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MICROBIAL LOCAL ADAPTATION

Soft selective sweeps in fungicide resistance evolution: recurrent mutations without fitness costs in grapevine downy mildew

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Abstract

Adaptation produces hard or soft selective sweeps depending on the supply of adaptive genetic polymorphism. The evolution of pesticide resistance in parasites is a striking example of rapid adaptation that can shed light on selection processes. *Plasmopara viticola*, which causes grapevine downy mildew, forms large populations, in which resistance has rapidly evolved due to excessive fungicide use. We investigated the pathways by which fungicide resistance has evolved in this plant pathogen, to determine whether hard or soft selective sweeps were involved. An analysis of nucleotide polymorphism in 108 field isolates from the Bordeaux region revealed recurrent mutations of *cytb* and *CesA3* conferring resistance to quinone outside inhibiting (QoI) and carboxylic acid amide (CAA) fungicides, respectively. Higher levels of genetic differentiation were observed for nucleotide positions involved in resistance than for neutral microsatellites, consistent with local adaptation of the pathogen to fungicide treatments. No hitchhiking was found between selected sites and neighbouring polymorphisms in *cytb* and *CesA3*, confirming multiple origins of resistance alleles. We assessed resistance costs, by evaluating the fitness of the 108 isolates through measurements of multiple quantitative pathogenicity traits under controlled conditions. No significant differences were found between sensitive and resistant isolates, suggesting that fitness costs may be absent or negligible. Our results indicate that the rapid evolution of fungicide resistance in *P. viticola* has involved a soft sweep.

Keywords: aggressiveness, carboxylic acid amide, local adaptation, plant pathogen, *Plasmopara viticola*, quinone outside inhibiting

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Introduction

Parasites have been shown to rapidly adapt to spatial and temporal heterogeneities in host environments (Greischar & Koskella 2007), climatic conditions (Laine 2008; Mboup *et al.* 2012) or xenobiotics (Levy & Marshall 2004; Kretschmer *et al.* 2009; Ishii & Hollomon 2015). Drug and pesticide resistance provides a striking example of rapid adaptation in organisms with short generation times and high population sizes. Resistance is selected when resistant individuals are able to reproduce, whereas their sensitive competitors are eliminated by xenobiotics. The development of resistant parasites and the failure to control their spread are major issues in public health and agronomy, the seriousness of which may depend on the parasite and the active molecule considered. The implementation of effective management strategies impeding resistance evolution requires an accurate understanding of the emergence and development of resistance in parasite populations (REX Consortium 2013).

Several factors control the dynamics of resistance evolution: the mode of action and specificity of xenobiotics (e.g. site specific or multisite), the particular biological characteristics of the pathogen (ploidy, life cycle, reproduction system) and the genetic basis of resistance

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(number of required resistance mutations and their dominance status, the qualitative or quantitative nature of resistance). The past and current demographic histories of the pathogen are also key drivers of resistance evolution as the polymorphism available for selection depends on population size (Charlesworth 2009). In small populations, the waiting time for the appearance of beneficial mutations is long and adaptation is slow. In this case, adaptation results in hard selective sweeps where only one haplotype with a beneficial mutation reaches a high frequency (reviewed in Messer & Petrov 2013). Conversely, in large populations, adaptive mutations can occur in multiple genetic backgrounds, allowing for fast adaptation and resulting in soft selective sweeps when several haplotypes rise in frequency (Pennings & Hermisson 2006). Another important factor is the existence of biological costs of resistance (Andersson & Levin 1999; Melnyk *et al.* 2015; Mikaberidze & McDonald 2015). Resistance alleles might be expected to decrease fitness, as they often disrupt important physiological and biological processes (Milgroom *et al.* 1989; Berticat *et al.* 2008; Vila-Aiub *et al.* 2009; Andersson & Hughes 2010). However, fitness costs are difficult to demonstrate experimentally and should be explored in different environments in the light of the underlying molecular polymorphisms (Coustau & Chevillon 2000; Billard *et al.* 2012).

Grapevine downy mildew is caused by the oomycete *Plasmopara viticola*, a diploid heterothallic organism (i.e. sexual reproduction occurs between individuals with different mating types), reproducing clonally during the growing season and sexually in the fall to produce overwintering oospores. It has been a threat to viticulture since the middle of the 19th century, when it was introduced into Europe from North America (Millardet 1881; Fontaine *et al.* 2013). This biotrophic plant pathogen is known to rapidly adapt to new evolutionary challenges such as new host plants (Rouxel *et al.* 2013, 2014), resistant cultivars (Peressotti *et al.* 2010; Delmotte *et al.* 2014; Delmas *et al.* 2016) or fungicides (Chen *et al.* 2007; Blum *et al.* 2010). Among fungicides, two types of site-specific molecules are particularly well studied: quinone outside inhibitors (QoIs), which inhibit mitochondrial respiration, and carboxylic acid amides (CAAs), which affect cellulose synthesis (Gisi & Sierotzki 2008). QoIs and CAAs were introduced at the end of the 1990s (Gisi 2002), and resistance to both groups of fungicides has been increasing ever since (Corio-Costet 2015). Resistance monitoring conducted by ANSES (French Agency for Food and Environmental Safety) reveals that QoI resistance is now widespread in most French vineyards while the proportion of fields showing CAA resistance have increased from 25% to 89% (Magnien *et al.* 2012). The rapid evolution of fungicide resistance to QoIs and CAAs in *P. viticola* field populations and the identification of specific substitutions conferring resistance (reviewed in Gisi & Sierotzki 2015) provide a unique opportunity to investigate the evolutionary pathways to fungicide resistance in a plant pathogen species. Most studies in *P. viticola* have been based on the monitoring of resistance alleles in bulked populations, either using bioassays that only detect the presence/absence of resistance in populations or using a PCR-based approach. Here, we used an individual-based approach combining genetic and phenotypic analyses to assess whether the rapid evolution of fungicide resistance in *P. viticola*, which forms large populations during epidemics, followed a hard or a soft sweep scenario. Using 108 field isolates from Bordeaux vineyards, we assessed the nucleotide polymorphisms in genes involved in QoI and CAA resistance and the neutral genetic structure of *P. viticola* populations. We also experimentally assessed pathogen fitness for these 108 isolates in controlled conditions, to assess the cost of resistance to the fungicides considered, on different host plants.

Materials and methods

Pathogen sampling

In 2012, we collected 108 *Plasmopara viticola* isolates (single sporulating lesions) early in the growing season, from six wine regions in the Bordeaux area (Fig. 1, Table 1): Saint-Estèphe ($n = 4$ vineyards), Pauillac ($n = 4$ vineyards), Listrac ($n = 4$ vineyards), Entre-Deux-Mers West ($n = 3$ vineyards), Entre-Deux-Mers East ($n = 5$ vineyards) and Côte de Bordeaux ($n = 9$ vineyards). We collected a mean of four isolates from each vineyard (range: 1–7).

Information on the fungicide spray programmes in each sampled population was not available. However, a large survey of fungicide treatments from 110 winegrowers in the Bordeaux region was carried out in 2012 (L. Delière, unpublished data). The number of CAA treatments ranged from 0 to 4 (41% of winegrowers applied 0 treatment; 35%: 1; 23%: 2; 1%: >3). The number of QoI treatments ranged from 0 to 3 (28% of winegrowers applied 0 treatment; 42%: 1; 26%: 2; 4%: 3). Fungicide application dates were highly variable, ranging from May to August with a peak of treatments in June.

Genetic analysis

DNA extraction. For each isolate, we retained one inoculated leaf disc (cv. Cabernet sauvignon) for DNA extraction after sporangium collection (see Aggressiveness phenotyping below). Leaf discs were freeze-dried

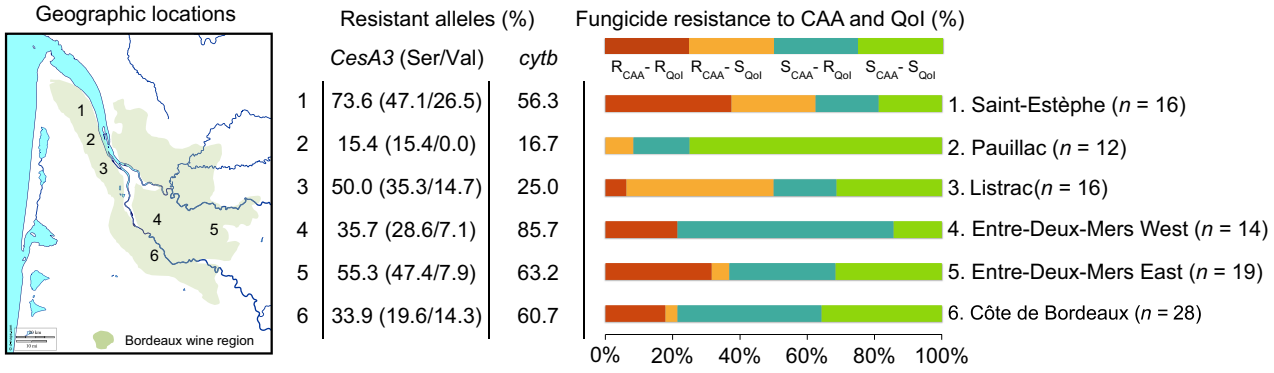

Fig. 1 Geographic locations of the six populations of *Plasmopara viticola*, percentage of resistance alleles and percentage of isolates resistant and sensitive to CAA and QoI fungicides per population. Fungicide resistance categories were determined by sequencing of the *cytb* and *CesA3* genes: R_{CAA}-R_{QOI}: resistant to CAA and QoI; S_{CAA}-S_{QOI}: sensitive to CAA and QoI; R_{CAA}-S_{QOI} resistant to CAA and sensitive to QoI; S_{CAA}-R_{QOI}: sensitive to CAA and resistant to QoI. The number of isolates sampled per population is indicated in parentheses.

Table 1 Genetic diversity in *Plasmopara viticola*, according to wine region of origin and isolate resistance to CAA and QoI fungicides. Wine regions are numbered as presented in Fig. 1

	N	A	H_e	F_{IS}	HW
Wine region					
1. Saint-Estèphe	17	2.32	0.34	0.014	ns
2. Pauillac	13	2.10	0.33	−0.034	ns
3. Listrac	17	2.37	0.37	−0.002	ns
4. Entre-Deux-Mers West	14	2.39	0.35	−0.034	ns
5. Entre-Deux-Mers East	19	2.21	0.33	−0.015	ns
6. Côte de Bordeaux	28	2.31	0.36	0.041	ns
Fungicide resistance					
R_{CAA}-R_{QOI}	21	2.39	0.35	0.028	ns
R_{CAA}-S_{QOI}	14	2.40	0.35	0.009	ns
S_{CAA}-R_{QOI}	35	2.44	0.35	−0.019	ns
S_{CAA}-S_{QOI}	35	2.29	0.35	0.044	ns

N: number of isolates, A: allelic richness (based on a sample size of 10 individuals for wine regions and 12 for fungicide resistance category), H_e: unbiased expected heterozygosity, F_{IS}: inbreeding coefficient (Weir & Cockerham 1984), HW: departure from Hardy–Weinberg expectations (ns: nonsignificant), S: sensitive, R: resistant.

overnight and DNA was extracted by the standard CTAB–phenol–chloroform method, with precipitation in isopropanol as described in Delmotte *et al.* (2006). DNA was resuspended in 150 μL of sterile water.

Sequencing of cytb and CesA3. In *P. viticola*, resistance to QoIs is conferred by mutations of the mitochondrial gene encoding cytochrome b (cytb), and resistance to CAA is conferred by recessive mutations of the nuclear gene encoding cellulose synthase 3 (CesA3). Mutations of cytb leading to the replacement of a glycine by an alanine residue at position 143 (Gly143Ala) prevent QoI fungicides from inhibiting the cytochrome bc1 enzyme complex of the respiratory chain in mitochondria (Bartlett *et al.* 2002). This substitution has been detected in QoI-resistant isolates of many other plant pathogens (Sierotzki *et al.* 2000; Ishii *et al.* 2001; Kim *et al.* 2003; Fontaine *et al.* 2009; Gisi & Sierotzki 2015) and has occurred several times in *P. viticola* (Chen *et al.* 2007). A second amino acid substitution in the cytb protein, Phe129Leu, confers a lower level of QoI resistance than Gly143Ala in *P. viticola* (Gisi & Sierotzki 2015). The frequency of this mutation is very low in France (<0.4%, Chen *et al.* 2007) and it was, therefore, not studied here. CAA resistance is conferred by missense mutations of CesA3, resulting in the replacement of a glycine by a serine or a valine residue (Sierotzki *et al.* 2011; Blum *et al.* 2012) at position 1105 in the protein. We amplified and sequenced short fragments of *cytb* and *CesA3*, including the codons for which mutations confer fungicide resistance (codon 143 for *cytb*, codon 1105 for *CesA3*). We also investigated the relationship between the haplotypes identified in analyses of the short region of *CesA3* (see Results), by amplifying and sequencing a longer fragment including the end of the coding sequence and 320 bp downstream from the gene, for a smaller number of selected isolates (n = 25). This larger *CesA3* fragment was also sequenced for four North American isolates from which DNA was extracted in a previous study (Rouxel *et al.* 2014). For the short fragments, we used the primers CB 279F and CB 865R for *cytb* (Chen *et al.* 2007) and Pcesa3f1 and Pcesa3r2 for *CesA3* (Blum *et al.* 2010). For the long *CesA3* fragment, we used Pcesa3f1 and a new primer (Pcesa3r3, 5′-GTACGTG CAGTAGGAACCTG-3′) designed on the basis of a recently published draft genome sequence (GenBank Accession no.: MBPM00000000, Dussert *et al.* 2016).

PCR was performed in a volume of 15 μL (1 μL DNA, 1.5 mM $MgCl_2$, 1× reaction buffer (Eurogentec, Angers, France), 0.2 U Silverstar *Taq* polymerase

(Eurogentec) for both genes; 0.13 mM dNTP and 0.13 mM primer, for each primer, for *cytb*; 0.27 mM dNTP and 0.2 mM primer, for each primer, for *CesA3*) in a Mastercycler EP gradient (Eppendorf, Montesson, France). The program consisted of initial denaturation for 4 min at 96 °C, followed by 38 cycles of 40 s at 96 °C, 50 s at 50 °C for *cytb* and 52 °C for *CesA3*, 1 min at 72 °C and a final elongation for 10 min at 72 °C. PCR products were sequenced by Beckman Coulter Genomics (Danvers, MA, USA) with the corresponding forward primer for the short fragments and with the two primers for the long fragment.

SSR genotyping. We amplified 35 microsatellite loci by multiplex PCR: Pv7, Pv14, Pv16, Pv17, Pv31, Pv39 (Delmotte *et al.* 2006); Pv61, Pv67, Pv74, Pv76, Pv83, Pv87, Pv88, Pv91, Pv101, Pv103, Pv124, Pv127, Pv134, Pv138, Pv139, Pv140, Pv141, Pv142, Pv143, Pv146, Pv147, Pv148 (Rouxel *et al.* 2012) and ISA (Gobbin *et al.* 2003). PCR was performed in a volume of 6 μL containing 2.5 μL H_2O, 1.5 μL Multiplex Master Mix (Qiagen, Hilden, Germany), 0.5 μL primer mix and 1.5 μL DNA in an Eppendorf Mastercycler EP gradient (initial denaturation for 15 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 55 °C, 45 s at 72 °C, and a final elongation for 30 s at 60 °C). PCR products (1–1.5 μL diluted 1:14) were mixed with 10 μL formamide and 0.14 μL internal lane size standard (GeneScan 600 LIZ, Thermo Fisher Scientific, Waltham, MA, USA) and analysed in an ABI3130 capillary sequencer (Applied Biosystems, Thermo Fisher Scientific), according to the manufacturer's instructions. Alleles were automatically scored with GENEMAPPER v4.0 (Applied Biosystems, Thermo Fisher Scientific). We discarded five loci (Pv7, Pv67, Pv74, Pv138 and Pv140), for which more than 20% of the data were missing, three loci with amplification issues (Pv124, Pv146 and Pv61) and four monomorphic loci (Pv76, Pv87, Pv126 and Pv134). The final analysis was carried out on 23 microsatellite markers.

Genetic structure and diversity analyses. Microsatellite multilocus genotypes were identified with GENODIVE (Meirmans & Van Tienderen 2004), using a stepwise-mutation model. Population genetics analyses were performed by wine region, with each region considered to correspond to a single population ($n = 6$ as presented in Fig. 1). We assessed the genetic diversity of these populations, of fungicide resistance categories (i.e. R_{CAA}-R_{QOI}: resistant to CAA and QoI; S_{CAA}-S_{QOI}: sensitive to CAA and QoI; R_{CAA}-S_{QOI} resistant to CAA and sensitive to QoI; S_{CAA}-R_{QOI}: sensitive to CAA and resistant to QoI), and of the whole sample, by calculating the allelic richness (A), using a rarefaction approach to account for different sample sizes (Mousadik & Petit 1996), and the unbiased expected heterozygosity (H_e, Nei 1978). F_{IS} values for each population and for the whole sample, pairwise F_{ST} values between populations and overall F_{ST} values (Weir & Cockerham 1984) were also calculated. The significance of F_{IS} values and pairwise F_{ST} values was assessed with 15 000 permutations followed by sequential Bonferroni correction (Holm 1979), and the 95% confidence interval (95% CI) of the overall F_{ST} was determined with 15 000 bootstraps. Diversity indices and *F*-statistics were calculated with FSTAT v2.9.3 (Goudet 2001). We investigated genetic structure through principal component analysis (PCA) with the adegenet package in R (Jombart 2008). We also inferred the population genetic structure of our sample with the model-based Bayesian algorithm implemented in STRUCTURE v2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003). We used a model with correlated allele frequencies, with a burn-in length of 100 000 iterations, followed by 500 000 iterations, in which we allowed the number of clusters (K) to range from 1 to 5 (10 replicates for each value of K). Membership coefficients were averaged across replicates for each K value, with CLUMPP v1.1.2 (Jakobsson & Rosenberg 2007) and displayed with DISTRUCT (Rosenberg 2004).

The allelic phases of the *CesA3* nucleotide sequences of heterozygous individuals were inferred with PHASE v2.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003). For the short *CesA3* and *cytb* sequences from the Bordeaux region, the number of haplotypes, total, synonymous and nonsynonymous nucleotide diversity (π, π_{syn} and $\pi_{non\text{-}syn}$) per site (Nei 1987) and Tajima's D values (Tajima 1989) were calculated with DNASP v5.10 (Librado & Rozas 2009). Haplotype networks were constructed by the median-joining method (Bandelt *et al.* 1999) implemented in POPART v1.7 (Leigh & Bryant 2015). For *cytb*, we included sequences of North American isolates (Chen *et al.* 2007) available from GenBank (Accession nos: DQ459459 to DQ459463) in the haplotype network analysis. The alignment sizes for *cytb* and the short and long *CesA3* fragments were, respectively, 542, 269 and 636 bp. We used PGDSPIDER v2.0.9.1 (Lischer & Excoffier 2012) to convert data files into appropriate formats for the different analyses.

Comparison of differentiation between microsatellite loci and fungicide resistance genes. Selection pressure in fungicide-treated fields should increase the frequency of resistant genotypes and lead to a directional selection signal. We investigated whether the allele frequencies of *CesA3* (short fragment) and *cytb* departed from neutral expectations, according to the F_{ST} -outlier approach of Beaumont & Nichols (1996), in which loci under spatially heterogeneous or balancing selection display stronger or weaker differentiation, respectively, than

neutral loci (Lewontin & Krakauer 1973). The differentiation between populations was estimated for all markers by calculating a standardized F_{ST} value, G'_{ST} (Hedrick 2005), because microsatellite loci and nuclear and mitochondrial sequences have different rates and modes of mutation and, thus, different heterozygosity levels. G'_{ST} values and their 95% CIs (1000 bootstraps) were computed with SMOGD (Crawford 2010). This analysis was carried out by taking the codons of the two genes as alleles, rather than as independent SNPs (i.e. the codons for Gly, Ser and Val at position 1105 in *CesA3* were considered as three alleles of the same locus), because fungicide susceptibility is known to be caused by different mutations of the same codon of *CesA3*. Additionally, for each microsatellite locus and gene position, overall F_{ST} values were computed and genetic differentiation was tested (50 000 randomizations) using the procedure of Goudet *et al.* (1996) implemented in FSTAT. Linkage disequilibrium between positions in genes was also tested with the same procedure (60 000 permutations for *cytb*, 210 000 for *CesA3*).

In vitro tests of sensitivity to CAA

We characterized the sensitivity of *P. viticola* isolates to a CAA fungicide (dimethomorph: 150 g/L, density of 1.08), by propagating 12 isolates on the leaves of *Vitis vinifera* cv. Cabernet sauvignon. We tested two isolates for each amino acid combination found at position 1105 in CesA3: Gly/Gly, Gly/Ser, Gly/Val, Ser/Ser, Val/Ser, Val/Val. The day before the bioassay, the leaves were rinsed to ensure that the sporangia (i.e. structures containing the zoospores produced during the asexual phase) studied were freshly produced. Sporangia were collected for each isolate and mixed with various dilutions of fungicide. The leaf disc was inoculated by placing three 15 μL droplets on its adaxial surface. We used 10 leaf discs for each CAA concentration (0, 0.03, 0.06, 0.1, 0.3, 0.6, 1, 3 mg/L for Gly/Gly, Gly/Ser, Gly/Val isolates – predicted to be sensitive and 0, 0.3, 0.6, 1, 3, 6, 10, 30 mg/L for Ser/Ser; Val/Val; Val/Ser isolates – predicted to be resistant). The leaf discs were incubated in Petri dishes under controlled conditions (21 °C, 12-h day/12-h night photoperiod). After 7 days, the sporulation in each droplet was recorded (scores from 0: no sporulation to 4: sporulating lesion with a larger diameter than the droplet) and compared with that on the control leaf disc (0 mg/L dimethomorph). A percentage efficacy was obtained, as follows: (100 × control score - dose score)/control score. For each isolate, we estimated the concentration inhibiting 50% of grapevine downy mildew sporulation (IC_{50}), using a logistic model fitted with PROC NLIN in SAS Studio (SAS University Edition; version 3.3; SAS Institute Inc., Cary, NC, USA) as follows: $Y_i = \frac{1}{1+\left(\frac{IC_{50}}{x}\right)^{slope}} + \varepsilon_i$ where x is the dose (see above) and Y_i is the fungicide efficacy divided by 100. We calculated resistance factors for each genotype as the ratio between the IC_{50} values of sensitive and resistant isolates.

Aggressiveness phenotyping

Plant material. We estimated pathogen aggressiveness under controlled conditions and compared the relative fitness of resistant (carrying resistance mutations) and sensitive isolates, in the absence of fungicides, by inoculating two host plants – the susceptible host *V. vinifera* cv. Cabernet sauvignon and the partially resistant grapevine host Cabernet carbon – with the 108 isolates. Cabernet carbon is a German variety, not cultivated in France, originating from interspecific breeding between Cabernet sauvignon and varieties descending from American resistant *Vitis* species (Vitis International Variety Catalogue; www.vivc.de). The aggressiveness of each isolate was assessed in these two hosts because they represent two contrasting host environments for the pathogen: an optimal environment in the case of the susceptible host and a suboptimal environment in the case of the partially resistant host. Plants were grafted onto the SO4 rootstock and grown simultaneously in a glasshouse, under natural photoperiod conditions.

Inoculation under controlled conditions. We generated the 108 inocula for this experiment by propagating each *P. viticola* isolate on detached leaves from glasshouse-grown *V. vinifera* cv. Cabernet sauvignon plants, in Petri dishes stored in controlled conditions (20 °C, 12-h day/12-h night photoperiod). On the day before the inoculation experiment, we gently washed the sporulating leaves to remove the sporangia. Leaves were placed in growth chambers for 1 day to ensure the production of fresh sporangia of the same age for all isolates. The inoculum for each isolate was obtained by collecting sporangia from leaves in sterile water. Its concentration was adjusted to 10 000 sporangia/mL with a portable particle counter (Scepter 2.0TM automated cell counter; Millipore).

We then prepared plant leaf discs for inoculation by collecting the third and fourth leaves below the apex of young shoots from each cultivar, at the ten-unfolded-leaf stage. Leaves were rinsed with distilled water, and leaf discs of 15 mm in diameter were excised with a cork borer. We used eight replicate leaf discs for the sensitive inoculated host, and six for the partially resistant inoculated host. We inoculated 1512 leaf discs in total, to study 216 plant–pathogen interactions (108 isolates × 2 host plants).

Finally, for each of these plant–pathogen interactions, leaf discs were floated on the surface of the inoculum, adaxial-side-up, for 4 h at 20 °C. Inoculated leaf discs were randomized and placed abaxial-side-up on damp filter paper in square Petri dishes (23 × 23 cm). The Petri dishes were sealed with cling film once the discs had dried, and were placed in a phytotron (type LMS 610 XAP; LMS Ltd; UK) with a 12-h (18 °C) light/12-h (15 °C) dark photoperiod, for 7 days.

Quantitative trait analyses. We first quantified four elementary traits of the pathogen life cycle: sporangium production, sporangium size, zoospore number per sporangium and latency period. Spore production, spore size and zoospore number per sporangium were assessed 7 days postinoculation (dpi), with a Multisizer 3 automatic particle counter (Coulter Counter® Multisizer™ 3; Beckman Coulter). We gently washed each leaf disc separately in 10 mL of saline (Isoton II, Coulter Corporation) to collect sporangia (t = 0 min; no zoospores released, as Isoton stops the pathogen cycle). Two discs per isolate and per inoculated host were washed separately in 4 mL of distilled water. The resulting suspensions were kept at 20 °C to promote zoospore release, and 11 mL of saline buffer was added at t = 100 min (some zoospores released), as described by Delmas *et al.* (2014).

We determined the number of sporangia per mm² (cumulative, over 7 days of infection), weighted sporangium size and zoospore number per sporangium, as described by Delmas *et al.* (2014). The latency period was estimated by visually checking leaf discs daily under a stereomicroscope and recording the day on which sporulation first occurred for each disc. The latency period was defined as the time interval between inoculation and the first recorded sporangia.

In the optimal environment (i.e. Cabernet sauvignon), we also estimated the sporulation dynamics of each infected leaf disc, using two traits: time to 50% of final sporulation (T_{50}) and sporulation rate, as described by Delmas *et al.* (2016). T_{50} and sporulation rate are model parameters estimated from the dynamics of pathogen infection monitored over time by image analysis. The estimation of these traits was independent of the estimation of final sporulation level. Cabernet sauvignon leaf discs with a sporulating area of more than 5% were included in the analysis to ensure the accurate estimation of sporulation by image analysis. Each day, from 1 to 7 dpi, we took pictures of leaf discs and analysed them in ImageJ (Peressotti *et al.* 2011) with a simple semi-automatic method in which sporulation was quantified as the number of pixels covered by sporangia as a proportion of the total number of pixels corresponding to the leaf disc. Sporulation is thus expressed as the proportion of the leaf disc area that is sporulating. We fitted a logistic model to these experimental data, with PROC NLIN in SAS Studio, as follows: $Y_{irx} = \frac{1}{1+(\frac{T_{50}}{x})^{slope}} + \varepsilon_{irx}$; where x is the time (from 0 to 7 dpi) and Y_{irx} is the relative sporulating leaf disc area (the proportion of the leaf area displaying sporulation of isolate i replicate r on day x divided by the proportion of the leaf area displaying sporulation of isolate i replicate r on day 7). Y_{irx} ranged from 0 to 1 and was independent of spore production 7 dpi. This approach made it possible to estimate two parameters: the time at which the pathogen reached 50% of its maximal sporulation at 7 dpi (T_{50}) and the slope of the curve at T_{50}. The parameters estimated were therefore independent of the final sporulation recorded 7 dpi.

We investigated whether quantitative pathogenicity traits differed between isolates resistant to one, two or none of the fungicides: R_{CAA}-R_{QOI}: resistant to CAA and QoI; S_{CAA}-S_{QOI}: sensitive to CAA and QoI; R_{CAA}-S_{QOI} resistant to CAA and sensitive to QoI; S_{CAA}-R_{QOI}: sensitive to CAA and resistant to QoI. A generalized linear model was generated for each quantitative trait and each inoculated host, with PROC GLM in SAS Studio. Data for leaf discs inoculated with the same isolate (replicates) were averaged. We accounted for spatial sampling effects, by including the effect of the region of origin in the model and its interaction with the effect of fungicide resistance. We performed similar analyses to compare isolates that were resistant and sensitive to one of the fungicides, regardless of their resistance status concerning the other fungicide. These analyses are presented in the supplementary data.

For all models, we plotted studentized marginal and conditional residuals, to check the normality, identity and independence of the residuals of each trait. For four of the six measured traits (spore production, latency period, zoospore number per sporangium and the slope at T_{50}), log-transformation of the data was required to satisfy these requirements.

Results

Fungicide resistance

By contrast to the bulk analysis usually performed for resistance monitoring, the molecular individual-based approach used here made it possible to estimate the frequencies of the different resistance alleles precisely (Fig. 1). The sequencing of *cytb* revealed the expected polymorphism responsible for the Gly143Ala substitution conferring QoI resistance. Overall, in the Bordeaux region, 53.3% of isolates were resistant (n = 56) and 46.7% were sensitive to QoI (n = 49). Resistant isolates were found in all regions, but frequency of

QoI-resistant isolates differed considerably between geographic areas, ranging from 16.7% for Pauillac to 85.7% for Entre-Deux-Mers West (Fig. 1). The sequencing of *CesA3* revealed the polymorphisms responsible for amino acid substitutions at position 1105 of the protein: the GGC codon encoding glycine (Gly), the AGC codon encoding serine (Ser), and the GTC codon encoding valine (Val). No other nonsynonymous polymorphism was observed.

The CAA bioassay indicated that the minimum inhibitory concentration of dimethomorph and the concentration inhibiting sporulation by 50% (MIC and IC_{50}; Table 2) clearly distinguished between two categories of isolates: sensitive (Gly/Gly, Gly/Ser, Gly/Val) and resistant isolates (Ser/Ser, Val/Val, Ser/Val). One of the Val/Val isolates was excluded from the analyses as it was too well controlled by the first dose tested (0.3 mg/L), making it impossible to fit the logistic model. MIC and IC_{50} of sensitive genotypes tend to be lower for homozygous Gly/Gly isolates than for heterozygous Gly/Ser and Gly/Val isolates (Table 2; Fig. S1, Supporting information). For resistant genotypes, MIC and IC_{50} were higher for Ser/Val and Val/Val isolates than for Ser/Ser isolates (Table 2; Fig. S1, Supporting information).

CesA3 sequencing revealed that 33.3% of isolates in the Bordeaux region were resistant to CAA (16.7% Ser/Ser, $n = 18$; 12% Val/Ser, $n = 13$ and 4.6% Val/Val, $n = 5$). An analysis of the sensitive isolates showed that 44.5% were Gly/Gly ($n = 48$), 18.5% Gly/Ser ($n = 20$) and 3.7% Gly/Val ($n = 4$). Resistant isolates were found in all regions and all geographic areas, and the frequency of CAA-resistant isolates ranged from 7.7% for Pauillac to 64.7% for Saint-Estèphe.

Overall, 20% of the isolates were resistant to both QoI and CAA (R_{CAA}-R_{QoI}), 33.33% to QoI only (S_{CAA}-R_{QoI}), 13.33% to CAA only (R_{CAA}-S_{QoI}) and 33.33% were susceptible to both fungicides (S_{CAA}-S_{QoI}). In summary, QoI- and CAA-resistant *P. viticola* isolates were found in vineyards from all geographic areas of the Bordeaux region, and isolates resistant to both fungicides were found in all populations except Pauillac (Fig. 1).

Table 2 Frequency and sensitivity to CAA of the different genotypes found at codon 1105 of *CesA3* in *Plasmopara viticola*. Bioassays were performed with dimethomorph, to determine the range of minimal inhibitory concentrations (MIC, mg/L) and fungicide concentrations inhibiting growth by 50% (IC_{50}, mg/L) for each genotype

Codon 1105	Amino acid	*N*	MIC	IC_{50} (SEM)	R/S
GGC/GGC	Gly/Gly	2	0.3–0.6	0.28 (0.05)	S
GGC/AGC	Gly/Ser	2	0.6–1	0.52 (0.003)	S
GGC/GTC	Gly/Val	2	0.6–1	0.49 (0.13)	S
AGC/AGC	Ser/Ser	2	3–6	3.14 (0.057)	R
AGC/GTC	Ser/Val	2	6–10	6.28 (0.26)	R
GTC/GTC	Val/Val	1	6–10	5.90	R

N, number of isolates tested in bioassay; Gly, glycine; Ser, serine; Val, valine; S, sensitive; R, resistant; SEM, standard error of the mean.

Multiple independent mutations of cytb and CesA3 conferring fungicide resistance

The global nucleotide diversity π for *cytb* in the Bordeaux region was 0.0024 substitutions per site ($\pi_{syn} = 0.0065$). Only four haplotypes (IS, IR, IIS, IIR) were observed (Table S1, Supporting information). Consistent with the findings of Chen *et al.* (2007), haplotype network analysis showed that the resistance allele had appeared independently at least twice, in the IR and IIR haplotypes (Fig. S2, Supporting information).

For the Bordeaux region, the nucleotide diversity π of the short *CesA3* fragment was 0.0081 substitutions per site ($\pi_{syn} = 0.0247$), and 10 haplotypes were observed (Table S1, Fig. S3, Supporting information). We found four CAA-resistant haplotypes: a single haplotype with the mutation responsible for the Gly1105Val substitution (Val1 haplotype, 12.5% of the sequences) and three haplotypes with the mutation causing the Gly1105Ser change (Ser1, Ser2 and Ser3, respectively, 17.1%, 13.9% and 0.5% of the sequences). None of the North American haplotypes contained a resistance allele (Fig. S3, Supporting information). The haplotype network for the short *CesA3* fragment was not informative enough to determine the relationship between haplotypes. We therefore performed the same analysis with a larger sequenced fragment. We were unable to obtain long sequences for the Gly7 and Gly8 haplotypes, and we discarded one sequence (Gly9) that was almost certainly a recombinant haplotype giving rise to spurious results (data not shown). Our analysis confirmed that the Gly1105Ser mutation was present in three different haplotypes (Fig. 2), suggesting that there were at least three independent mutations conferring fungicide resistance at this position. It was not possible to exclude definitively a role of recombination in this pattern, but visual inspection of the sequences showed that multiple recombination events located close to this position and on either side of it would be required to explain the presence of the mutation in three haplotypes. Such multiple recombination events appear unlikely.

Lack of neutral population genetic structure and greater differentiation at the CesA3 and cytb loci

We identified 108 different multilocus genotypes with 23 microsatellite loci. All of the isolates were, therefore,

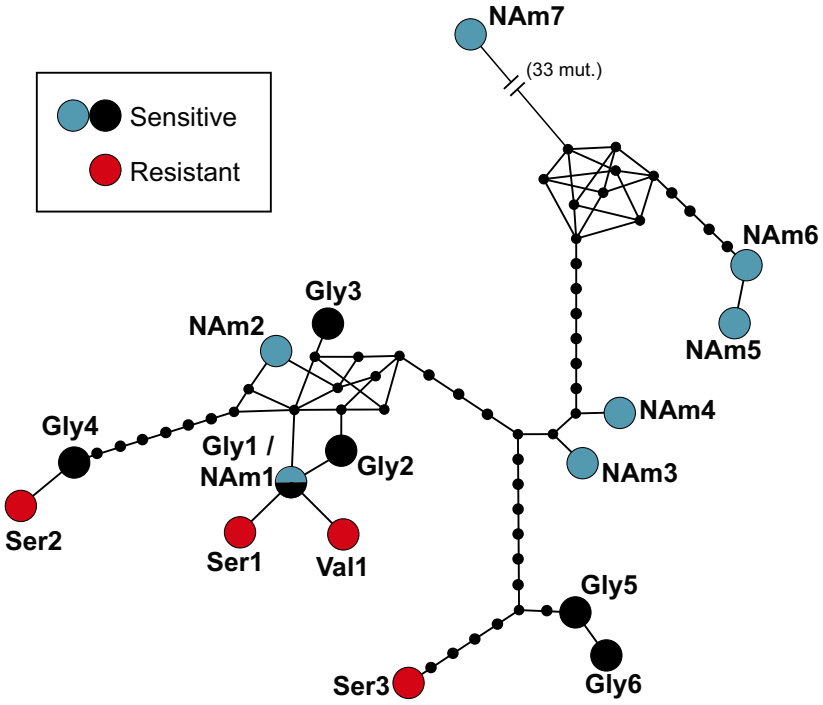

Fig. 2 Median-joining haplotype network for the long *CesA3* fragment. Haplotypes with mutations in codon 1105 of *CesA3* conferring resistance to CAA fungicides (Ser and Val haplotypes) are represented in red, sensitive haplotypes are shown in dark blue (Gly haplotypes from the Bordeaux region) or light blue (North American haplotypes). Black dots along edges represent missing haplotypes, except for one very long edge, where the number of mutations is indicated.

genetically different. Overall, 73 alleles were detected, with two to seven alleles per locus. The whole sample showed a low genetic diversity, with a mean number of alleles per locus of 3.19 (SD: 1.46) and a mean H_e of 0.35 (SD: 0.23), and was at Hardy–Weinberg equilibrium ($P = 0.37$). The six different regions of origin and the four resistance categories (R_{CAA}-R_{QOI}, R_{CAA}-S_{QOI}, S_{CAA}-R_{QOI}, S_{CAA}-S_{QOI}) displayed very similar levels of genetic diversity (H_e and A), with no significant departure from Hardy–Weinberg expectations (Table 1).

There was no neutral genetic structure in the sample. Axes 1 and 2 of the PCA on microsatellite data accounted for 6.3% and 5.5% of the total genetic variability, respectively. The PCA did not group isolates by fungicide resistance category (Fig. S4, Supporting information) or by area of origin (data not shown). The overall F_{ST} between geographic populations was very low, at 0.002 (95% CI: 0.000-0.008), and none of the pairwise F_{ST} values differed significantly from 0 after sequential Bonferroni correction (Table S2, Supporting information). This lack of genetic structure was confirmed by the Bayesian clustering analysis (Fig. S5, Supporting information).

We compared differentiation levels between microsatellite markers and fungicide resistance genes. The G'_{ST} values at codons conferring fungicide resistance in *CesA3* and *cytb* (0.18 and 0.36, respectively) were higher than those of microsatellites (ranging from 0.00 to 0.07), and 95% CI included 0 for all but the resistance positions (Fig. 3). Overall F_{ST} displayed the same pattern, with higher values for resistance positions (0.07 for *CesA3*, 0.14 for *cytb*) than for microsatellites (ranging from 0.00 to 0.04, Table S3, Supporting information). Finally, differentiation tests were significant for the two resistance positions ($P = 0.005$ for *CesA3*, $P = 0.004$ for *cytb*), with only two microsatellite loci with P-values lower than 0.05 (Pv83: $P = 0.034$, Pv135: $P = 0.038$; Table S3, Supporting information). There was, therefore, a signal for spatially heterogeneous selection at resistance positions. However, the 95% CI for G'_{ST} was large for all markers, indicating a lack of precision in our estimations, and the 95% CI for codons conferring resistance overlapped with many of the CIs of microsatellite markers. Other positions in the two genes had very low or null G'_{ST} values and did not show any genetic linkage with resistance positions (Fig. 4), indicating the absence of a hitchhiking signal.

Pathogen aggressiveness and the cost of resistance

The inoculation of the two host plants (Cabernet sauvignon, Cabernet carbon) with 108 isolates led to bioassays on 1512 leaf discs. We discarded 165 leaf discs with brown coloration (premature degeneration of plant tissues after inoculation). The final sample consisted of 1357 leaf discs corresponding to 214 plant–pathogen interactions.

On the susceptible host, *V. vinifera* cv. Cabernet sauvignon, the first sporangia were observed a mean of (±SEM) 4 ± 0.009 dpi (latency period), and final

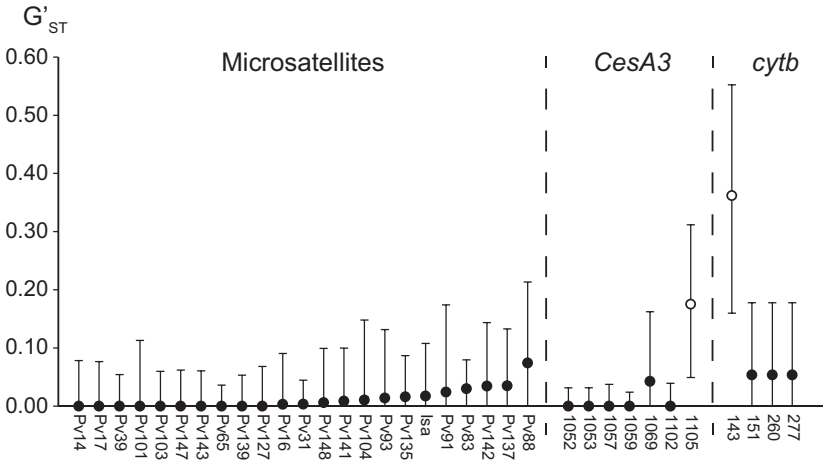

Fig. 3 G'_{ST} values for microsatellites markers and the *CesA3* (short fragment) and *cytb* genes for *Plasmopara viticola* populations in the Bordeaux region. For genes, values are given for codons with nucleotide polymorphism, and codons are named after the position of the amino acid encoded. Positions with alleles conferring fungicide resistance are shown in white. The error bars show the 95% confidence intervals calculated from 1000 bootstraps.

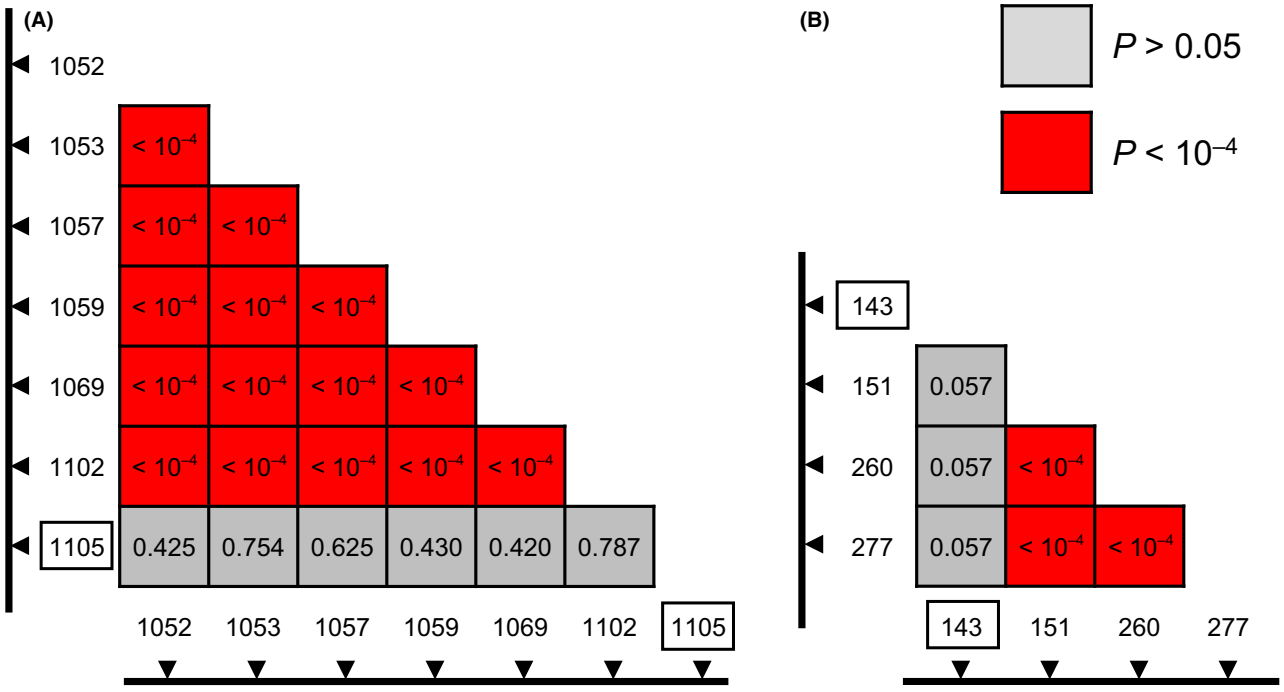

Fig. 4 Tests of pairwise linkage disequilibrium in the short fragment of *CesA3* (A) and in *cytb* (B). *P*-values computed using 210 000 or 60 000 permutations (for *CesA3* and *cytb*, respectively) are indicated in the cells. Positions are named after the amino acid they code in the protein, and positions with mutations conferring fungicide resistance are outlined in black boxes. Grey: *P*-values > 0.05, red: *P*-values < 10^{-4}.

sporangium production on each leaf disc at 7 dpi reached, on average, 737 ± 24 sporangia/mm^2. The sporangia measured, on average, 10.82 ± 0.028 µm, and the mean number of zoospores per sporangium was 6.58 ± 0.26. An analysis of sporulation dynamics on the susceptible host indicated that the mean time taken to achieve 50% the final level of sporulation observed on 7 dpi (T_{50}) was 5.07 ± 0.009 days.

The first sporangia appeared slightly later on the partially resistant host, Cabernet carbon, than on the susceptible host (4.7 ± 0.027 dpi, on average), final sporulation levels on the leaf disc 7 dpi were lower (142 ± 9 sporangia/mm^2 on average), sporangia were smaller (11.85 ± 0.028 µm), and the number of zoospores per sporangium was higher (7.18 ± 0.20). Finally, 30 leaf discs presented no sporulation 7 dpi on this host.

Quantitative traits were compared between multiresistant (resistant to both CAA and QoI), singly resistant (resistant to CAA or QoI) and sensitive isolates on both Cabernet sauvignon and Cabernet carbon, taking into account a potential wine region effect. On the

susceptible host, no significant differences were found between the various isolate types, for any of the traits considered (Table 3; Figs 5 and S6, Supporting information). The multiresistant isolates had an aggressiveness (i.e. sporangium production, sporangium size, number of zoospores per sporangium and latency period) similar to that of the singly resistant and sensitive isolates (Fig. S6, Supporting information). Furthermore, sporulation dynamics (T_{50} and slope at T_{50}) did not differ significantly between the different categories of isolates defined on the basis of fungicide resistance (Table 3). In separate analyses for the two types of fungicide, no difference in aggressiveness was found between the isolates resistant and sensitive to each fungicide (Tables S4 and S5; Fig. S6, Supporting information). On the partially resistant host, Cabernet carbon, there was also no significant difference in aggressiveness for any of the traits considered, between multiresistant isolates, singly resistant and sensitive isolates (Table 3; Figs 5 and S6, Supporting information). When fungicides were considered independently on this host, sporangium production levels were significantly higher ($P = 0.03$; Table S4; Fig. S6, Supporting information) and the number of zoospores per sporangium tended to be slightly lower ($P = 0.08$; Table S4; Fig. S6, Supporting information) for isolates resistant to CAA than for isolates susceptible to this fungicide. Sporangium size and latency period did not differ significantly between CAA-resistant and CAA-sensitive isolates. No significant difference in quantitative traits was found between QoI-resistant and QoI-sensitive isolates (Table S5; Fig. S6, Supporting information).

Discussion

Multiple origins of fungicide resistance alleles leading to soft selective sweeps

This study provides compelling evidence for the recent evolution of fungicide resistance alleles through soft rather than hard sweeps in *P. viticola* populations of the Bordeaux region. Our data support the emergence of alleles conferring resistance to QoI and CAA fungicides on several occasions in *P. viticola*. The multiple origins of QoI resistance have previously been documented (Chen *et al.* 2007). However, we provide the first demonstration that the Val and the Ser resistance mutations are present in one and three different haplotypes, respectively, indicating that mutations responsible for CAA resistance occurred independently at least four times.

Soft sweeps could have originated because adaptive alleles were already present at the onset of fungicide treatment or from de novo mutations afterwards. Polymorphisms conferring resistance could have been present in the North American isolates infecting wild grapevine populations and from which European *P. viticola* isolates originated. However, none of the

Table 3 Statistical analyses of the effects of resistance to two fungicides (fungicide resistance, FR) and of wine region of origin (WR) on quantitative pathogenicity traits in *P. viticola*. Isolates were used to inoculate *V. vinifera* cv. Cabernet sauvignon (susceptible) and Cabernet carbon (partially resistant)

	Cabernet sauvignon			Cabernet carbon		
	Fungicide resistance	Wine region	FRxWR	Fungicide resistance	Wine region	FRxWR
Main pathogenicity traits						
Sporangium production*	$F_{3,77} = 1.34$ $P = 0.27$	$F_{5,77} = 0.69$ $P = 0.63$	$F_{13,77} = 1.62$ $P = 0.10$	$F_{3,78} = 2.56$ $P = 0.61$	$F_{5,78} = 0.57$ $P = 0.72$	$F_{13,78} = 0.43$ $P = 0.196$
Sporangium size	$F_{3,77} = 1.10$ $P = 0.35$	**$F_{5,77} = 2.80$** **$P = 0.02$**	$F_{13,77} = 1.31$ $P = 0.23$	$F_{3,78} = 1.95$ $P = 0.13$	**$F_{5,77} = 4.41$** **$P = 0.001$**	$F_{13,78} = 1.29$ $P = 0.24$
Zoospores/sporangium*	$F_{3,75} = 0.65$ $P = 0.59$	$F_{5,75} = 2.18$ $P = 0.07$	$F_{13,75} = 0.96$ $P = 0.50$	$F_{3,78} = 2.36$ $P = 0.08$	$F_{5,78} = 1.95$ $P = 0.09$	$F_{5,78} = 0.78$ $P = 0.68$
Latency period*	$F_{3,77} = 0.19$ $P = 0.91$	$F_{5,77} = 1.80$ $P = 0.12$	$F_{13,77} = 0.93$ $P = 0.53$	$F_{3,78} = 1.77$ $P = 0.16$	$F_{5,78} = 0.94$ $P = 0.46$	$F_{13,78} = 1.01$ $P = 0.45$
Sporulation dynamics						
T_{50}	$F_{3,69} = 1.28$ $P = 0.29$	$F_{5,69} = 1.59$ $P = 0.17$	$F_{13,69} = 1.10$ $P = 0.37$	—	—	—
Sporulation rate*	$F_{3,69} = 0.95$ $P = 0.42$	$F_{5,69} = 1.39$ $P = 0.24$	$F_{13,69} = 0.61$ $P = 0.84$	—	—	—

–: T_{50} and sporulation rate were not estimated.
Statistically significant results (P-values < 0.05) are shown in bold.
*Log transformed.

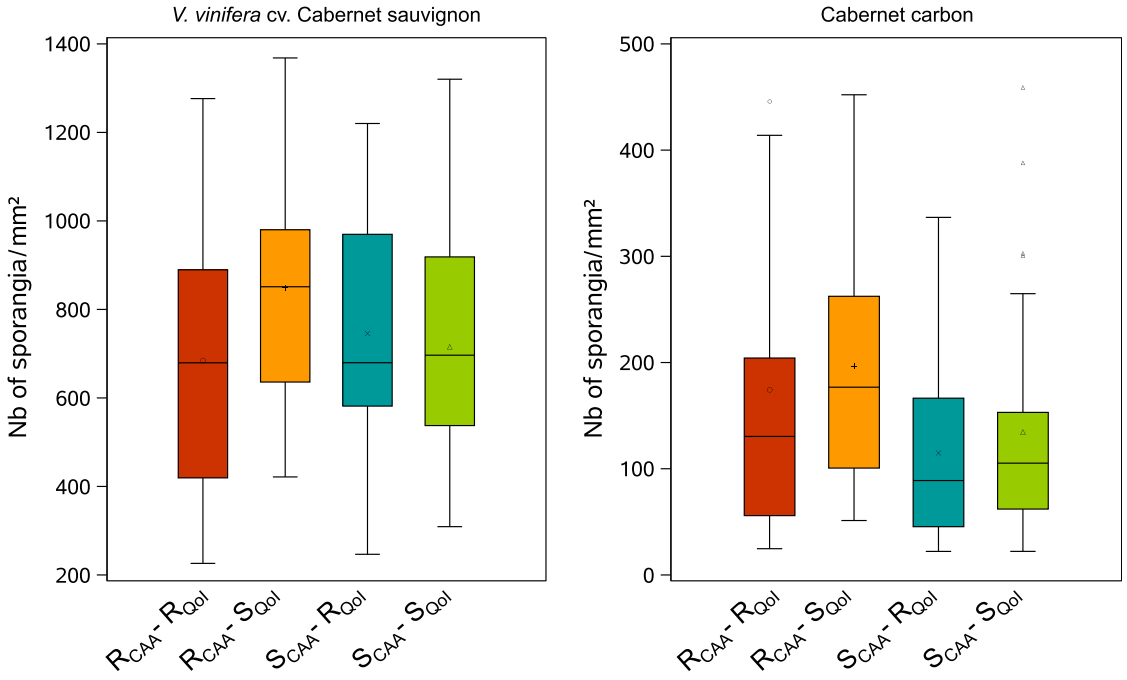

Fig. 5 Sporangium production of *Plasmopara viticola* isolates resistant (R) and sensitive (S) to CAA and QoI fungicides. Isolates were used to inoculate *V. vinifera* cv. Cabernet sauvignon (susceptible host) and Cabernet carbon (partially resistant host). Note that the *y*-axes of the two boxplots have different scales. The other quantitative traits are presented in Fig. S6 (Supporting information).

North American isolates contained resistance alleles. A large-scale study of the genetic diversity of *P. viticola* populations in their area of origin would help to test this hypothesis. Alternatively, de novo mutations in European populations may be responsible for the appearance of resistance alleles. A high population size and/or a high mutation rate is required for recurrent mutations of such small targets (Pennings & Hermisson 2006).

Fontaine *et al.* (2013) estimated the long-term population effective size of *P. viticola* at <1000 individuals in Western Europe. This low value is most likely linked to recurrent bottlenecks, associated with (i) the recent introduction of the species in Europe (Fontaine *et al.* 2013), (ii) intra- and interannual variations of climatic conditions and fungicide use, and (iii) the existence of a yearly sexual phase acting as a bottleneck for this heterothallic organism. During downy mildew epidemics, the number of infected plants in a given fungicide-treated vineyard can be very high, resulting in large population sizes. This gives the opportunity for multiple advantageous mutations to emerge in different haplotypes and be selected (Barton 2010), leading to soft selective sweeps (Messer & Petrov 2013). The selection of independent mutations despite recurrent changes in population size is more likely for strong selection pressures (Wilson *et al.* 2014), which is the case for fungicides. In summary, adaptation in *P. viticola* is probably not mutation-limited during epidemics, explaining a rapid adaptation to single-site fungicides and resulting in soft selective sweeps.

Soft sweeps have been described in many organisms including *Plasmodium falciparum*, common fruit flies, sticklebacks, mice, humans and HIV (see Messer & Petrov 2013 for a review), and theoretical studies showed they can be prevalent in a large number of situations (Hermisson & Pennings 2005; Wilson *et al.* 2014). Our data imply that soft sweeps are an important adaptation mechanism in *P. viticola*, and we can assume this is likely the case for many plant pathogens. However, as resistance alleles were not fixed in our populations, we may be observing an ongoing process. It is therefore possible that only one haplotype will eventually rise to fixation, especially if mutations or haplotypes have not the same selective effects (Jensen 2014). Following this idea, our bioassays suggested that the Val allele provided a higher level of resistance than the Ser allele, but these results require confirmation using a larger number of isolates.

Lack of neutral genetic structure and higher differentiation at fungicide resistance loci

An analysis of microsatellite polymorphism showed that the populations were at Hardy–Weinberg

equilibrium, with an absence of population genetic structure and low levels of genetic diversity (low H_e for polymorphic loci and the presence of four monomorphic loci) at the regional scale. These results are consistent with those of Gobbin *et al.* (2003, 2006), Fontaine *et al.* (2013) and Delmas *et al.* (2016). The lack of neutral genetic structure may be due to recent introduction into Europe from a single source population (Fontaine *et al.* 2013) and high levels of gene flow between vineyards. In addition, no genetic clustering of isolates by fungicide resistance category was observed, and genetic diversity was very similar between these categories. This may be due to the multiple occurrences of resistance alleles in different genetic backgrounds, as discussed above.

Our F_{ST} outlier approach showed that there was much more differentiation at positions involved in fungicide resistance than at microsatellite loci, consistent with a role for local adaptation, that is spatially heterogeneous selection, in the variation of allele frequencies between areas. Indeed, the differentiation levels for these positions were 2.6 and 5.1 times higher than for the microsatellite locus with the highest G'_{ST} value. The absence of hitchhiking signal for neighbouring sites is most likely due to the existence of the resistance mutations in different haplotypes. The high level of differentiation at resistance positions reflects high levels of heterogeneity in fungicide spraying between vineyards, resulting in considerable differences in selection pressure across the region studied. Indeed, spatial and temporal patterns of fungicide treatments are managed at the vineyard scale in response to local epidemiological and climatic conditions.

This heterogeneity of treatments can also explain why resistance alleles are not fixed in populations, with a global frequency of resistance alleles of only 53% for QoI and 44% for CAA. In addition, QoI and CAA fungicides are used in association with multisite fungicides. Theoretical studies have shown that the heterogeneity of sprays and the use of mixtures of different molecules favour the coexistence of sensitive and resistant isolates (Parnell *et al.* 2005, 2006; van den Bosch & Gilligan 2008; Mikaberidze & McDonald 2015). Another explanation relies in the genetic determinism of resistance. The recessive nature of CAA resistance may have a direct impact on the evolution of resistance to this fungicide. Indeed, recessive alleles are hidden from selection if heterozygous (Gly/Ser, Gly/Val), resulting in slower changes in allele frequency during selection events (Haldane 1927; Teshima & Przeworski 2006). This is, however, not relevant for the mitochondrially inherited QoI resistance. More generally, the maintenance of sensitive strains in *P. viticola* populations suggests that there may be hidden costs of resistance.

Is there a cost of fungicide resistance?

Resistance alleles have been predicted to be disadvantageous in the absence of pesticides, in many organisms (Bergelson & Purrington 1996; Andersson & Levin 1999; Bourguet *et al.* 2004; Melnyk *et al.* 2015; Mikaberidze & McDonald 2015). However, experimental data are less clear-cut, and fitness costs depend on many factors, including the mechanism underlying resistance (Coustau & Chevillon 2000; Lalève *et al.* 2014). Here, we found no significant differences between the aggressiveness of resistant and sensitive isolates to QoI or CAA fungicides, in optimal (susceptible host) and suboptimal (partially resistant host) environments. This lack of fitness cost for resistance to QoI or CAA fungicides was evidenced even in isolates resistant to both CAA and QoI (20% of isolates). Our data may reflect a real absence of cost, but they may also result from a lack of detection of fitness costs in the experimental conditions used. Resistance costs may be apparent only under a specific set of environmental conditions, or the traits affected by resistance acquisition may not be considered in the analysis. We analysed the main pathogenicity traits relating to pathogen dispersal and infection. However, fitness costs may occur mostly during the winter sexual reproduction phase, which was not taken into account in this study. Such fitness costs have been reported for *P. infestans* during host adaptation (Montarry *et al.* 2010). Modelling the spatio-temporal distribution of fungicide resistance alleles in vineyards might provide a better assessment of fitness costs as this approach integrates the whole life cycle of the pathogen (Orr 2009; Rieux 2011). Finally, compensatory mutations, counteracting fitness penalties due to a mutation conferring resistance, have been widely documented (Kimura 1990; Andersson & Levin 1999; Vallières *et al.* 2011). This is the most likely explanation in grapevine downy mildew characterized by a large supply of adaptive genetic polymorphism. In addition, resistant pathogens have been evolving for more than 15 years under selective pressure due to fungicide applications, allowing compensatory mutations to occur.

Fitness costs linked to QoI resistance have been shown to be variable among plant pathogens (reviewed in Fernández-Ortuño *et al.* 2008; Hollomon 2015). For example, no fitness cost was found in *Erysiphe graminis* (Chin *et al.* 2001), *Alternaria alternata* (Karaoglanidis *et al.* 2011) or in the studied pathogen *P. viticola* (Corio-Costet *et al.* 2011), whereas fitness penalties were detected in *Botrytis cinerea*, *Cercospora beticola* and *Ustilago maydis* (Ziogas *et al.* 2002; Malandrakis *et al.* 2006; Markoglou *et al.* 2006). It has been suggested that the presence of an intron after the Gly143 codon in the *cytb* genes of several species is responsible for the observed difference in fitness cost between plant pathogens (Fisher & Meunier 2008). The

Gly143Ala mutation impedes intron splicing and is, therefore, deleterious when *cytb* contains this intron. The *cytb* gene of grapevine downy mildew does not possess this intron (Grasso *et al.* 2006; Sierotzki *et al.* 2007), probably accounting for the lack of fitness penalty found here.

The lack of fitness cost associated with CAA resistance in grapevine downy mildew is reported for the first time here. We found that the resistant genotypes (Ser/Ser, Val/Val, Ser/Val) had a phenotype (i.e. aggressiveness) similar to that of the sensitive genotypes. Our results even suggested that resistant isolates produced more spores in suboptimal host environments (Cabernet carbon). The few studies addressing the cost of CAA resistance in oomycetes used isolates obtained by experimental evolution under fungicide pressure (Wang *et al.* 2010; Pang *et al.* 2014) or UV mutagenesis (Zhu *et al.* 2008). Their results were variable, ranging from no fitness cost (Pang *et al.* 2014), variable fitness costs depending on pathogenicity trait or strain (Wang *et al.* 2010) to a lower pathogenicity of resistant isolates (Zhu *et al.* 2008).

Towards a durable fungicide management

In *P. viticola*, the evolution of resistance is not limited by mutation because of large population sizes during epidemics or by fitness costs. The most recent approaches in medicine for managing viruses or bacteria focus on high treatment heterogeneity that is associated with a slower evolution of drug resistance (Bal *et al.* 2010; Feder *et al.* 2016). The combination of molecules having different modes of action should therefore always be favoured to increase the long-term efficiency of fungicides (multiple intragenerational killing, REX Consortium 2013). However, this strategy may not be successful when resistance alleles have already increased in frequency, such as for CAA- and QoI-resistant alleles. By extension, we suggest that the combination of fungicide treatments with other agronomical practices for pathogen control may be a relevant management strategy to slow down the evolution of resistance (REX Consortium 2016). The use of resistant grapevine varieties is a promising strategy to control grapevine downy mildew (up to 90% of sporulation reduction; Delmas *et al.* 2016). A key challenge for viticulture is therefore to design new cropping systems combining disease natural resistance of grapevine with fungicide treatments to achieve a durable control of the pathogen.

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Data accessibility

Trait data (quantitative traits of pathogenicity of *Plasmopara viticola* isolates), genetic data (gene haplotypes and microsatellite genotypes), geographic sampling locations for each isolate and DNA sequences can be found in the following Dryad package: doi:10.5061/dryad.vp8n9.

DNA sequences : GenBank Accession nos KY403517–KY403621 (*Cytb*), KY403622–KY403671 (*CesA3*, long fragment), KY403672–KY403887 (*CesA3*, short fragment).

C.E.L.D., L.D. and F.D. designed the research; C.E.L.D., L.D., C.C., I.D.M., S.R.C. and F.D. performed the experiments; C.E.L.D. and Y.D. analysed the data; C.E.L.D., Y.D. and F.D. wrote the manuscript; and all authors revised the final version of the manuscript.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Dose-response curves for the treatment with dimethomorph, a CAA fungicide, of six sensitive *Plasmopara viticola* isolates and five resistant isolates collected in the Bordeaux wine region in 2012.

Fig. S2 Median-joining haplotype network for *cytb*.

Fig. S3 Median-joining haplotype network for the short fragment of *CesA3*.

Fig. S4 Principal component analysis on microsatellite data for *Plasmopara viticola* strains from the Bordeaux region.

Fig. S5 Bayesian clustering analysis of *Plasmopara viticola* in the Bordeaux region.

Fig. S6 Quantitative pathogenicity traits of *Plasmopara viticola* isolates.

Table S1 Genetic diversity of *cytb* and *CesA3* for *Plasmopara viticola* in the Bordeaux region

Table S2 Pairwise F_{ST} values between populations (wine region of origin). Upper diagonal: F_{ST} values, lower diagonal: *P*-values calculated from 15 000 permutations.

Table S3 Overall F_{ST} values and differentiation tests for microsatellite loci and polymorphic positions in the *CesA3* and *cytb* genes.

Table S4 Statistical analyses of the effects of resistance to CAA fungicides and of wine region of origin on quantitative pathogenicity traits in *P. viticola*.

Table S5 Statistical analyses of the effects of resistance to QoI fungicide and of wine region of origin on quantitative pathogenicity traits in *P. viticola*.