels of genotypic diversity of *P. viticola* populations were observed in São Paulo State. Specifically, only 55 distinct MLGs were identified in the 516 isolates sampled. In contrast, Li *et al.* (2016) detected 193 genotypes among 206 isolates analysed in China; in European and North American populations, Rouxel *et al.* (2012) found 89 MLGs among their 96 isolates; and in Europe, Gobbin *et al.* (2006) reported 3910 genotypes among 8991 isolates collected. Among the MLGs identified in São Paulo, 34.5% were clonal and represented 93.0% of the isolates sampled. Four dominant genotypes were present in at least five different locations, corresponding to 65.7% of the sampled isolates. The SSR markers used in this study could have identified a large number of different allele combinations (4×10^5 per marker), providing sufficient resolution to distinguish many more genotypes than observed if they had been present in the population. Overrepresentation of the same MLG is strong evidence of clonality, especially when one or a few MLGs are observed at different locations or multiple times (Tibayrenc *et al.*, 1991). High values of clonal fraction (>0.58) confirmed the limited genotypic diversity of the populations sampled. In environmental conditions unsuitable for oospore production and/or germination, downy mildew epidemics occur mainly by multiple clonal infections caused by a few genotypes, resulting in limited genetic variation (Rumbou & Gessler, 2004, 2006). Dominant genotypes shared among populations might have a genetic advantage (Rumbou & Gessler, 2004), such as high fitness or ability to adapt to that particular environment. In the present study, the majority of *P. viticola* isolates were sampled from vineyards where fungicides were used to control downy mildew. Fungicide applications are an important factor reducing *P. viticola* diversity (Koopman *et al.*, 2007).

Only five private alleles were observed in two specific locations (Atibaia and Porto Feliz, both traditional areas of grape-growing in Sao Paulo), showing that the majority of the identified alleles were shared among

populations. Significant pairwise values of F_{ST} existed among many populations; however, most of the pairwise comparisons had low to moderate F_{ST} values. Low genetic differentiation among populations was also revealed by AMOVA. Similarly, analysis of principal coordinates showed no distinguishable clusters, indicating that *P. viticola* populations in São Paulo were not subdivided.

Negative values of F_{IS} were observed for three of the loci analysed, indicating excess of heterozygosity, while positive values were observed for the other four loci, indicating deficiency of heterozygosity. In strictly clonal populations, heterozygosity tends to be fixed in all loci; however, in populations with low levels of sexual reproduction and recombination, values of F_{IS} among loci are variable; some loci are fixed for heterozygosity and others for homozygosity (Balloux *et al.*, 2003). Deviations from HWE were verified for many loci and populations. Deviations from HWE can have many causes, such as new founder populations, presence of null alleles, presence of loci linked to genes under selection, immigration events, or occurrence of genetic bottlenecks (Gobbin *et al.*, 2006) and can also be a consequence of asexual reproduction (Tibayrenc *et al.*, 1991; Milgroom, 2015). Indeed, asexual survival of *P. viticola* from one season to another has been reported from Greek islands and in South Africa (Rumbou & Gessler, 2006; Koopman *et al.*, 2007). In these cases, it is assumed that pathogen survival occurs by the presence of mycelium in shoots and in dormant buds, or by sporangia in green tissue. Due to mild winters in these regions, green leaves that did not abscise during the winter can support survival of sporangia (Rumbou & Gessler, 2006). In Brazil, in tropical and subtropical areas where viticulture is practised, grape production is staggered and, consequently, green tissue is available throughout almost the entire year, which may serve as a mechanism for asexual survival of *P. viticola* (Angelotti *et al.*, 2012). In the current study, isolates from Votuporanga were collected 3–4 months after those from other locations, yet the majority of the MLG observed in Votuporanga were also observed in other locations. This is strong evidence that *P. viticola* can survive asexually in São Paulo, although the occurrence of genotypes over multiple years at the same location was not monitored. However, the occurrence of unique MLGs in the sampled populations is evidence that sexual reproduction occurs at least in some locations. Future research is needed to determine the occurrence of the two mating types and of oospores of the pathogen across the sampling region.

No IBD was observed, indicating that high levels of gene flow or migration are occurring among populations. Indeed, repeated MLGs were observed in many locations, with distances between them reaching up to 463 km. Recent studies showed that typical distances of sporangial dispersal of *P. viticola* range from 20 to 130 m (Rumbou & Gessler, 2004; Gobbin *et al.*, 2006). However, long-distance dispersal of sporangia has been reported for *P. viticola* (Lafon & Clerjeau, 1988), as well as for other downy mildew pathogens, such as *Bremia lactucae*, the sporangia of which can be dispersed for distances up to 3 km (Wu *et al.*, 2001) and *Peronospora tabacina*, with sporangia easily carried by the wind from sources several hundred kilometres away (Main *et al.*, 2001). Therefore, long-distance dispersal of sporangia cannot be excluded for grapevine downy mildew in São Paulo, Brazil. In the study region, winds typically come from the southeast (Secretaria de Energia do Estado de São Paulo, 2012) and there are no major geographical barriers that would restrict movement of airborne sporangia.

Results from this study revealed an overall limited genetic diversity of *P. viticola* populations in São Paulo, although there was some variation among locations that appeared to be associated with the length of time grapes had been grown in the area. The presence of a single cryptic species of *P. viticola* may be evidence of the restricted genetic variability of this pathogen. The predominance of clonal MLGs among different locations suggests high levels of gene flow or migration and limited occurrence of sexual reproduction. A genetically homogenous population present in a large region may be experiencing widespread dispersal among populations (Milgroom & Fry, 1997). Hence, epidemics are driven by asexual reproduction composed of a few dominant genotypes, as seen for downy mildew epidemics in certain regions of Australia and South Africa (Hug, 2005; Koopman *et al.*, 2007). Future studies should focus on whether and, if so, how the pathogen overwinters asexually *in situ*. The presence of a single *P. viticola* cryptic species and of limited genotypic diversity may facilitate disease management, including the adoption of warning systems that are already well established to control downy mildew in other countries (Madden *et al.*, 2000; Caffi *et al.*, 2010).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Map of São Paulo State showing locations of sampling for *Plasmopara viticola*-infected grape leaves during the 2015/16 growing season.

Table S1. Isolate code, location of origin, host, species and GenBank accession numbers for isolates of *Plasmopara viticola* used in this study.