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At Least Two Origins of Fungicide Resistance in Grapevine Downy Mildew Populations[▽]

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Quinone outside inhibiting (QoI) fungicides represent one of the most widely used groups of fungicides used to control agriculturally important fungal pathogens. They inhibit the cytochrome bc_1 complex of mitochondrial respiration. Soon after their introduction onto the market in 1996, QoI fungicide-resistant isolates were detected in field plant pathogen populations of a large range of species. However, there is still little understanding of the processes driving the development of QoI fungicide resistance in plant pathogens. In particular, it is unknown whether fungicide resistance occurs independently in isolated populations or if it appears once and then spreads globally by migration. Here, we provide the first case study of the evolutionary processes that lead to the emergence of QoI fungicide resistance in the plant pathogen *Plasmopara viticola*. Sequence analysis of the complete cytochrome *b* gene showed that all resistant isolates carried a mutation resulting in the replacement of glycine by alanine at codon 143 (G143A). Phylogenetic analysis of a large mitochondrial DNA fragment including the cytochrome *b* gene (2,281 bp) across a wide range of European *P. viticola* isolates allowed the detection of four major haplotypes belonging to two distinct clades, each of which contains a different QoI fungicide resistance allele. This is the first demonstration that a selected substitution conferring resistance to a fungicide has occurred several times in a plant-pathogen system. Finally, a high population structure was found when the frequency of QoI fungicide resistance haplotypes was assessed in 17 French vineyards, indicating that pathogen populations might be under strong directional selection for local adaptation to fungicide pressure.

Preventing and delaying resistance adaptation to fungicides in pathogen populations is a major goal in sustainable plant pathogen management. The development of effective strategies to control pathogen populations requires precise understanding of the conditions under which fungicide resistance alleles appear, spread, and are maintained in natural populations. Among the factors that influence the evolution of resistance, the rate of mutation in fungicide resistance-associated genes plays an important role. It is still uncertain whether fungicide resistance-associated mutations commonly arise independently or whether pest adaptation occurs by convergent evolution through recurrent mutations at selected sites in the genes coding for the fungicide target. In other words, do alleles conferring fungicide resistance arise once before spreading across populations, or do they arise independently in each locality, and are they favored by large local population sizes of the pathogen population?

Numerous experimental and phylogenetic studies in extensive geographical contexts have already examined the pattern of the evolution of genes coding for antimicrobial drugs or pesticide targets in animals, plants, and bacteria. For instance, the question of parallel genetic adaptation to drugs and pesticides has already been addressed for various insecticide resistances (2–4, 14, 42, 55), for herbicide resistance (11, 16), for resistance to a nematocide (17), and for resistance to antibiotics (8, 32). However, to our knowledge, no study has ever addressed the question of the number of origins of fungicide resistance alleles in plant pathogen populations. To study the evolutionary process that leads to the onset of fungicide resistance alleles, we investigated the evolution of the target site for quinone outside inhibiting (QoI) fungicides in the plant pathogen *Plasmopara viticola*.

Launched in the late 1990s, strobilurin and the other fungicides belonging to the group of QoI fungicides are an important class of chemicals for the management of a broad range of fungal diseases in agricultural systems. These fungicides are effective against three major groups of plant pathogens: oomycetes, basidiomycetes, and ascomycetes (9, 34). The QoI fungicides act as mitochondrial respiration inhibitors by binding to the ubiquinol oxidation site (Qo) formed by the cytochrome *b* and iron-sulfur protein domains within the cytochrome bc_1 complex (10, 25). By compromising ATP production in mitochondria, QoI fungicides affect the energy-demanding stages of pathogen development, such as spore germination.

The first QoI fungicide (azoxystrobin) was launched for use in agricultural pest management in 1996. One year later, the development of field-reduced sensitivity to azoxystrobin in populations of the plant pathogen *Mycosphaerella fijiensis* was first observed (49). As for other single-site inhibitors, the longevity of QoI fungicides was thus quickly challenged in the field, and resistant isolates have been described for numerous

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plant pathogen species, such as *Alternaria solani*, *Blumeria graminis*, *Mycosphaerella graminicola*, *Plasmopara viticola*, *Podosphaera fusca*, *Puccinia* species, *Magnaporthe oryzae*, and *Venturia inaequalis* (5, 21, 22, 30, 33, 35, 38, 44, 49, 50, 52, 54, 56). In most cases, resistance to QoI fungicides is conferred by a point mutation in the mitochondrial cytochrome *b* gene, giving rise to a substitution from glycine to alanine at position 143 (G143A) of the amino acid sequence (25, 29, 35). In addition, another mutation at amino acid sequence position 129 of cytochrome *b* resulting in a substitution from phenylalanine to leucine (F129L) has also been reported to confer resistance to QoI fungicides in species such as *Pythium aphanidermatum*, *Magnaporthe oryzae*, and *Alternaria solani*, although the level of resistance is lower than that conferred by the G143A substitution (25, 35, 44).

P. viticola (Berkl. and Curt.) Berl. and de Toni., the causal agent of grapevine downy mildew disease, is a native species from North America that was introduced several times into Europe in the late 1870s (28). The disease spread rapidly through most of continental Europe to become one of the most important vineyard diseases (23). Chemical control is the most effective measure currently used to protect grapes from downy mildew disease. QoI fungicides were introduced into France for pest management in viticulture in 1998. Only 2 years later, QoI fungicide resistance was detected in *P. viticola* populations of most French vineyards (39), and the nonsynonymous G143A substitution was detected using a quantitative PCR method (48, 51). Moreover, the results of a recent comparative survey of the cytochrome *b* gene structure in various plant pathogen species suggest that the F129L substitution of the cytochrome *b* gene could also be involved in resistance to QoI fungicides in *P. viticola* (29).

The recent selection of well-characterized mutations associated with QoI fungicide resistance in natural populations of *P. viticola* provides a unique opportunity to understand the mechanisms leading to the evolution of fungicide resistance in a plant-pathogen system. Because the mitochondrial genome does not recombine, QoI fungicide resistance is a good model to investigate the number of origins of resistance alleles. In this respect, we address three fundamental questions concerning the evolution of QoI fungicide resistance haplotypes. (i) How many mutations are involved in resistance to QoI fungicides in grapevine downy mildew populations? (ii) How often do these mutations arise in natural populations of grapevine downy mildew populations, i.e., is resistance the result of a single mutation in an allele that spreads subsequently to different areas or rather the result of multiple convergent acquisitions of the same mutation in the gene coding for the cytochrome *b* target? (iii) What is the genetic structure of QoI fungicide resistance alleles in French *P. viticola* populations after 5 years of use of this group of fungicides?

Based on the complete sequence of the cytochrome *b* gene, nucleotide polymorphism variations were analyzed in a broad range of sensitive and resistant isolates of *P. viticola* collected in European and American vineyards. Patterns of nucleotidic polymorphisms confirmed the occurrence of a unique resistance mutation in the cytochrome *b* gene, while phylogenetic analyses of the complete fragment provided evidence for at least two independent origins of QoI fungicide-resistant haplotypes in natural populations of *P. viticola*. In addition, the genetic structure of QoI fungicide-resistant mitochondrial haplotypes in 17 French populations is discussed in relation to local fungicide selective pressures.

MATERIALS AND METHODS

***P. viticola* sampling and collection.** *P. viticola* is a heterothallic diploid oomycete (Oomycota, Stramenopiles) that undergoes several asexual generations during the grape growing season and one sexual cycle in autumn. In spring, oospores resulting from sexual reproduction germinate and release zoospores that give rise to primary infections. Under favorable weather conditions, asexual reproduction leads to the secondary infection through the production of sporangia containing zoospores that spread to the leaves and berries by splashing rain or wind.

A total of 819 downy mildew lesions on *Vitis vinifera* were collected. Each sample consisted of 1 cm^2 of a fresh leaf colonized by sporulating downy mildew (also called oil spots). Since previous studies have shown that a single lesion rarely includes more than one multilocus genotype (27), we treated each lesion (oil spot) as a single strain.

Samples came from 51 localities in France ($n = 799$); 20 localities in Italy, Switzerland, Germany, Portugal, Spain, Greece, and Romania ($n = 51$); and 1 locality in the United States ($n = 14$) (Table 1). In France, sampling was carried out during June and July of the grapevine growing season in 2003 (Table 1). For samples collected abroad, the years of collection ranged from 1997 to 2004.

Among the 51 populations sampled in France, 17 populations (representing a total of 676 samples) were used to further analyze the genetic structure of mitochondrial haplotypes. These 17 populations included 12 fields not treated with QoI fungicide in 2003 and 5 fields that were treated in 2003. The isolates from treated fields were sampled after at least one QoI fungicide treatment. Plots ranged in size from 100 to 300 vines (3 to 5 rows) included in vineyards under fungicidal management. In a field, each sample was collected on a different vine.

DNA extraction. Oil spots were freeze-dried overnight, and DNA was extracted from each of them (containing infected grapevine leaf tissue and sporangia) according to the standard cetyltrimethylammonium bromide-phenol-chloroform method described previously (58) with a slight modification (phenol-chloroform was replaced with isoamyl alcohol and chloroform at a dilution ratio of 24:1). After extraction, DNA was precipitated with isopropanol, and the pellet was washed with 75% ethanol and resuspended in 50 μl of sterile water.

Fungicide in vitro sensitivity tests. To characterize *P. viticola* isolate sensitivity to the QoI fungicides, 27 single lesions were multiplied onto leaves of *Vitis vinifera* cv. Cabernet-Sauvignon. Sporangia were collected and suspended in sterile water at 4°C. The inoculation was done by depositing fifteen 10-μl droplets onto the adaxial face of each leaf. The leaves were incubated in petri dishes under controlled conditions (21°C) with a 16 h photoperiod. After 7 days, freshly produced sporangia were harvested to inoculate the test units.

Six discriminatory doses (10, 100, 300, 600, 1,000, 1,500 mg $liter^{-1}$) of an aqueous solution of famoxadone fungicide (96%) were applied in the bioassay. For sensitive isolates that did not grow at the fungicide concentration of 10 mg/liter, eight other fungicide doses were applied to determine the MIC: 0.1, 0.2, 0.5, 0.8, 1, 2, 5, and 10 mg $liter^{-1}$. For each concentration (including a control), 3 ml of fungicide was sprayed using a hand-held sprayer (Ecospray) onto the lower side of 10 detached grapevine leaf disks placed in a petri dish. The upper sides of the leaves were placed in contact with filter paper impregnated with 4 ml of water. After the surfaces were dried at room temperature, the leaf disks were inoculated with 3 droplets of 10 μl per disk of an inoculum of 20,000 sporangia per ml. After 7 days of inoculation, development of mildew on each disk was visually estimated as the proportion of leaf area with sporulation (24). For each fungicide concentration, 10 leaf disks were tested. The average score for each fungicide concentration was converted to a percentage of inhibition by comparison with the untreated disks. The fungicide concentration inhibiting 50% of growth (IC_{50}) and the MIC of growth (MIC) discriminated two classes of isolates according to their sensitivity to famoxadone: famoxadone-sensitive isolates (IC_{50} and MIC <10 mg $liter^{-1}$) and highly resistant isolates (IC_{50} and MIC >100 mg $liter^{-1}$). For all isolates, DNA was extracted for subsequent sequencing to characterize molecular variations in the cytochrome *b* gene sequence.

Isolation of cytochrome *b* gene and flanking regions. Initial amplification of the mitochondrial genome fragment was obtained by performing a PCR with degenerate primers (P1) designed from two oomycete sequences (*Phytophthora infestans*, NC002387, and *Phytophthora megasperma*, L16863) and a reverse primer (P2) specific to *P. viticola* cytochrome *b* (partial sequence, AX577570) (Table 2; Fig. 1). Each reaction was carried out in a PCR of 30 μl containing 0.2 μM of each primer, 0.33 mM of each deoxynucleoside triphosphate (dNTP), 1×

TABLE 1. Geographic origin and sampling year of the 865 samples of *Plasmopara viticola* collected in European vineyards and in the United States[g]

Country and vineyard location(s)	Samples used for sequencing[h]	Samples used for SNP typing collected in 2003
France		
Bordeaux and southwestern area	**AUB** (2)[a], **BIE** (1)[a], BLA(4)[b], **BOM** (6)[b], BON (1)[f], **CAZ** (1/3)[b], CHA (1)[b], CIS (1)[f], **CGU** (3)[b], **COU** (7)[b], CUS (2)[b], GAL (3)[b], **GF** (3)[b], JUI (1)[b], **LEV** (1)[b], **LEO** (2)[b], LIS (3)[b], MER (1)[f], MON (3)[b], **MOR** (3)[b], **PAR** (6)[b], **PAU** (3)[b], PEI (2)[f], **PCN** (2)[b], **PIC** (5)[b], POR (5)[b], PVC (3)[b], **SAL** (3)[b], SAU (1)[f], SJB (3)[b], VER (1)[b], VIG (4)[b], **ZAN** (3)[b]	BLA (50), COU (86), GAL (45), GCU (44), MON (41), PAU (44), PCN (36), ZAN (43)
Bourgogne and Rhône Valley	**BEA** (3)[a,b], **FEM** (2)[b], GAR (1)[f], MCO (1)[f], NIM (1)[a], PIZ(3)[a], SAV(3)[a]	NIM (81)
Champagne	**AVI** (2)[b], CRE (2)[b], **EPE** (2)[b]	AVI (35), BAR (32), CRE (18), PLU (34)
Loire Valley	BOS (2)[b], **TOU** (2)[b], LDV (2)[b], **MAR** (2)[b], **MAI** (1)[b], **MTL** (2)[b], VAU (1)[f]	BOS (11), LDV (16), MAR (30), TOU (30)
Germany	A (1)[e], **ERB** (3)[c], **FRE** (4)[b], **GUN** (3)[b], **LOR** (3)[c]	
Greece	**THE** (3)[c]	
Italy	**ALB** (3)[c], **MAL** (1)[e], I (1)[d,e], **NAV** (2)[c]	
Portugal	**P** (2)[c], **PTG** (3)[a]	
Romania	**ARA** (4)[a], **HER** (4)[a], **REC** (1)[a]	
Spain	**E** (3)[c], **MAD** (1)[a]	
Switzerland	**CUT** (5)[c], **PER** (3)[c], **STA** (3)[c]	
United States	**USA** (14)[b]	

[a] Samples collected in 2004.
[b] Samples collected in 2003.
[c] Samples collected in 2000.
[d] Samples collected in 1999.
[e] Samples collected in 1998.
[f] Samples collected in 1997.
[g] Localities are coded with one to three letters, and the total number of samples analyzed (sequenced and/or SNP typed) is indicated in parentheses. Localities of the 70 samples for which we sequenced the complete mitochondrial fragment (2,281 bp) are indicated in bold.
[h] A, Beilstein Amalienhof; ALB, Piémont; ARA, Arad; AUB, Aubertin; AVI, Avize; BAR, Barbonne; BEA, Beaune; BIE, Bielle; BLA, Blanquefort; BOM, Bommes; BON, Bonzac; BOS, Bonny-sur Loire; CAZ, Cazaugitat; CIS, Cissac; CGU, Cadaujac; CHA, Charente; COU, Couhins; CRE, Crezancy; CUS, Cussac; CUT, Cugnasco; E, Pontevedra; ERB, Erbach; EPE, Epernay; FEM, Puligny; FRE, Freiburg; GAL, Monbazillac; GAR, Monfrin; GCU, Chateau Guiraud; GF, Grande-Ferrade; GUN, Guntersblum; HER, Herculane; I, Emilia Romagna; JUI, Juillac le coq; LDV, Angers; LEO, Léognan; LEV, Les lèves; LIS, Listrac; LOR, Lorch; MAD, Madrid; MAI, St-Mélaine; MAL, Chianti; MAR, Martigné le grand; MCO, St-Marie; MER, Mérignac; MON, Caussens; MOR, Morizes; MTL, Montlouis sur Loire; NAV, Navicello; NIM, Nîmes; P, Braga; PAR, Parempuyre; PAU, Pauillac; PCN, Pomerol; PEI, Perpignan; PER, Perroy; PIC, Latresne; PIZ, Villefranche sur Saone; POR, Pomport; PTG, Refois do Lima; PVC, Cenon; REC, Recas; SAL, Salleboeuf; SAU, Sauternes; SAV, Savigny les Beaune; SJB, St-Jean de Blaignac; STA, Stäfa; THE, Thessalie; TOU, Tours; USA, Finger Lakes region (Geneva, NY); VAU, Le Vau Delnay; VER, Verrière; VIG, Vignonet; ZAN, Rauzan.

reaction buffer, 1.5 mM of $MgCl_2$, 0.33 mM of each dNTP, 0.5 U of *Taq* polymerase, and 1 μl of genomic DNA. PCR cycles consisted of 95°C for 4 min, 35 cycles of 95°C for 45 s, 57°C for 45 s, and 72°C for 50 s, and a final extension step of 72°C for 7 min. The PCR products were purified and used for sequencing.

TABLE 2. Primers used for the amplification, sequencing, and SNP typing of the mtDNA of *Plasmopara viticola* isolates

ID[a]	Primer name	Sequence (5′–3′)
P1	ND9 394F	ACN GAT TAY GGT TTT GAA GGW CA
P2	CB 505R	GAA TCC ACC CCA TAA CCA GTC A
P3	ATP9 132F	AGA ATT AAT DAG AAC TGC TAT T
P4	ND9 537R	TAA ATT TTT ATA CCA AGG ACT A
P5[b]	ND9 529F	TGA ATT TAA TAG TCC TTG GTA
P6[b]	CB 773F	AAG CAA ATC CTA TGA AAA CTC C
P7	CB 279F	TAT ACA TAT TTT TAG GGG TTT G
P8[b]	CB 485F	ATT AAT TGG AAA AGA AGT TGT TGA
P9	CB 865R	ACA TAG CGA TAA CAC CAC CAA T
P10[b]	ND5 247R	TAT TAC GAC CAG CAT TAT CAT TGT
P11[b]	CB 1003R	CAT TGA CCA ACC CAT CCT AAT AA
P12[b]	ATP9 187F	TTT TGT TTA ATG ATG GCT TTT T
P13[b]	CB 100R	CTA ACA TRA TAC CGG CTA ATG A
P14[c]	SNP 0R	CCA AGG CAA AAC ATA ACC CAT AA
P15[c]	SNP 1R	AAT AAA TTT GTA ATA ACT GTT GCA
P16[c]	SNP 1Rbis	TTC CAA TTA ATG GGA TAG CCG A
P17[c]	SNP 2F	TCA TCC GGA TAA TTA TAT TGA AGC

[a] ID, primer identity.
[b] Working primers used to systematically amplify and sequence the mtDNA fragment.
[c] Extended (SNP) primers.

The unknown flanking sequence was determined at both ends by a primer walking procedure to obtain the entire cytochrome *b* gene sequence and that of its neighboring regions. The upstream sequence was successfully determined according to the same procedure as described above using a degenerate forward primer (P3, located at the 3′ end of ATP9 gene) and a reverse primer (P4) specific to the template *P. viticola* sequence (Table 2; Fig. 1). A genome walking strategy as implemented in the Universal GenomeWalker kit (BD Biosciences) was subsequently used to obtain the unknown downstream cytochrome *b* sequence. A large quantity of genomic DNA was extracted from sporangia produced on several leaves infected by a single strain of *P. viticola* (BEA 18). Pools of uncloned adaptor-ligated DNA fragments of *P. viticola* were produced by digestion with a set of seven restriction enzymes (PvuII, HincII, ScaI, Bst1107 I, KspAI, MssI, and Eco105I). After DNA library construction, two successive PCRs were performed: the first amplification used the outer adaptor primer AP1 and the gene-specific primer P8. This primary PCR mixture was diluted to be used as a template for the second nested PCR amplification with adaptor primer AP2 and primer P6 (Table 2; Fig. 1). More details about the protocol and PCR conditions are described in the manual of the Universal GenomeWalker kit (BD Biosciences).

Mitochondrial sequence diversity. A mitochondrial fragment of 2,281 bp including the cytochrome *b* gene was amplified and sequenced for 56 European and 14 American samples (Table 1). In addition, a partial sequence was generated on the 5′ end, the middle part, and the 3′ end of the complete fragment (length ranging from 700 bp to 1,500 bp) for 119 additional samples. This resulted in a data set of 182 mitochondrial DNA sequences that allowed the detection of 23 polymorphic sites (single nucleotide polymorphisms [SNPs]). Primers used for the sequencing are presented in Fig. 1 and Table 2.

SNP typing. SNP typing was performed by using a primer extension genotyping assay with fluorescence polarization (FP) detection (12) implemented in the AcycloPrimeTM-FP SNP detection kit (PerkinElmer Life Science, Inc.) from a preamplified 630-bp SNP-containing fragment synthesized using primers P7 and P9 (Table 2; Fig. 1). The PCR was performed in a 30-μl volume containing 0.13

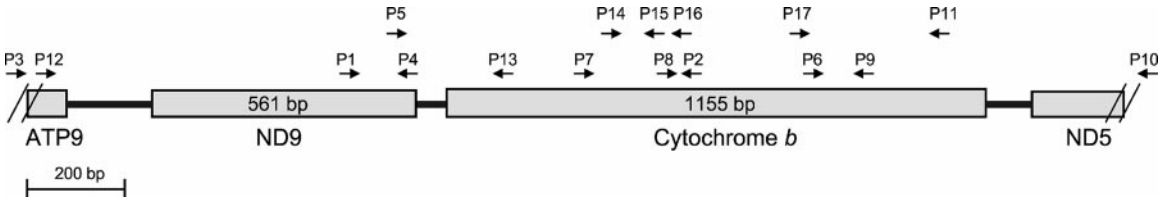

FIG. 1. Schematic representation of primer localities and gene organization of the mitochondrial fragment isolated and used for sequence analyses in this study. The open reading frames of genes are indicated by gray boxes. The bold line between genes represents intergenic regions. Arrows above the fragment indicate different primers used in this study.

μM of each primer, 0.2 mM of each dNTP, 1× reaction buffer, 1.5 mM of $MgCl_2$, 0.33 mM of each dNTP, 0.5 U of *Taq* polymerase (Silverstar; Eurogentec Inc.), and 1 μl of genomic DNA. Subsequent procedures were conducted as follows: (i) the AcycloPol polymerase was used to extend by one base a SNP primer (P15 to 17) (Table 2; Fig. 1) that ends immediately upstream of a targeted SNP position using one of two fluorescent dye-labeled terminators, depending on the SNP profile previously determined, and (ii) the identity of the terminal base added was determined by the increased FP of its linked dye (scanned and analyzed by Vector3TM, PerkinElmer Life Science, Inc.). PCR cycles for the primer extension reaction consisted of 95°C for 6 min and 35 cycles of 95°C for 15 s and 55°C for 30 s. We used a lower T_m temperature (51°C) for the assay using P16 so as to optimize the result. The reaction was prepared on a 96-well dark plate and subsequently visualized on a Vector3TM scanner.

Intraspecific phylogenetic analyses. Two graphical representations obtained by the following methods were used to illustrate the evolutionary relationships among haplotypes derived from 70 mitochondrial sequences of *P. viticola* samples from Europe and North America. First, a phylogram was generated from phylogenetic analysis as implemented in PAUP version 4.0b10 (53) using the criterion of maximum parsimony (MP) under the exhaustive search option. The resultant MP tree was rooted using the midpoint method as implemented in PAUP. Statistical support of nodes was evaluated using MP bootstraps (19) with 1,000 replicates. Second, a haplotypic network was constructed using the median-joining network approach (7) as implemented in the program NETWORK, version 4.111 (available at http://www.fluxus-engineering.com/sharenet.htm).

Population genetic analysis. The genetic structure was examined by analysis of molecular variance (AMOVA) using the Arlequin 2.001 software package (18). This method was used to partition the genetic variance within populations, among populations within groups, and among groups. The populations were grouped into regions according to their geographical origin. Levels of significance were determined through 1,000 random permutation replicates. Fisher exact tests for population differentiation were performed using Genepop version 3.1b (47).

Nucleotide sequence accession numbers. The nucleotide sequences detected in this study have been deposited in GenBank under the accession numbers AY696297 and DQ459459 to DQ459469.

RESULTS

Mitochondrial DNA cloning and sequencing. A fragment consisting of 2,281 nucleotides of *Plasmopara viticola* mitochondrial DNA was successfully obtained. The sequence includes two complete genes (cytochrome *b* and NAD9), two partial genes (ATP9 and NAD5), and three complete intergenic regions (Fig. 1). The cytochrome *b* gene of *P. viticola* coding for the QoI fungicide target was characterized by 1,155 nucleotides encoding 384 amino acids with 1 and 3 amino acids in addition to those of *Phytophthora infestans* (36) and *Saprolegnia ferax* (31), respectively. Comparison of the gene order in *P. viticola* mitochondrial DNA (mtDNA) with *P. infestans* showed that gene organization upstream from the cytochrome *b* gene (represented by ATP9 and NAD9 genes and two intergenic regions) was the same, whereas the downstream region in *P. viticola* was followed by an intergenic region and the NAD5 gene (Fig. 1) instead of the ATP1 gene in *P. infestans* (43). No intron sequence was found in the *P. viticola* cytochrome *b* gene.

Mitochondrial sequence variability. A set of 70 complete mitochondrial sequences with 2,281 nucleotides and 119 partial sequences (between bp 700 and 1500) was examined representing 71 different localities in European countries and 14 samples from the United States. Twenty-three polymorphic sites were detected among all the sequences (Table 3). Except for one nonsynonymous mutation in cytochrome *b* (position 1256), the 22 polymorphic sites were either synonymous substitutions or mutations in noncoding regions. Ten polymorphic sites were parsimony informative, and 11 different mitochondrial haplotypes were identified (Table 3).

Five haplotypes were found among the 14 sequences of samples from the United States, while only six haplotypes were detected from the 56 samples from France, Germany, Italy, Spain, Portugal, Switzerland, Greece, and Romania (Fig. 2; Table 1). Analysis of the 119 additional partial mitochondrial sequences in European samples confirmed the existence of six main haplotypes in Europe. No American haplotypes were found in European samples, and there was no association between geographic sites and haplotype distribution in European populations. A strong correlation was found between haplotype and QoI fungicide sensitivity, QoI fungicide-resistant isolates exhibiting a nonsynonymous substitution resulting in the replacement of a glycine by an alanine at position 143 (Fig. 1; Table 4). Among the 175 European sequences analyzed, the frequency of European haplotypes IS, IR, IIS, and IIR was 56%, 21.7%, 19.4%, and 2.85%, respectively. Two haplotypes (IS′ and IIS′) were rare and represented less than 0.5%. Haplotypic diversity was 0.81 in the American population and 0.617 for the whole European data set.

Famoxadone sensitivity and resistance mutation at amino acid position 143. QoI fungicide sensitivity assays of 27 isolates demonstrated two distinct groups of sensitivity in *P. viticola* (Table 3). The first group represents isolates sensitive to famoxadone with a mean MIC (± the standard deviation) of 0.68 ± 0.23 mg $liter^{-1}$. The second group comprises isolates resistant to famoxadone with MICs >1,000 mg $liter^{-1}$. The mean resistance factor between the two groups of sensitivity was >2,040 (Table 4).

Table 4 demonstrates that the mutation at amino acid position 143 is associated with the high level of famoxadone resistance in *P. viticola*. Moreover, the analysis of 189 complete or partial mitochondrial sequences from European and American samples failed to detect any mutation at amino acid position 129 of the cytochrome *b* gene. This result was also confirmed

TABLE 3. Polymorphic sites of the mitochondrial fragment (2,281 bp) including cytochrome *b* gene identified among 189 *P. viticola* isolates in American and European populations

Accession no.	Geographic origin	*n*[a]	Mt haplotype[b]	Nucleotide at position																						
				79	168	570	993[c]	1011[c]	1074[c]	1137[c]	1212[c]	1256[c,d]	1281[c]	1284[c]	1456[c]	1467[c]	1473[c]	1590[c]	1608[c]	1623[c]	1659[c]	1743[c]	1818[c]	2062	2254	2281
DQ459459	North America	2	A	T	T	C	T	A	C	C	A	G	T	G	T	C	A	G	A	C	T	T	C	T	G	G
DQ459460	North America	5	B			T		G			G			A			G	A				C		C		
DQ459461	North America	3	C	C						T								A								
DQ459462	North America	1	D												C			A								
DQ459463	North America	3	E															A								A
DQ459464	Europe	98	IS						T				C			T						T				
DQ459465	Europe	1	I′S						T				C			T						T		A		
DQ459466	Europe	34	IR						T			C	C			T						T				
DQ459467	Europe	38	IIS		C											T			G		C					
DQ459468	Europe	1	II′S		C											T			G		C			A		
DQ459469	Europe	5	IIR		C							C				T			G		C					

[a] *n*, number of mitochondrial haplotypes.
[b] Mt, mitochondrial.
[c] Nucleotide positions of cytochrome *b* gene.
[d] Position 1256 corresponds to the nonsynonymous amino acid mutation conferring QoI fungicide resistance (G143A).

by SNP typing assays on 87 additional samples (data not shown) using the extended SNP primer P14 designed to detect this putative mutation.

Evolutionary relationships between mitochondrial haplotypes. MP and median-joining network analyses were performed to infer the evolutionary relationships among 11 mitochondrial haplotypes identified from 70 complete sequences of the mitochondrial fragment including the cytochrome *b* gene (Fig. 2; Table 1). Only one MP tree with a tree length of 25 was found (Fig. 2A). The tree splits into two major clades (I and II) consisting of European haplotypes. These clades are supported by robust MP bootstrap values of 91 and 92, respectively (Fig. 2A). Both phylogenetic analyses yielded genealogy patterns revealing an unambiguous differentiation between European and American populations. The distance from either European clade to the most central missing intermediate among all

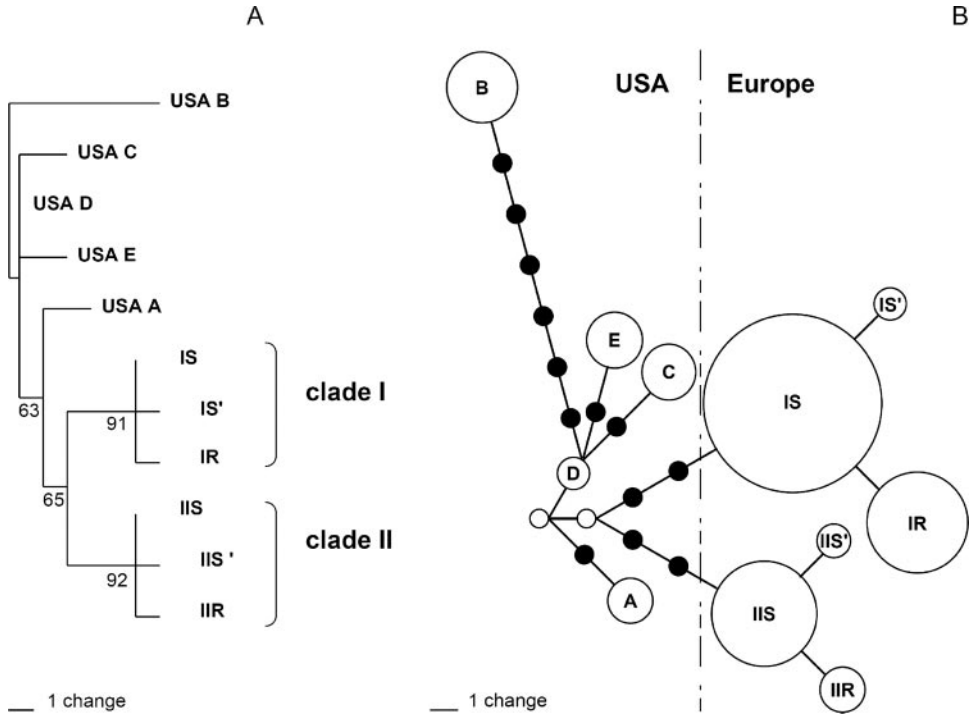

FIG. 2. MP tree (A) and median-joining network (B) depicting phylogenetic relationships and geographical assignment among mitochondrial haplotypes based on analyses of 70 mitochondrial sequences of *P. viticola* samples from Europe and North America (USA). Numbers represent node supports inferred from MP bootstraps. The branch lengths of the MP tree are proportional to the inferred substitution changes. The size of each circle in the network is proportional to the corresponding haplotype frequency. Missing intermediates are indicated by black circles except for central missing intermediates (white circles). Each branch between two (sampled or missing) haplotypes indicates a single mutational step.

TABLE 4. Relationship between mitochondrial polymorphism and fungicide sensitivity to famoxadone in 27 isolates of *Plasmopara viticola*

Isolate[a]	Mt haplotype[c]	Amino acid in cytochrome *b* gene[b]		MIC (mg/liter)
		Position 129	Position 143	
Sensitive isolates				
COU 05	IS	Phe	Gly	0.8
EPE 09	IS	Phe	Gly	0.68
FEM 03	IS	Phe	Gly	0.2
LEO 03	IS	Phe	Gly	0.6
MAI 17	IS	Phe	Gly	1
PCN 26	IS	Phe	Gly	0.7
SAL 19	IS	Phe	Gly	0.9
BOM 13	IIS	Phe	Gly	0.4
BOM 06	IIS	Phe	Gly	0.8
BOM 03	IIS	Phe	Gly	0.8
CAZ 22	IIS'	Phe	Gly	0.9
COU 15	IIS	Phe	Gly	0.5
MOR 22	IIS	Phe	Gly	0.8
PIC 21	IIS	Phe	Gly	0.4
Resistant isolates				
CGU 20	IR	Phe	Ala	>1,100
COU 12	IR	Phe	Ala	>1,500
FRE 01	IR	Phe	Ala	>1,350
GF 10	IR	Phe	Ala	>1,500
LEV 05	IR	Phe	Ala	>1,500
MIC 11	IR	Phe	Ala	>1,500
MOR 08	IR	Phe	Ala	>1,500
PAR 29	IR	Phe	Ala	>1,500
PAU 32	IR	Phe	Ala	>1,500
PIC 59	IR	Phe	Ala	>1,500
ZAN 06	IR	Phe	Ala	1,000
COU 23	IIR	Phe	Ala	>1,500
BEA 18	IIR	Phe	Ala	>1,000

[a] Geographic origins of the isolates and sampling year are described in Table 1, footnote *h*.
[b] Nonsynonymous mutation sites of the cytochrome *b* gene described as conferring the QoI fungicide resistance phenotype in previous studies on different fungal pathogens.
[c] Mt, mitochondrial.

TABLE 5. Frequency of the four major mitochondrial haplotypes (IS, IR, IIS, and IIR) obtained by SNP typing of 17 French populations of *Plasmopara viticola*

Region and locality[b]	*n*	QoI fungicide treatment[a]	% of European mitochondrial haplotypes			
			IS	IR	IIS	IIR
Bordeaux and southwestern vineyards						
COU	86	T	57.0	31.4	9.3	2.3
GAL	45	T	48.9	15.6	35.6	0
PAU	44	T	38.6	56.8	2.3	2.3
MON	41	T	29.3	43.9	26.8	0
PCN	36	T	72.2	2.8	16.7	8.3
BLA	50	NT	44	14.0	34.0	8.0
CGU	44	NT	45.5	36.4	13.6	4.6
ZAN	43	NT	37.2	25.6	32.6	4.7
South area of Rhône Valley						
NIM	81	NT	81.5	6.2	12.4	0
Champagne						
CRE	18	NT	77.1	20.0	2.9	0
PLU	34	NT	73.5	23.5	2.9	0
BAR	32	NT	75	25.0	0	0
AVI	35	NT	77.8	16.7	5.6	0
Loire Valley						
TOU	30	NT	66.7	6.7	26.7	0
MAR	30	NT	73.3	10.0	16.7	0
LDV	16	NT	62.5	12.5	25	0
BOS	11	NT	81.8	9.1	9.1	0
Total	676					
Mean			58.0	22.2	16.3	3.5
SE			20.4	14.7	11.3	9.0

[a] Isolates from treated fields were sampled after at least one QoI fungicide treatment. T, treated; NT, not treated.
[b] Geographic origins of the isolates and sampling year are described in Table 1, footnote *h*. PLU, Plumecoq.

haplotypes was found to be four mutational steps, whereas the distance between two European clades was six mutational steps (Fig. 2B). In contrast to the looser connection between American haplotypes, three haplotypes within each European clade were connected by a single mutational step (Fig. 2B).

At least two origins of QoI fungicide-resistant alleles. Since the European QoI fungicide-sensitive (S) and -resistant (R) isolates were distributed between two well-supported groups separated by six mutational steps (clade I and clade II) (Fig. 2), we concluded that there are four major European haplotypes: IS and IIS for sensitive haplotypes and IR and IIR for resistant haplotypes (Fig. 1 and 2). No difference in famoxadone sensitivity was found between IS and IIS or between IR and IIR in bioassays (Table 4). According to the phylogenetic analyses, the two resistance haplotypes (IR and IIR) derived by one mutational step from the two QoI fungicide-sensitive haplotypes IS and IIS, respectively. At least two origins of QoI fungicide resistance haplotypes were thus revealed by the presence of several resistant alleles that differed from their potential susceptible progenitors only by the presence or absence of the resistance-associated mutation itself.

Mitochondrial haplotype distribution in French vineyards. An SNP typing assay was used to survey the geographical distribution of various mitochondrial haplotypes from a panel of 676 *P. viticola* isolates collected in 17 French localities (Table 5). Depending on the downy mildew epidemics, the number of samples collected and genotyped ranged from 11 to 86, with a mean of 40 (±19) samples per population. Primers P7 and P9 (Fig. 1; Table 2) were used for DNA amplification and yielded a product of 630 bp. European clades I or II were established with extended SNP primers P16 and P17, and the extended primer P15 was used to detect the G143A mutation (Table 2). The four major European haplotypes, IS, IR, IIS, and IIR, were typed as GCA, CCA, GTG, and CTG, respectively, at nucleotidic positions 1256, 1281, and 1608 (Table 3). The same assay also differentiated between American and European haplotypes, since all American isolates shared the same specific haplotypic pattern (GTA), while no European isolate showed the American haplotypic pattern.

The frequency of the four main European haplotypes was estimated in 17 populations using this SNP typing procedure (Table 5). The average frequency of IS, IR, IIS, and IIR haplotypes in French populations was 58.0%, 22.2%, 16.3%, and 3.5%, respectively. Haplotypes from the European clade I

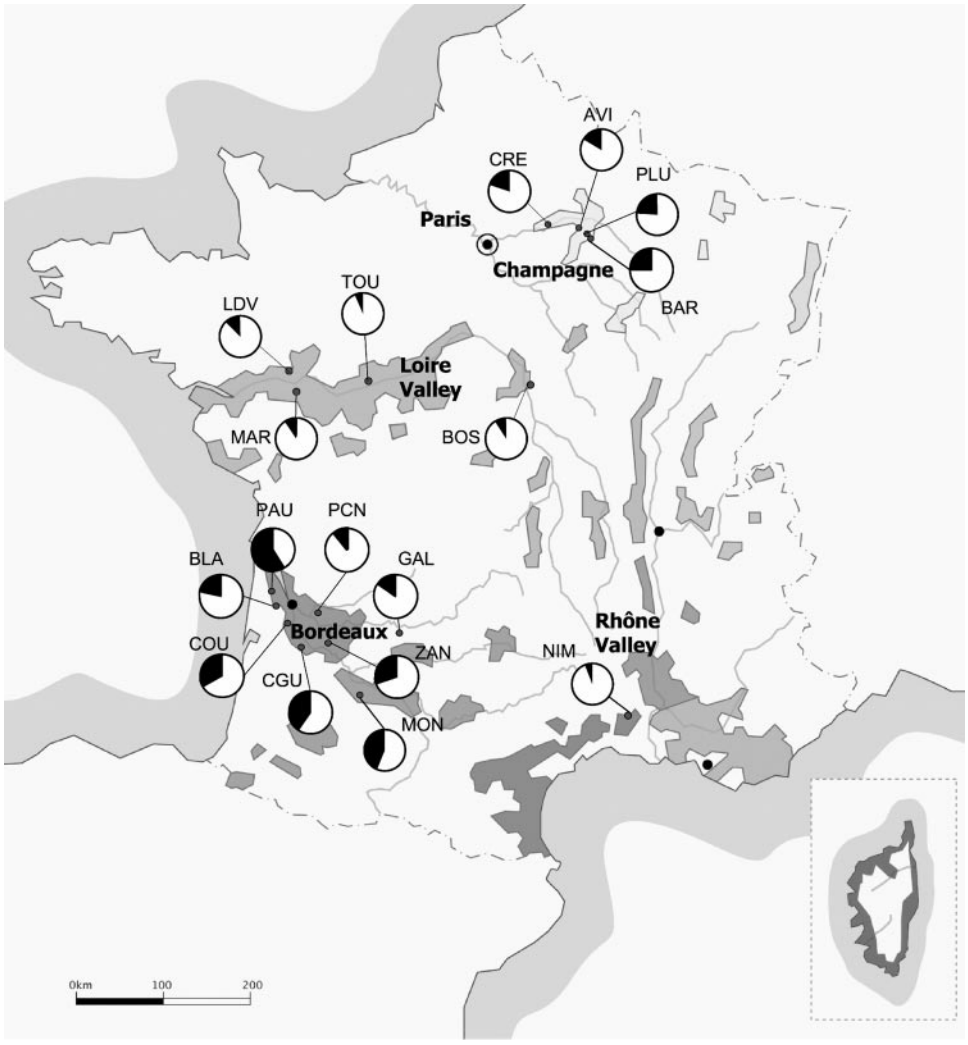

FIG. 3. Frequency of QoI fungicide-resistance haplotypes among 17 French populations of *P. viticola* collected in 2003. Within the circles, QoI fungicide resistance haplotypes (IR and IIR) are represented by black, and sensitive haplotypes (IS and IIS) by white. Major French wine-producing regions are shown in different gray patterns.

(IS and IR) were on average fourfold more common than clade II haplotypes (IIS and IIR). Interestingly, haplotype II was significantly ($P < 0.001$) less common in the Champagne vineyard (2.8%) than in any other regions (25.7% and 19.4% in Bordeaux and the Loire Valley, respectively), which could indicate a restriction to gene flow at the regional level.

Concerning QoI fungicide resistance, at least one of the haplotypes conferring resistance was found in all populations sampled. Overall, the percentage of resistant haplotypes (IR and IIR) was 24.3% (±15.6%) in French populations but with considerable local differences, as indicated by the high standard deviation (Fig. 3). For instance, Loire and Rhone Valley populations are still apparently less penetrated by QoI fungicide resistance alleles (<10%), whereas in the southwestern and Champagne vineyards, the frequency of resistance haplotypes is rather high (32.1% and 21.3%, respectively). The effect of QoI fungicide treatments on the mitochondrial genetic structure was tested by comparing the latter in treated and untreated populations in Bordeaux vineyards. The Fisher exact test indicated that populations treated with QoI fungicides in 2003 presented a significantly higher number of resistance haplotypes than those not treated ($P = 0.022$).

AMOVA confirmed the considerable heterogeneity of haplotype distribution at the different hierarchical levels of analysis. Consistently, all components of variance partitioning (among groups, among populations within groups, and within populations) were significant at the different geographical levels (Table 6). Most of the genetic variation was found within populations (88.6%, $P < 10^{-5}$), a result also evidenced by the highly significant F_{ST} (fixation index for alleles from different individuals within the same population [18]) value ($F_{ST} = 0.115$). Significant variation was also found among populations within regions (7.54%, $P < 0.001$), and a slightly lower amount of variability was found between regions (3.93%, $P < 0.001$). To test the impact of QoI fungicide treatments on the spatial heterogeneity observed within regions, we performed an AMOVA including only the 12 populations that were untreated with QoI fungicides during the year of sampling (Table

TABLE 6. AMOVA of *Plasmopara viticola* haplotypes with regions composed of populations of the same vineyards (see Table 5)[a]

Source of variation	All populations ($n = 17$)			Populations not treated with QoI fungicides ($n = 12$)		
	Variance components	% of variation[b]	Fixation indices[c]	Variance components	% of variation[b]	Fixation indices[c]
Among regions	0.01156	3.93***	$F_{CT} = 0.0425$	0.02857	9.83***	$F_{CT} = 0.0983$
Among populations within region	0.02214	7.54***	$F_{SC} = 0.0753$	−0.0017	−0.59, NS	$F_{SC} = -0.0065$
Within populations	0.2601	88.53***	$F_{ST} = 0.115$	0.2637	90.75***	$F_{ST} = 0.0925$

[a] The analysis was conducted for the complete data set (17 populations) and for a reduced data set including only the 12 populations not treated with QoI fungicides in 2003.

[b] ***, $P < 0.001$; NS, not significant.

[c] F_{CT}, estimated fixation index among different regions; F_{SC}, estimated fixation index within a region; F_{ST}, estimated global fixation index within populations.

5). Removing populations that had been treated with QoI fungicide resulted in an increase in the genetic structure between regions, together with a decrease in the percentage of genetic variability among populations of a region. For the latter scale, the genetic structure became nonsignificant (Table 5), underlining that QoI fungicide treatments are responsible for the heterogeneous genetic variability among populations at the regional level.

To test whether the frequency of the resistance allele was identical in mitochondrial clades I and II, we performed Fisher exact tests on the distribution of mitochondrial haplotypes in each population. Except for two populations in France (Blanquefort and Cadaujac), no significant differences were found, indicating that the frequency of resistant haplotypes (IR and IIR) did not differ according to whether they belonged to one clade or another.

DISCUSSION

How many mutations are responsible for QoI fungicide resistance in grapevine downy mildew? Two nonsynonymous substitutions in the cytochrome *b* gene have been described to be involved in resistance to QoI fungicides: (i) the G143A substitution, which confers a strong resistance, has been identified in most plant pathogens in which QoI fungicide resistance has become a threat to disease control (25), and (ii) the F129L substitution, which is thought to confer weaker resistance to QoI fungicides, was recently detected in some pathogen species such as *Pythium aphanidermatum*, *Magnaporthe oryzae*, *Alternaria solani*, and *Plasmopara viticola* (25, 29, 35, 48).

Confirming previous findings (25, 29, 48, 51), we found that one major mutation at amino acid position 143 in cytochrome *b* provides a high level of resistance to QoI fungicide in *P. viticola*. However, contrary to Grasso et al. (29), we did not find any supplementary mutation at amino acid position 129 of the cytochrome *b* gene in 276 *P. viticola* isolates screened in 17 French vineyards. The lack of detection of the F129L substitution indicates that the frequency of this allele is below 0.36% in our sample, an estimate in agreement with monitoring data obtained by Sierotzki et al. (48), who evaluated the F129L mutation frequency to be 0.4% in French *P. viticola* populations in 2003.

Origins and spread of QoI fungicide resistance haplotypes. Accumulation studies on QoI fungicide resistance have shown that a single replacement in a single gene occurred across a wide range of plant pathogens, clearly indicating parallel evolution of the resistance phenomena in different species (29). However, genetic data on the rate at which adaptive substitutions arise at the infraspecific level are still lacking. For the first time in a plant-pathogen system, we used phylogenetic arguments to demonstrate that a selected substitution (G143A) in the cytochrome *b* gene has occurred at least twice during the course of evolution of QoI fungicide resistance alleles in European populations of *P. viticola*. This finding confirms that parallel evolution of resistance to fungicide is acting not only between but also within species. This result is in agreement with expectations from the mutation-selection balance theory. The equilibrium allele frequency under mutation-selection balance for a haploid is expressed by μ/s, where μ is the mutation rate and s is the selection coefficient. Assuming complete selection ($s = 1$) against the resistant haplotype, the equilibrium frequency of a mutation will be that of the neutral substitution rate per site for mtDNA, which has been estimated to be approximately 2.10^{-8}. Given that the population size of grapevine downy mildew must readily exceed 10^8, this calculation, while extremely rough, shows that it would not be surprising to find multiple independent origins of QoI fungicide resistance haplotypes in populations of *P. viticola*.

The double origins of resistance haplotypes estimated from our data set could be a low estimate of the number of recurrent substitutions that might have occurred at the selected site of the cytochrome *b* gene. Indeed, mtDNA genetic variability of *P. viticola* was extremely low in Europe. Two hypotheses could explain this. First, given the absence of normal recombination in the mitochondrial genome, any advantageous mutation such as the G143A will lead to the fixation of all other polymorphisms in the population by a process known as hitchhiking (40). This hypothesis is unlikely, since we did not find more haplotypes in European populations that never received QoI fungicide applications (data not shown). The alternative explanation is that the bottlenecking of populations resulting from the recent and multiple introduction of *P. viticola* in Europe (i.e., since 1878) (28) has greatly reduced mitochondrial variability in European populations. This lack of variability may have reduced our ability to detect phylogenetic events resulting in the emergence of the different resistance haplotypes. To improve our estimation of the rate of these recurrent mutations (G143A), it would be useful to analyze the mitochondrial variability of native populations of *P. viticola* (in the United States) that present a higher level of nuclear and mitochondrial genetic diversity, as illustrated by our results. Analysis of the mitochondrial polymorphism and the derived phylogenies of

sensitive and resistant haplotypes from American isolates would certainly allow more precise evaluation of the number of independent replacements of the amino acid conferring resistance to QoI fungicides in *P. viticola*. The relevance of this approach is enhanced by the fact that grapevine downy mildew populations have recently been described to develop resistance to QoI fungicides in the United States (A. Baudoin, personal communication). Although recurrent mutation is probably an important initial force driving the evolution of QoI fungicide resistance-associated point mutations in this pathogen species, the role of migration in the spread of resistance haplotypes across vineyards should not be underestimated. Indeed, when combined with directional selection, long-distance migration of sporangia (by passive migration or by human activities) could be a primary determinant of the spread of these kinds of adaptive mutations over broadly distributed vineyards. The impact of gene flow between distant populations on the final distribution of resistance alleles has been well illustrated in *Culex pipiens*: Raymond et al. (46) showed that frequent migration events, including passive transportation, have resulted from the worldwide spread of resistance alleles to organophosphorous insecticides.

Patterns of genetic variation of mtDNA. Mitochondrial genetic structure estimated by SNP typing of 17 populations was high ($F_{ST} = 0.11, P < 10^{-3}$); most of the genetic variability was found within populations, and genetic variability was loosely structured by geography at the regional scale. The high mitochondrial genetic structure observed contrasts with previous estimates of population differentiation of *P. viticola* obtained using microsatellite markers (15, 26). Indeed, population differentiation observed on a scale similar to that found in our study has provided evidence for a low genetic structure ($F_{ST} = 0.018$ among French and Swiss populations, $P < 10^{-3}$). The difference between these two F_{ST} estimates is not surprising, since it has been demonstrated that the mitochondrial genome is often more structured than the nuclear genome, owing to its smaller effective size (45) and to small fitness differences between mtDNA genomes (6). In our case, the differences observed between nuclear and mitochondrial markers likely resulted from differences in selective regimen. Indeed, population differentiation by adaptive genetic variations results from selection and genetic drift, while population differentiation by neutral polymorphism is only driven by genetic drift. Given that mtDNA variations in *P. viticola* influence pathogen fitness, populations of the pathogen might be under strong directional selection for local adaptation to fungicide pressure. In agreement with this, we found a significant increase in resistance haplotype (IR and IIR) frequency in populations treated with QoI fungicides during the sampling year. Similarly, removing treated populations from the AMOVA resulted in a drastic reduction of genetic differentiation among populations within regions. These results demonstrate that, in the same year, the mitochondrial genetic structure responds to the spatial heterogeneity resulting from fungicide treatments. The ability of *P. viticola* to quickly adapt to fungicide pressure is likely enhanced by its ability to undergo clonal amplification of resistant genotypes by several asexual generations during the growing season.

Even though we detected a significant global effect of fungicide treatments on mitochondrial population differentiation, it is still rather difficult to establish a solid relation between resistance haplotype frequencies in populations and the selective pressure exerted by fungicides. In fact, selection pressure might not be uniform among vineyards because many factors contribute to fungicide treatment efficacy, such as disease pressure, local climatic conditions, timing, intervals, and rates of spraying programs. Thus, selection by fungicides leads to a pattern of local adaptation of the pathogen and, as evidenced by the high genetic structure, this pattern is not homogenized by gene flow among populations during the grapevine growing season (26).

Another significant result of this study is that all populations either treated or not treated with fungicides presented a large proportion of QoI fungicide-resistant isolates. This large proportion of isolates could result from local migration, because control plots should be considered as small, untreated islands surrounded by treated vineyards in which selection for resistance is operating. Moreover, the untreated plots in this study likely received fungicide applications during the years preceding the sampling year. Thus, resistant genotypes may have remained in control plots from one year to another. The latter hypothesis raises the question of the factors determining the maintenance of resistance haplotypes in the field, in particular when chemical control is suspended. Fungicide resistance alleles are frequently costly, and their cost is variable (13). When resistance alleles carry a fitness cost, the rise in resistance is followed by a fall when chemical control is suspended. Maintenance of resistance alleles in untreated plots could indicate an absence of cost for QoI fungicides. However, another hypothesis could be that the initial fitness cost gradually decreases, owing to subsequent mutations at secondary loci that compensate for the cost (37). Unfortunately, reliable data on the existence of a cost associated with the QoI fungicide resistance mutations in the absence of fungicide pressure are lacking for *P. viticola*. Clearly, more studies are now needed to understand the complex interaction between mutations, migration, and competition between these QoI fungicide resistance haplotypes.

Implications for the evolution of resistance. Many interacting factors contribute to the appearance and spread of fungicide resistance alleles in pathogen populations. The degree of risk of resistance appearance and development is dependent on the mode of action of the fungicide, the selective pressure resulting from its application, the local conditions for disease development, and the evolutionary potential of the pathogen target (migration rate, mutation rate, and effective population size) (41). In this study, we show that the large population size of *P. viticola* combined with strong selective pressure has resulted in at least two independent origins of QoI fungicide resistance alleles. We contend that the recurrent appearance of single resistant-associated mutations may be an important way in which pathogen or pest species respond to the challenge imposed by chemical selection. This process is probably linked with the kind of genomic modification involved in the resistance, with SNPs associated with target site insensitivity being more readily subject to recurrent mutations than more complex genome rearrangements (gene amplification or gene regulation). In accordance with this hypothesis, multiple origins of resistance alleles have already been described for numerous resistances to pesticides and drugs in plants, animals, protozoa,

and bacteria. A parallel evolution has been evidenced for resistance to cyclodienes, pyrethroids, and dichlorodiphenyltrichloroethane insecticides in several insect species (1–4, 20, 42, 55), for resistance to herbicides inhibiting acetyl-CoA carboxylase in plants (16), for resistance to chloroquine in malaria (57), and for resistance to beta-lactamate in bacteria (8, 32). There is now growing evidence for independently acquired adaptive substitutions during the evolution of resistances to pesticides and drugs. This finding will certainly help to define the scale at which resistance evolves and will provide valuable insights into our models in order to devise appropriate resistance management strategies for microbial populations.

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