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Research

Phylogenetic and experimental evidence for host-specialized cryptic species in a biotrophic oomycete

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Summary

- Assortative mating resulting from host plant specialization has been proposed to facilitate rapid ecological divergence in biotrophic plant pathogens. Downy mildews, a major group of biotrophic oomycetes, are prime candidates for testing speciation by host plant specialization.
- Here, we combined a phylogenetic and morphological approach with cross-pathogenicity tests to investigate host plant specialization and host range expansion in grapevine downy mildew. This destructive disease is caused by *Plasmopara viticola*, an oomycete endemic to North America on wild species and cultivated grapevines.
- Multiple genealogies and sporangia morphology provide evidence that *P. viticola* is a complex of four cryptic species, each associated with different host plants. Cross-inoculation experiments showed complete host plant specialization on *Parthenocissus quinquefolia* and on *Vitis riparia*, whereas cryptic species found on *V. aestivalis*, *V. labrusca* and *V. vinifera* were revealed to be less specific. We reconstructed the recent host range expansion of *P. viticola* from wild to cultivated grapevines, and showed that it was accompanied by an increase in aggressiveness of the pathogen.
- This case study on grapevine downy mildew illustrates how biotrophic plant pathogens can diversify by host plant specialization and emerge in agrosystems by shifting to cultivated hosts. These results might have important implications for viticulture, including breeding for resistance and disease management.

Introduction

Host shift speciation has been shown to be responsible for the diversification of plant parasites (Refregier *et al.*, 2008; Tellier *et al.*, 2009). Host plants and their parasites exhibit intimate physiological interactions, which lead to the evolution of host-specific adaptations following host shifts. Certain life history traits and specificities in the life cycles of plant pathogens may facilitate rapid ecological divergence by reducing the constraints that usually impair speciation. Indeed, the production of numerous propagules, the linkage of traits experiencing selection, together with the strong selection imposed by the hosts and the gene exchange occurring within hosts, are key factors that strongly favor ecological speciation (Giraud *et al.*, 2010). Within-host mating and selection are especially important in biotrophic plant pathogens that strictly depend on the plant to survive and that undergo sexual reproduction on the host plant. In this case, mutations providing adaptation to a new host pleiotropically affect mating patterns, providing one of the most favorable scenarios for ecological speciation (Gavrilets, 2004). The rapid ecological divergence experienced by plant pathogens implies that many may, in fact, be complexes of sibling species, an idea put forward by Crous & Groenewald (2005): 'Show me a plant pathogen, and I will show you a species complex'. Since then, there has been abundant phylogenetic evidence supporting the view that many current names of well-known plant pathogens actually mask complexes of cryptic species (O'Donnell *et al.*, 2000; Steenkamp *et al.*, 2002; Fournier *et al.*, 2005; Le Gac *et al.*, 2007; Garcia-Blazquez *et al.*, 2008; Choi *et al.*, 2011). In addition to the divergent selective pressures caused by specialization on the host, plant pathogens have evolved in response to the agro-ecosystem. Cultivated crops indeed represent large naïve targets for pathogens shifting from wild hosts. Genetic and environmental uniformity, coupled with vast plantations of cultivated plants, are expected to favor more aggressive pathogens (Anderson & May, 1982; Stukenbrock & McDonald, 2008). Confirming this hypothesis, evidence for an increase in aggressiveness resulting from domestication has been documented for the main

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pathogens of rice (Couch *et al.*, 2005), apple (Lê Van *et al.*, 2012) and wheat (Stukenbrock *et al.*, 2011).

The pathosystem *Vitis–Plasmopara viticola* is a prime candidate for the study of host specialization in biotrophic plant pathogens. The modern grapevine originated from the domestication of a unique Eurasian species (*V. vinifera* L.; Zecca *et al.*, 2012). It has been cultivated since ancient times, with the earliest evidence for winemaking dating back to 7400–7000 BP in the Caucasian area (McGovern *et al.*, 1996). The history of viticulture parallels the development of western civilizations, and its cultural significance persists today in centuries-old viticultural countries. From the middle of the 19th century, viticulture has been threatened by grapevine downy mildew, a disease that was introduced into Europe from northern America (Millardet, 1881). Grapevine downy mildew, caused by the obligate biotrophic oomycete *P. viticola*, is considered to be one of the most destructive grapevine diseases worldwide (Viennot-Bourgin, 1949). Grapevine (*V. vinifera*) cultivars are highly susceptible to the disease and currently require an intense chemical management programme to control the disease. In northeastern America, the native range of *P. viticola*, the Vitaceae family presents a higher diversity than in Europe. Therefore, *P. viticola* has been described on several *Vitis* species in the Vitaceae: in addition to *V. vinifera*, it has been found on other cultivated grapes (e.g. *Vitis labrusca* L. (fox grape)), as well as on wild grapes, such as *V. riparia* Michx. (river bank grape) and *V. aestivalis* Michx. (summer grape), and Virginia creeper (*Parthenocissus quinquefolia* L. Planch; Bush & Meissner, 1883).

Several studies have addressed the question of host plant specialization in the interaction between *P. viticola* and the Vitaceae. First, several decades ago, Savulescu & Savulescu (1951) and Golovina (1955) independently proposed the existence of different specialized forms of *P. viticola* on the Vitaceae based on the morphology of spores; however, as for many plant pathogens, morphological characteristics alone are too variable and/or simple to allow clear species delineation, and these specialized forms of the pathogen could never be further confirmed. Second, a comparison of disease resistance screens of *Vitis* germplasm performed in different laboratories with different isolates showed a lack of concordance between the different studies (Cadle-Davidson, 2008); this observation could, in part, be explained by the occurrence of race-specific isolates in *P. viticola*, in particular for resistant cultivars and advanced breeding lines. Third, the genetic analysis of 14 *P. viticola* isolates, including nine North American isolates, led Schröder *et al.* (2011) to propose the existence of several genetic lineages in grapevine downy mildew; however, because of the limited sampling of this study, the results were not conclusive on the status of the lineages described, thus making it difficult to assess the association between the pathogens and their host plants. A more comprehensive study is therefore required to address the existence of specialized cryptic species in grapevine downy mildew.

Different criteria have been employed to recognize species: typological, which emphasize morphological divergence; biological (Mayr, 1942), which emphasize reproductive isolation; and phylogenetic, based on genetic divergence. As a result of the biotrophic nature of *P. viticola*, crossing strains of this pathogen and obtaining viable progenies are very challenging, as they require cumbersome and time-consuming bioassays (Scherer & Gisi, 2006; Gisi *et al.*, 2007). This clearly impedes the use of biological procedures for the recognition of species in grapevine downy mildew. Taylor *et al.* (2000) advocated the use of the analysis of multiple genes as a criterion to identify phylogenetic species within fungal pathogens, and introduced the term 'genealogical concordance phylogenetic species recognition' (GCPSR). GCPSR uses the phylogenetic concordance of multiple unlinked genes to indicate a lack of genetic exchanges, and thus the evolutionary independence of lineages. This approach has proved to be the most convenient for fungal species delimitation, because it is more finely discriminating than the other criteria, and also because it is applicable to fungal species that cannot be cultivated or mate in control conditions (O'Donnell *et al.*, 2000; Steenkamp *et al.*, 2002; Pringle *et al.*, 2005; Le Gac *et al.*, 2007; Giraud *et al.*, 2008; Henk *et al.*, 2011).

In the present study, we have addressed the existence of host plant-specialized species in grapevine downy mildew by combining a phylogenetic approach based on the concordance of multiple genealogies, analyses on sporangia morphology and controlled cross-inoculation experiments. We collected downy mildew samples on three wild species (*P. quinquefolia*, *V. riparia*, *V. aestivalis*) and several cultivated varieties (*V. labrusca* cv Niagara and Concord, *V. vinifera*, interspecific hybrids) in the states of Michigan, Ohio, New York and Virginia (USA). We analyzed the polymorphism at four different nuclear loci (*actin*, *tubulin*, *internal transcribed spacer* and *nrLSU*) of these isolates, and assessed their pathogenicity and aggressiveness by cross-inoculating a subset of isolates on six host plants. This allowed us to address the following specific questions. Are there genetically isolated lineages within *P. viticola* that have not been distinguished on the basis of morphological criteria? What is the level of morphological divergence among *P. viticola* collected on different hosts? Are *P. viticola* isolates able to infect a single host species or a broad range of *Vitis* spp.? Has the expansion from wild to cultivated hosts modified the life history traits of the pathogen (pathogenicity, aggressiveness)?

Materials and Methods

Background

Plasmopara viticola (Berk. & M.A. Curtis) Berl. & De Toni, 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 secondary infection through the production of sporangia containing zoospores that spread to the leaves and berries by splashing rain or wind. In this study, each sample of *P. viticola* collected from the field consisted of 1 cm^2 of a fresh leaf colonized by sporulating downy mildew.

Plant material

Six host plants were used for the inoculation experiments: *V. vinifera* cv Chardonnay (Double A vineyard nursery, Fredonia, NY, USA), *V. riparia* Michx. (Cold Stream Farm nursery, Free Soil, MI, USA), *P. quinquefolia* L. Planch. (growing wild in Lansing, MI, USA), *V. aestivalis* (accession REM59-77; USDA-ARS Plant Genetic Resources Unit, Geneva, NY, USA), *V. labrusca* cv Niagara (Double A vineyard nursery) and Chancellor (Seibel 7053; Double A vineyard nursery). Plants were grown in a growth chamber at 25°C, with a 16 h : 8 h light : dark photoperiod.

Plasmopara viticola sampling and collection

Downy mildew isolates were collected from naturally infected cultivated and wild grapes (*V. labrusca*, *V. vinifera*, *V. riparia*, *V. aestivalis*, *Vitis* interspecific hybrids), as well as from virginia creeper (*P. quinquefolia*) in Michigan, Ohio, New York and Virginia.

Isolates for phylogenetic study Downy mildew samples were collected from geographically separated locations for a given host plant. A total of 114 isolates was sampled in 18 locations on three wild species (*P. quinquefolia*, *V. riparia*, *V. aestivalis*) and cultivated varieties (*V. labrusca*, *V. vinifera*, hybrids; Fig. 1, Supporting Information Table S1).

Live isolates A collection of live isolates derived from natural infection of *P. viticola* was established. A first set of live isolates consisted of four bulk samples created by pooling the spores of several sporulating lesions collected in different locations, but on the same host plant. These bulk samples were made from isolates collected on *V. vinifera* (bulkVIN), *V. labrusca* (bulkLAB), *V. riparia* (bulkRIP) and *P. quinquefolia* (bulkPAR). In addition, 19 isolates were established from individual infected leaves from *V. aestivalis* ($n=2$), *V. vinifera* ($n=5$), *V. labrusca* ($n=4$), *V. riparia* ($n=4$) and Chancellor ($n=4$) collected at different locations (Fig. 1, Table S2). All the isolates were genetically characterized using the four nuclear markers described in the Genetic characterization section.

Genetic characterization and phylogenetic analysis

DNA extraction Oil spots were freeze–dried overnight, and DNA was extracted from each according to the standard cetyltrimethyl-ammonium-bromide (CTAB) and phenol–chloroform methods described in Delmotte *et al.* (2006) and Chen *et al.* (2007).

DNA amplification and sequencing Four different gene regions were selected for molecular characterization. Specific primers were designed for the internal transcribed spacer region 1 (ITS), a fragment of the 28S gene of the ribosomal RNA (28S), a fragment of the gene encoding β-tubulin (TUB) and a fragment

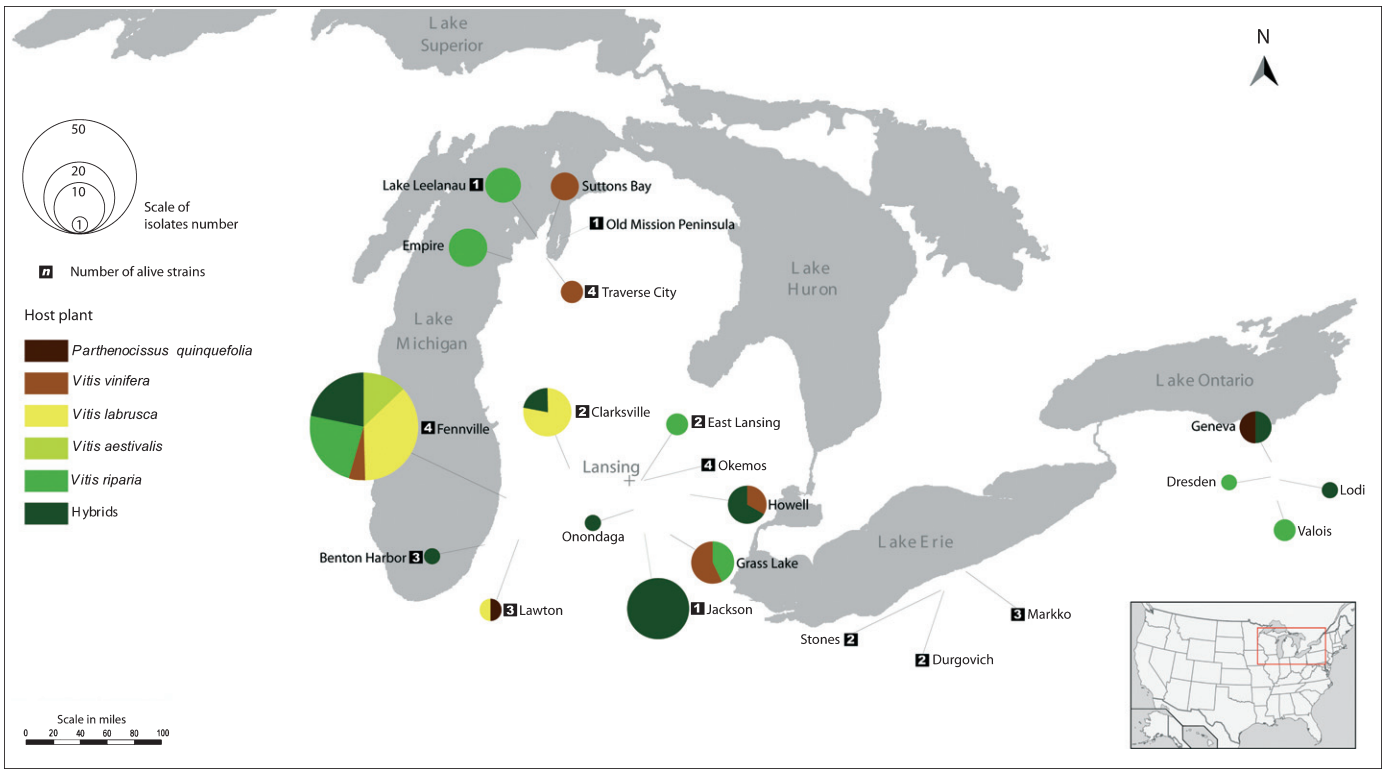

Fig. 1 Geographical origin and source host plants of *Plasmopara viticola* isolates. A detailed description of the samples is presented in Supporting Information Tables S1 and S2.

of the actin gene (ACT) (Table S3). PCRs were carried out in a final volume of 15 μl containing 1 μl of 1 : 3 dilution of genomic DNA, 2 mM $MgCl_2$, 150 μM of each deoxynucleoside triphosphate (dNTP), 4 pmol of each primer and 0.3 U T*aq* Silverstar DNA polymerase in reaction buffer. Thermocycling conditions were as follows: 95°C for 4 min, 40 cycles of 95°C for 40 s, 58°C for 45 s, 72°C for 90 s, followed by 72°C for 10 min. Sequencing of PCR amplicons was outsourced at Genoscope, the French National Sequencing Center (Evry, France).

Sequence assembly and allele inference Forward and reverse sequences were imported into CodonCode Aligner (v. 2.0.6; Codon Code Corporation, Centerville, MA, USA), assembled into contigs and visually checked for errors. All the polymorphic sites were confirmed by manual examination of the electropherogram. For sequences that presented heterozygote sites, gametic phase estimation was performed using the ELB algorithm implemented into Arlequin v3.5 (Excoffier *et al.*, 2005). Using this method, we have determined for each isolate the two alleles at each of the four loci (Table S1). Aligned sequences for each gene region and for the concatenated dataset were analyzed in DnaSP v. 4.00.6 (Rozas *et al.*, 2003) for nucleotide polymorphisms.

The sequences of the alleles obtained have been deposited in GenBank: ITS hap1–4 (JF897779–JF897782), 28S hap1–8 (JF897848–JF897855), β-tubulin hap1–32 (JF897816–JF897847), actin hap1–32 (JF897783–JF897815).

Phylogenetic analyses Data from each genomic region were first analyzed alone, and then all regions were combined in a single analysis (concatenated dataset). The different sets of alleles were aligned using Muscle implemented in Seaview (Gouy *et al.*, 2010). Phylogenetic relationships among alleles (and individual samples) were inferred using maximum likelihood (ML) methods implemented in PhyML (Guindon & Gascuel, 2003) and maximum parsimony (MP) implemented in PAUP (Swofford, 1993). MP genealogies were constructed using the heuristic search function with 1000 random addition replicates and tree bisection and reconstruction (TBR) using a branch swapping algorithm. Gaps were treated as fifth characters and all characters were unordered and of equal weight. Insertions/deletions (indels), irrespective of their size, were each treated as one evolutionary event and weighted as one base substitution. To estimate branch support, bootstrap values were determined using 1000 bootstrap replicates for MP and ML.

The best-fit models of nucleotide substitution were selected using MODELTEST v. 3.06 (Posada & Crandall, 1998) based on likelihood scores for 88 different models and the Akaike information criterion (AIC). *Phytophthora sojae* was used as an outgroup for the phylogenetic analyses.

Species tree analyses A species tree inference approach was used to resolve the relationship among the lineages identified. We estimated the species tree of *P. viticola* using Bayesian methods under the *BEAST v1.7.3 software package (Drummond *et al.*, 2012). A single tree was reconstructed from the four independent loci (ITS, 28S, ACT, TUB), implementing the recommended substitution models for each locus as obtained from MODELTEST. All samples with sequence data for every locus were used. A Yule speciation process was specified as it is the most appropriate when comparing relationships between species. We ran Monte Carlo Markov chains (MCMCs) for 10 million generations, sampling trees every 1000 generations and using a strict molecular clock. We used Tracer v1.5 (Rambaut & Drummond, 2007) to assess likelihood stabilization and convergence for BEAST analyses, and discarded the first 10% of trees as burn-in.

Polymorphism, recombination, differentiation For each lineage, standard population genetic analyses were performed on samples presenting distinct genotypes (combination of alleles) at a given location. This was performed to avoid a misleading interpretation resulting from the clonal amplification of isolates that can occur in one geographical site (field). We calculated the number of segregating sites, the number of alleles, haplotypic diversity and π, the average number of nucleotide differences between pairs of sequences (Nei, 1987), the number of parsimony informative sites and Tajima's D using the program DnaSP 4.0 (Rozas *et al.*, 2003). Divergence among lineages was estimated in DnaSP as the average pairwise number of nucleotide differences per site, D_{xy} (Tajima, 1983). Differentiation between lineages was also tested using the K_{ST}* statistic (Hudson *et al.*, 1992).

Cross-inoculation experiments

For each *Vitis* spp., the fourth and fifth youngest leaves from growing shoots were collected, rinsed with sterile water and dried. Leaf disks were excised, bulked and distributed abaxial face up over Petri dishes covered by wet filter paper. Inoculum of downy mildew isolates was prepared as follows: infected leaves from the field were rinsed with water and placed in a growth chamber overnight in order to obtain new sporulation; sporangia on the surface were collected, resuspended in sterile water and kept in ice. The sporangia concentration of each sample was measured using a hemocytometer, and inocula were diluted to obtain a final concentration of 20 000 spores ml^{-1}. Inoculations were performed by placing a 20-μl drop of inoculum on the center of the abaxial face of each leaf disk, that is *c.* 400 spores per drop. After incubation overnight in the dark at 21°C, leaf drops were dried. Petri dishes were sealed and incubated at 21°C, with a 16 h : 8 h light : dark photoperiod.

In a first experiment, the four bulk samples of isolates (bulkVIN, bulkLAB, bulkRIP, bulkPAR) were inoculated on *V. vinifera* (cv Chardonnay), *V. labrusca* (cv Niagara), *V. riparia* and *P. quinquefolia*, leading to 16 host–pathogen interactions. Each interaction consisted of 21 leaf disks distributed in three circular 90-mm Petri dishes.

In a second experiment, 19 isolates collected on five source hosts (*V. vinifera*, *V. labrusca*, *V. riparia*, *V. aestivalis* and Chancellor) were inoculated on the same five host plants. A total of 95 host–pathogen interactions, each replicated seven times, was assessed. This consisted of 665 leaf disks placed in seven 23-cm square dishes, each including the 95 combinations of the experiment.

For both experiments, leaf disks were observed from 3 to 7 d post-inoculation (dpi) and scored for the presence of sporulation and necrosis (hypersensitive reaction of the plant). For the second experiment, sporulation of each leaf disk was quantified using the Scepter 2.0TM automated cell counter (Millipore). The cell size range used for counting sporangia was 8–25 μm.

Quantitative data were analyzed by nonparametric analysis of variance (Kruskal–Wallis test) followed by *post hoc* pairwise Wilcoxon tests for comparisons among isolates grouped by lineages and source host plant. Isolates coming from the same source host plant and belonging to the same lineage were considered as a random effect in the analysis. Therefore, data from these isolates were pooled, resulting in six groups of isolates: CHA (lineage A), RIP (lineage A), AES (lineage B), LAB (lineage B), VIN (lineage B), VIN (lineage C). Statistical tests were conducted in R (http://www.r-project.org/).

Morphological diversity

Sporangia size was assessed directly from natural infection collected in the field, as well as from infection resulting from cross-inoculations. For field samples, 40 samples were chosen among the isolates collected for the phylogenetic study. Plant tissue samples were stored in a fixative solution until analysis. Ten sporangia per isolate were measured under the microscope using a stage micrometer. For samples derived from the controlled inoculation, we assessed the morphology of sporangia from each leaf disk that exhibited sporulation. The size of sporangia was measured using the automated cell counter as described in the Cross-inoculation experiments section. Data were analyzed using a nonparametric analysis of variance implemented in R software.

Results

Phylogenetic analysis

Sequences were obtained from 112 *P. viticola* isolates for ITS, 113 for 28S, 107 for ACT and 103 for TUB (Table S1). All isolates for which a sequence was obtained were revealed to be homozygous for ITS, whereas, for ACT, TUB and 28S, heterozygous sites were detected. Gametic phase estimation was performed for these three gene regions, and the two alleles present at each locus were reconstructed for each sample (Table S1).

We obtained four alleles for ITS (ITS1–ITS4), nine for 28S (LS1–LS9), 33 for ACT (ACT1–ACT33) and 33 for TUB (TUB1–TUB33). For coding regions (ACT, TUB), all variable sites identified resulted from synonymous substitutions. A summary of the polymorphism and diversity of each gene region is presented in Table 1. Globally, the most polymorphic gene region was TUB ($\pi = 0.053907$) and the least polymorphic was 28S ($\pi = 0.010547$). Four indels of one nucleotide were found to be located in ITS ($n = 3$) and 28S ($n = 1$). The numbers of parsimony informative sites (excluding gaps) were 2, 13, 42 and 72 for ITS, 28S, ACT and TUB, respectively. Once concatenated, the four regions resulted in a dataset showing 161 polymorphic sites and 129 parsimony informative sites.

Table 1 Global polymorphism of the nucleotide alignments of *Plasmopara viticola* sequences for the four genomic regions analyzed

Taxon	Locus	n^a	bp[b]	S^c	$n_A{}^d$	$Pi_A{}^e$	$h_d{}^f$	π^g	D^h
All species	ITS	50	238	15	4	2	0.699	0.0197	–
	28S	60	702	20	8	13	0.775	0.0105	–
	ACT	88	455	52	33	42	0.941	0.0350	–
	TUB	70	519	79	32	72	0.953	0.0539	–
A	ITS	22	231	0	1	0	–	–	–
	28S	22	701	0	1	0	–	–	–
	ACT	44	455	21	13	15	0.827	0.0136	0.920
	TUB	22	519	9	11	6	0.827	0.0049	0.139
B	ITS	12	231	0	1	0	–	–	–
	28S	17	702	1	2	0	0.382	0.0005	0.566
	ACT	16	455	8	6	5	0.683	0.0072	0.739
	TUB	22	519	18	10	8	0.853	0.0074	−0.808
C	ITS	12	233	0	1	0	–	–	–
	28S	15	702	1	2	0	0.248	0.0003	−0.399
	ACT	24	455	15	13	7	0.935	0.0087	−0.043
	TUB	22	519	22	9	9	0.840	0.0075	−1.31
D	ITS	4	233	0	1	0	–	–	–
	28S	6	702	3	3	0	0.733	0.0020	0.338
	ACT	4	455	0	1	0	–	–	–
	TUB	4	519	1	2	0	0.667	0.0013	1.63

The dataset analyzed here was constructed by removing multicopy alleles within populations.
[a]Sample size (n).
[b]Total number of sites (bp).
[c]Number of segregating sites (S).
[d]Number of alleles (n_A).
[e]Number of parsimony informative site between alleles (Pi_A).
[f]Haplotypic (allelic) diversity (h_d).
[g]Average number of differences per site (π).
[h]Tajima's D (D).

MODELTEST indicated that the best model for the data was the General Time Reversible (GTR) model for each of the four gene regions. The phylogenies obtained from the sequence data of the gene regions were first determined separately. The main relationships among the alleles were topologically identical in the MP and ML consensus trees; therefore, we chose to show the ML tree with bootstrap values given for the well-supported branches with both methods (Fig. 2).

For each gene tree, four monophyletic lineages (A, B, C and D), each including the same isolates, were well supported (Figs 2, S1, Table S1). All pairwise comparisons of differentiation between *P. viticola* lineages measured by K_{ST} were significant at $P < 10^{-3}$ (Table S4). In the *tubulin* tree only, additional groups were supported within lineages B and C, but they did not correspond to any biological characteristics. All trees (ML, MP) generated with the different gene regions and the concatenated dataset produced congruent topologies, that is, for a given isolate, the corresponding alleles obtained by sequencing the four genes were always assigned to the same lineage (Table S1, Figs 2, S1).

Although our data provided evidence that each of the four lineages was monophyletic in each locus, the gene trees showed differing relationships between the four recognized lineages. The concatenated tree (Fig. S1) and the species tree (Fig. 3) inferred from the multilocus dataset recovered the same interspecific relationships: lineages C and D were the most closely related taxa

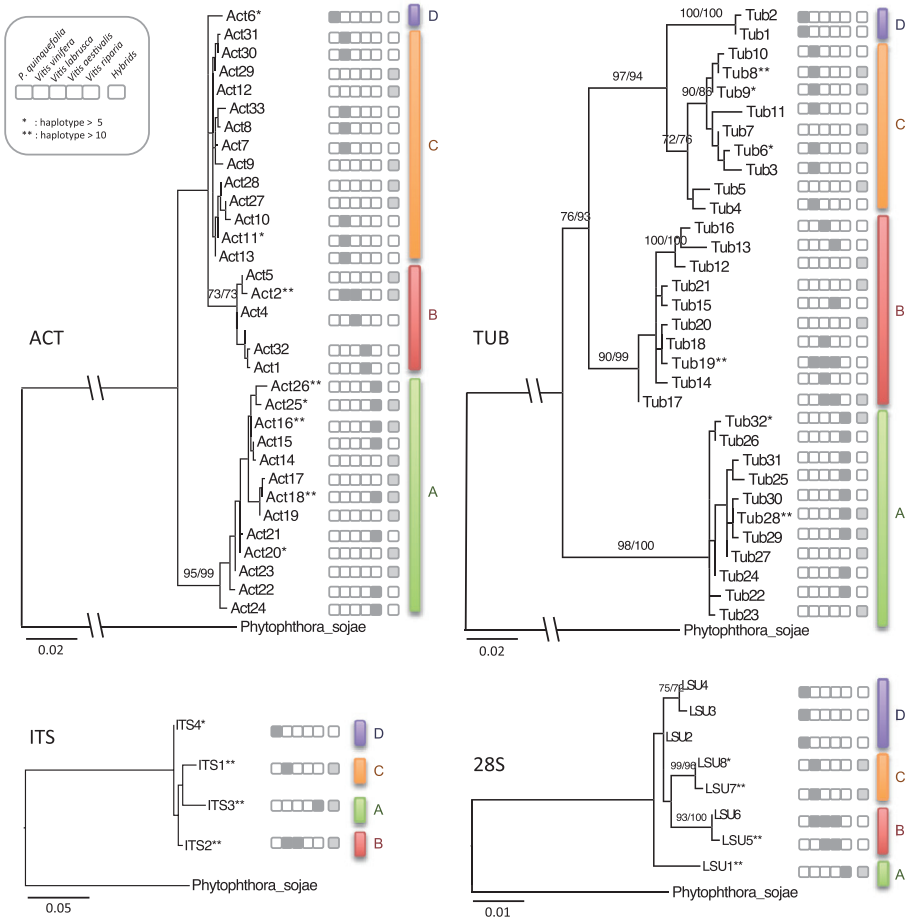

Fig. 2 Maximum likelihood reconstruction of allele relationships using partial *actin* (ACT), *tubulin* (TUB), *internal transcribed spacer* (ITS) and *nrLSU* (28S) sequences. Asterisks on alleles indicate their prevalence in the dataset: no asterisk, < 5; *, ≥ 5; **, ≥ 10. Bootstrap values are given when superior to 70% for both the maximum likelihood and parsimony analysis. The four *Plasmopara viticola* lineages identified are labeled on the trees (A, B, C, D). The host plant of origin for each allele is indicated with a shaded box next to the allele (legend for shaded boxes is shown in the inset).

and lineage A was the most divergent taxon. This is further confirmed by our results on fixed polymorphism and on the average number of nucleotide differences per site (Table S4). It is worth noting that the phylogenetic relationships between lineages reconstructed in the concatenated dataset are mainly driven by the *tubulin* phylogenetic signal. The small number of fossil records on oomycetes and their complete absence for downy mildews rendered impossible the calibration of the trees and the estimation of the time scale for the diversification of the lineages.

The lineages were strongly associated with the host plant collected (Figs 2, 3; Table 2). Lineage A corresponded to isolates collected on *V. riparia* ($n = 53$) and on hybrid Chancellor, and three isolates from other hybrids. Lineage B corresponded to isolates collected on *V. labrusca* and *V. aestivalis*, two isolates collected on *V. vinifera* and three isolates from hybrids. Lineage C included 11 isolates collected on *V. vinifera* and six isolates collected on three interspecific hybrids. Lineage D included the three isolates collected on *P. quinquefolia*. Except for samples collected on hybrids, samples collected from the same host plant species, but in different geographic regions, were genetically more similar than samples collected on different host species in the same fields (Table S1). Within *P. viticola* lineages collected on several host plants (A, B), no alleles were found to be associated with a given *Vitis* species, ruling out the existence of substructure driven by host plants within pathogen lineages.

Host specialization on *Vitis* spp

We assessed the ability of the *P. viticola* isolates from different *Vitis* hosts to colonize leaf tissue (pathogenicity) of wild and cultivated *Vitis* spp. In a first experiment, four bulk samples of isolates sampled from different hosts were inoculated on *V. vinifera* cv Chardonnay, *V. labrusca* cv Niagara, *V. riparia* and *P. quinquefolia* (Table S2). All bulk samples of isolates from a given source plant were found to grow on their host plant of origin. However, not all groups of isolates were able to infect all host plants (Fig. 4). bulkPAR was the only isolate able to infect *P. quinquefolia* leaves and was not able to colonize any other plants. Moreover, a hypersensitive response was observed when *P. quinquefolia* was inoculated with isolates from other host plants. Similarly, bulkRIP was able to infect *V. riparia*, but could not colonize leaf tissues of other host plants. Remarkably, bulkRIP induced a strong hypersensitive response on *V. vinifera* that produced numerous necrotic spots (Fig. 4a). Finally, bulkLAB and

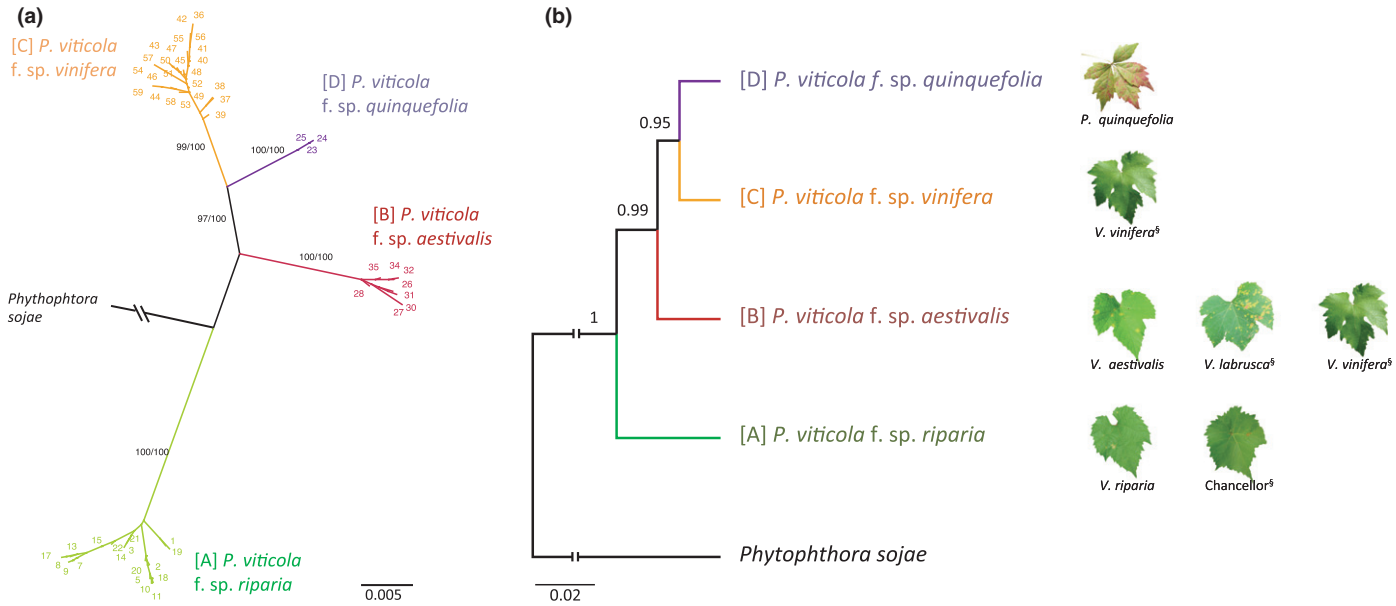

Fig. 3 Concatenated and species trees of *Plasmopara viticola* formae speciales. (a) Maximum likelihood tree of alleles obtained by concatenating *actin*, *tubulin*, *internal transcribed spacer* and *nrLSU* partial sequences. Bootstrap values of the branches are given for both the maximum likelihood and parsimony analyses. (b) Species tree topology recovered from the *BEAST analysis based on the same four loci. Posterior probabilities of the branches are given in the tree. Leaf pictures indicate the source host plants for each *P. viticola* formae speciales (§, cultivated hosts).

bulkVIN were able to infect all host plants, except *P. quinquefolia*. The molecular characterization of isolates constituting the bulk samples revealed that isolates from bulkRIP belonged to lineage A, isolates from bulkPAR belonged to lineage D and isolates from bulkVIN and bulkLAB belonged to lineage B (Table S2).

These results prompted us to perform a second experiment using individual isolates from the different groups and addressing the possible quantitative differences in growth between isolates and/or interactions. *Parthenocissus quinquefolia* and its derived isolates were not used for this experiment because of the strong specificity of the interaction observed in the previous experiment. Nineteen isolates were characterized at the four molecular markers and assigned to *P. viticola* lineages. We used four isolates from *V. riparia* (RIP) and four from Chancellor (CHA), all belonging to lineage A; two isolates from *V. aestivalis* (AES) and four from *V. labrusca* (LAB), all from lineage B; and five isolates from *V. vinifera* (VIN), three from lineage B and two from lineage C (Fig. 1, Table S2).

Isolates were inoculated on all source host plants. Results obtained with individual isolates confirmed the results of the previous experiment. Isolates collected on *V. riparia* or Chancellor (RIP, CHA) were able to infect *V. riparia* and Chancellor, but never grew on leaf tissues of other host plants (except for a very limited production of spores on *V. aestivalis* with necrosis) and induced a hypersensitive response in *V. vinifera*. In addition, isolates B and C (VIN, LAB) were able to infect all host plants. However, B isolates from *V. aestivalis* (AES) were not able to infect *V. vinifera* and *V. labrusca* in our experiment. This result must be interpreted with caution as it was difficult to obtain sporulation with isolates from *V. aestivalis*, even when inoculated on their own host plant. A summary of the qualitative results from both experiments is presented in Table 3.

Quantitative analysis revealed an interesting variability in the sporulation of isolates of different source hosts when inoculated on the five host plants (Figs 4b, S2). For a given group of isolates (same source host plant and lineage of *P. viticola*), we found significant effects of inoculated host plants on sporulation and, conversely (Kruskal–Wallis one-way analysis of variance, $P < 10^{-3}$), for a given inoculated host plant, we found a significant effect of the isolate group on sporulation (Kruskal–Wallis one-way

Table 2 Distribution of the *Plasmopara viticola* isolates following the host plant of origin and the phylogenetic lineage

		P. viticola lineage			
Host plant	Variety	A	B	C	D
Vitis riparia	Wild grape	29			
	Rootstock	1			
Vitis aestivalis	Wild grape		6		
Vitis labrusca	Niagara		24		
	Concord		2		
Vitis vinifera	Chardonnay		1	1	
	Gamay			2	
	Gewurtztraminer			1	
	Pinot noir		1	2	
	Riesling			4	
	Syrah			1	
Parthenocissus quinquefolia	Wild vine				3
Interspecific hybrids	Chancellor	24			
	NY-73.136			2	
	Table grape		2	1	
	Traminette			1	
	Vidal		1		
	Vignoles	1			
	Unknown	2		2	

analysis of variance, $P < 10^{-2}$). CHA, LAB and VIN isolates, which were able to infect several host plants in the experiment, were more aggressive on their source host plant than on other host plants (Fig. 4b).

Interestingly, we observed that sporulation of downy mildew isolates, when inoculated on their host plant of origin, was lower for wild species (*V. aestivalis* and *V. riparia*) than for cultivated hosts (Chancellor, *V. labrusca*, *V. vinifera*; Fig. S2).

We compared, on the one hand, the sporulation of lineage A isolates (RIP, CHA) on *V. riparia* (wild) and Chancellor (cultivated), and, on the other, the sporulation of lineage B isolates (AES, VIN, LAB) on *V. aestivalis* (wild), *V. vinifera* and *V. labrusca* (cultivated). Isolates from cultivated hosts were significantly more aggressive on cultivated hosts than were isolates from wild hosts. By contrast, no significant differences in aggressiveness between isolates were observed when inoculated on wild hosts (Fig. 5).

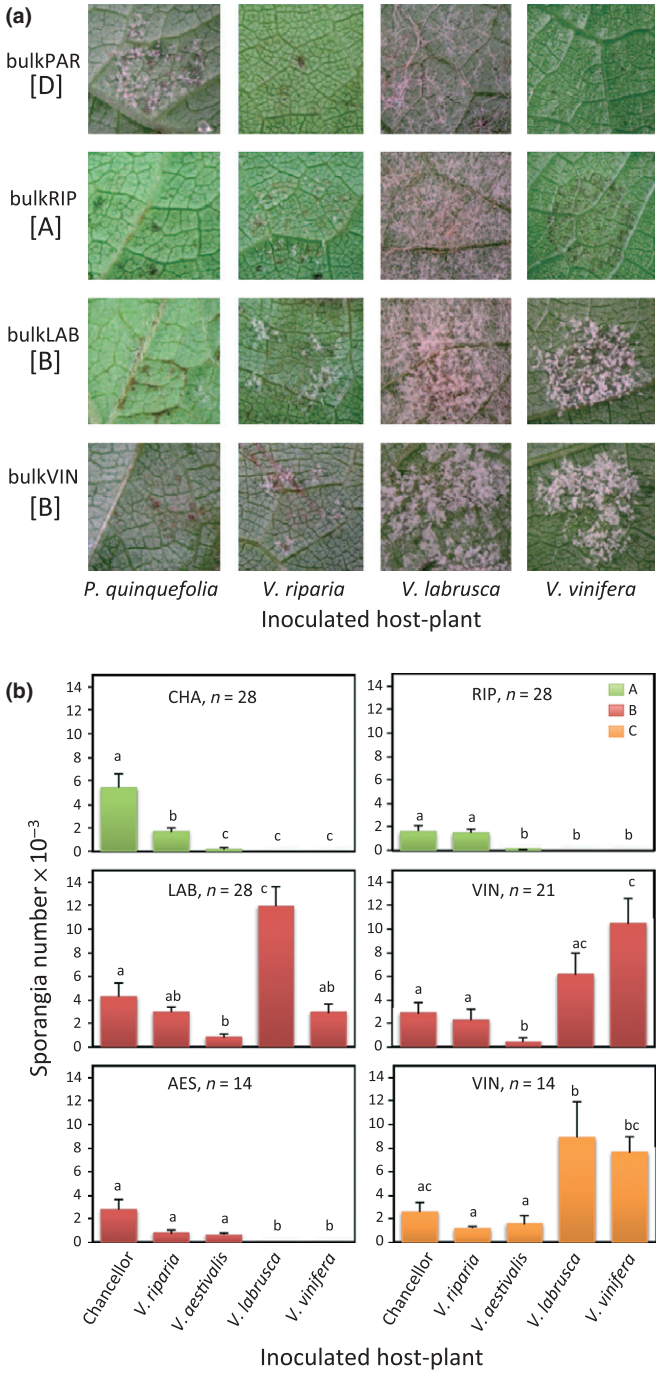

Fig. 4 Host specialization in the interaction between *Plasmopara viticola* and the Vitaceae. (a) Pathogenicity of *P. viticola* isolates on different plant hosts. Leaf disks from different Vitaceae species inoculated with bulks of isolates for each source host plant (bulkPAR, *Parthenocissus quinquefolia*; bulkRIP, *Vitis riparia*; bulkLAB, *V. labrusca*; bulkVIN, *V. vinifera*). Pictures are representative of results obtained for each host–isolate combination at 7 d post-inoculation (dpi). *Vitis labrusca* leaves present white hairs, and sporulation on *V. labrusca* was only found for bulkLAB and bulkVIN. (b) Sporulation of *P. viticola* isolates on different plant hosts. Each graphic represents a group of isolates based on their source host plant (CHA, hybrid chancellor; RIP, *V. riparia*; AES, *V. aestivalis*; LAB, *V. labrusca*; VIN, *V. vinifera*) and lineages (A, B, C). Data show the mean value (± SEM) of the number of sporangia per group of isolates (*n*, number of isolates from each group multiplied by the number of replicates). Identical letters do not differ from each other at the significance level of $P < 0.05$ ($\alpha = 5\%$) according to a Kruskal–Wallis test followed by a *post-hoc* pairwise Wilcoxon test.

Morphological analysis

We found that sporangial size differed significantly among the four phylogenetic lineages of *P. viticola*. Lineage D isolates were significantly smaller than all the others. Lineage A and lineage C isolates were not different in size. Isolates from lineage B were found to be intermediate between A/C and D (Fig. 6a). To rule out the possibility that the differences in sporangial size were caused by the host plant, and were not intrinsic to the isolate group, we measured the size of sporangia from cross-inoculation experiments. As shown in Fig. 6(b), the size differences between different isolate groups were independent of the inoculated host plant. It is worth noting that the sporangia sizes assessed with the cell counter were different from those measured under a microscope. This is because the cell counter estimates the sporangia size by modeling a symmetric particle, whereas microscope inspection allows the measurement of the actual morphology of sporangia.

By combining phylogenetic and experimental results, we have proposed a most likely scenario for host range expansion of *P. viticola* lineages from wild to cultivated species (Fig. 7).

Discussion

Evidence for several host-specific independent cryptic species of *P. viticola*

Here, we have presented several lines of evidence leading to the proposal that grapevine downy mildew is not caused by a single species, but rather by a complex of cryptic species that have radiated on the Vitaceae. Application of the GCPSR criterion (Taylor *et al.*, 2000) provided evidence that *P. viticola* includes four independent evolutionary lineages evolving without gene

Table 3 Pathogenicity of *Plasmopara viticola* isolates from six Vitaceae source host plants inoculated on the same six host plants (the table summarizes the results of two independent cross-inoculation experiments)

Source host plant	*P. viticola* lineage	Inoculated host plant					
		V. riparia	Chancellor	*V. aestivalis*	*V. labrusca*	*V. vinifera*	*P. quinquefolia*
Vitis riparia	A	+[a]	+	+[a]	–	–[a]	–[a]
Chancellor	A	+	+	+[a]	–	–[a]	–[a]
V. aestivalis	B	+[a]	+	+[a]	–	–[a]	–[a]
V. labrusca	B	+	+	+[a]	+	+	–[a]
V. vinifera	B	+	+	+[a]	+	+	–[a]
V. vinifera	C	+	+	+[a]	+	+	–[a]
Parthenocissus quinquefolia	D	–[a]	–		–	–	+[a]

+, *P. viticola* sporulation in at least 15% of leaf disks; –, absence of sporulation.
[a]Presence of necrosis in at least one of the experiments.
Colors in the table correspond to the four different *P. viticola* lineages identified: green for A, red for B, orange for C, violet for D.

flow and showing molecular divergences that might reflect distinct species. Although some of the genes presented a low phylogenetic signal (ITS, 28S), the four monophyletic lineages are well supported by independent gene genealogies, the concatenated dataset and the species tree. Isolates of *P. viticola* collected from the same host plants, but in different geographic locations, were genetically closer than isolates collected on different host species in the same field. The strong association between lineages and host plants suggests a major role of specialization in the origin and maintenance of the *P. viticola* lineages, a hypothesis supported by cross-pathogenicity tests. Two of these lineages are highly specific to their host (lineages A and D on *V. riparia* and *P. quinquefolia*, respectively), whereas lineages B and C grow on multiple hosts and have an optimal aggressiveness on *V. labrusca* and *V. vinifera*, respectively. Finally, morphological analysis further supported the existence of the phylogenetic lineages: we observed differences in sporangial size between lineages that were independent of the host plant inoculated, indicating that this result was not caused by phenotypic plasticity of the pathogen, but rather that differences in sporangia size are genetically determined.

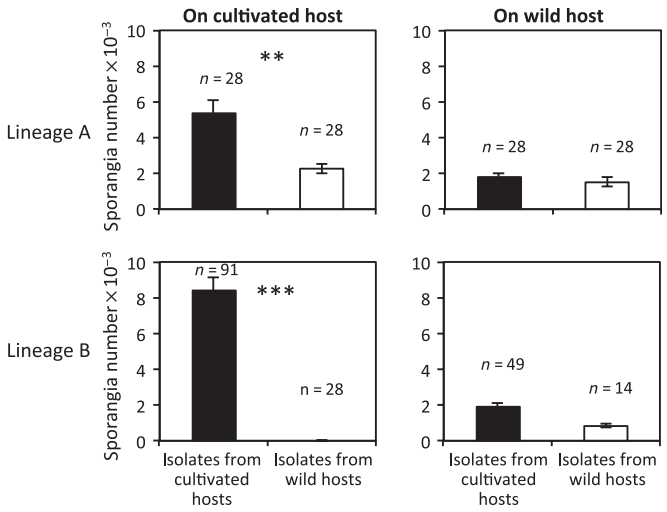

Fig. 5 Sporulation of *Plasmopara viticola* isolates from wild and cultivated source hosts interacting with their inoculated hosts. For lineage A isolates, the wild host is *Vitis riparia* and the cultivated host is Chancellor. For lineage B isolates, the wild host is *V. aestivalis* and the cultivated hosts are *V. vinifera* and *V. labrusca*. Data show the mean value (± SEM) of the sporangia number (*n*, number of isolates from each interaction multiplied by the number of replicates). Data were tested using a Kruskal–Wallis test: **, $P < 0.05$; ***, $P < 0.01$.

Therefore, *P. viticola,* an apparently well-known and widespread plant pathogen, actually hides several host-specific cryptic species that could be considered as formae speciales: *P. viticola* f. sp. *riparia* (lineage A occurring on *V. riparia* and some hybrids); *P. viticola* f. sp. *aestivalis* (lineage B found on *V. aestivalis, V. labrusca*, *V. vinifera* and some hybrids); *P. viticola* f. sp. *vinifera* (lineage C occurring on *V. vinifera* and some hybrids); *P. viticola* f. sp. *quinquefolia* (lineage D found on *P. quinquefolia*).

The results presented here are congruent with a previous phylogenetic study on *P. viticola*. Schröder *et al.* (2011) reported significant diversity among North American *P. viticola* isolates, giving a first hint to potential species boundaries in this species. As one of the markers used was shared with our study (*nrLSU*), we could establish that the lineages identified by Schröder *et al.* (2011) correspond to three of the cryptic species described here. However, the small number of isolates analyzed by Schröder *et al.* (2011) prevented these authors from reaching any conclusions concerning host specialization.

Ecological specialization promotes the evolution of cryptic species of *P. viticola*

Among oomycetes, biotrophic groups, such as downy mildews and albuginales, are prime candidates for ecological speciation via host specialization. Our results on grapevine downy mildew illustrate how biotrophic plant pathogens can diversify by host plant specialization. They are in agreement with phylogenetic studies that have recently reported the presence of distinct pathogen species on a specific host plant family, including *Albugo* and *Hyaloperonospora* on Brassicaceae (Goker *et al.*, 2009; Ploch *et al.*,

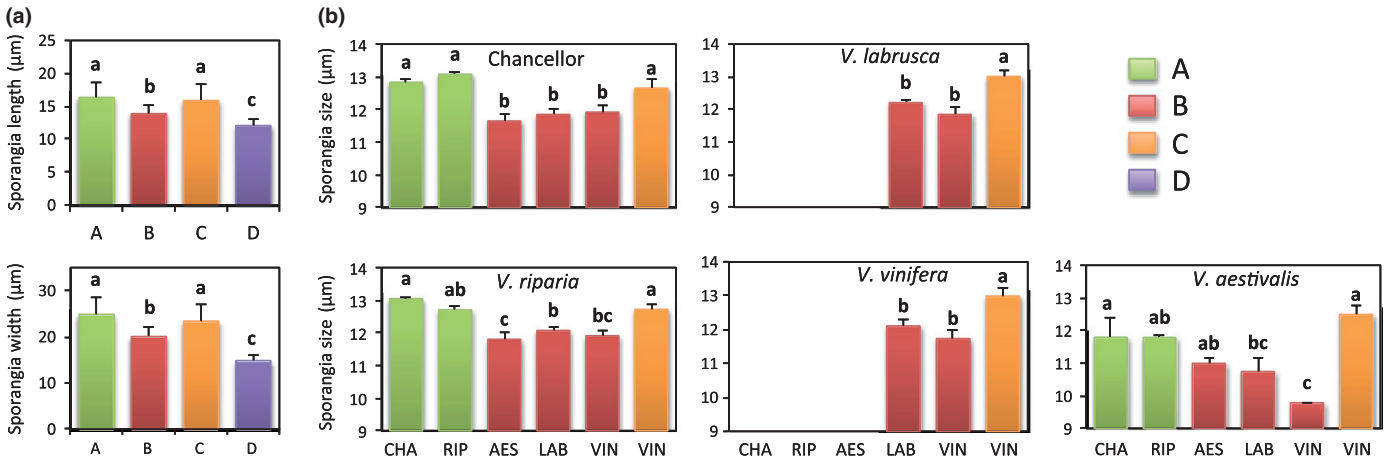

Fig. 6 Morphological analysis of sporangia of *Plasmopara viticola* isolates. (a) Samples derived from natural infections. Average (± SEM) length and width (µm) of sporangia of grapevine downy mildew samples assessed under the microscope using a stage micrometer. Samples are grouped by lineage (A, B, C, D). (b) Live isolates derived from the cross-inoculation experiment. Sporangia size (µm) was assessed using an automated cell counter. Isolates are grouped by source host plant (CHA, hybrid chancellor; RIP, *Vitis riparia*; AES, *V. aestivalis*; LAB, *V. labrusca*; VIN, *V. vinifera*) and by lineage (A, B, C, D). The title of each graphic indicates the inoculated plant. A mean size was calculated for each leaf disk. Bars indicate the mean size (± SEM) of seven replicates (seven leaf disks) of all isolates of each group: 28 values for CHA (lineage A), 28 for RIP (lineage A), 14 for AES (lineage B), 28 for LAB (lineage B), 21 for VIN (lineage B) and 14 for VIN (lineage C). Identical letters do not differ from each other at the significance level of $P < 0.05$ ($\alpha = 5\%$) according to a Kruskal–Wallis test followed by a *post-hoc* pairwise Wilcoxon test.

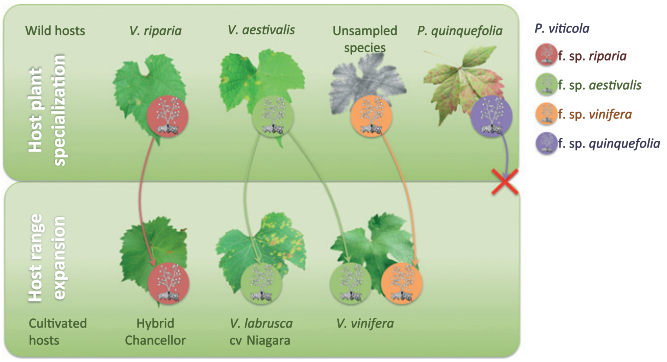

Fig. 7 Hypothetical scenario for host range expansion of *Plasmopara viticola* formae speciales from wild to cultivated hosts.

2010), *Bremia* on Asteraceae (Choi *et al.*, 2011), *Peronospora* on Fabaceae and on Lamiaceae (Garcia-Blazquez *et al.*, 2008; Choi *et al.*, 2009; Thines *et al.*, 2009) and *Peronosclerospora* on Poaceae (Telle *et al.*, 2011). Nevertheless, the proximal mechanisms responsible for such adaptive radiation in downy mildews remain unknown. It has been proposed that the evolution of sibling oomycete species may be driven by hybridization, as has been observed for *Phytophthora* species (Brasier, 2000). However, we can discard recent hybridization in this system because the heterozygosity of *P. viticola* isolates was low within species, a result that does not fit with hybridization events.

Although many studies have addressed the host plant specificity of downy mildews using phylogenetic approaches, very few have coupled it with an assessment of the specialization of the pathogens using cross-inoculation bioassays (Mitchell *et al.*, 2011; Runge & Thines, 2012). Yet, this is an important step towards understanding the interactions in a given pathosystem, because inoculation tests provide substantial information on the actual pathogenicity and aggressiveness of a given isolate. In this study, results of the cross-inoculation experiments were mostly in agreement with the distribution of *P. viticola* cryptic species observed in natural grapevine downy mildew populations. Cryptic species A and D only infected *V. riparia* and *P. quinquefolia*, respectively, and cryptic species B and C showed optimal development on *V. labrusca* and *V. vinifera*, respectively. The host range of cryptic species B and C appeared to be larger in controlled experiments than in natural infections: both colonized leaves of *V. riparia* and Chancellor in controlled conditions, but we did not observe these cryptic species on these host plants in the field; similarly, cryptic species C could infect *V. aestivalis* and *V. labrusca* in the laboratory, but not in the wild. This larger host range in the laboratory could be explained by the optimization of the conditions used in bioassays: interactions are indeed favored by the use of young leaves that are known to be more susceptible to the disease and by choosing the optimal temperature and hygrometry for the growth of the pathogen. By contrast, the combination of environmental and developmental factors in the field might affect eventual disease severity and may even lead plants to escape from infection.

Host range expansion and quantitative adaptation to cultivated hosts

Plasmopara viticola is endemic to North America, where it has coevolved with wild *Vitis* spp. (Bush & Meissner, 1883). Although we lack reliable historical records, the host shift of downy mildew from wild to cultivated grapes probably occurred during the 17th century with the development of viticulture by European colonists in America. The pathogen is now well established in vineyards where it over-seasons as oospores in the soil (Kennelly *et al.*, 2007). Our data suggest that species A, which

was found on the cultivated Chancellor hybrid, probably originated from *V. riparia*, whereas species B, which infected *V. vinifera* and *V. labrusca*, may have originated from *V. aestivalis*. Colonization on novel hosts can be caused by either host range expansion (the pathogen can still infect its host of origin) or host shifts (accompanied by a loss of the ability to infect the ancestral host). Here, we found that isolates collected on cultivated hosts all remained pathogenic on their ancestral (wild) hosts. Host range expansion from wild to cultivated hosts is usually associated with quantitative adaptation to the cultivated host, even in cases in which ecological specialization is not found (Lê Van *et al.*, 2012). In agreement with this hypothesis, the fitness of the isolates originating from cultivated varieties (CHA, VIN, LAB) was higher on their source hosts than on the other hosts. Previously, several authors have reported quantitative adaptation to the cultivar of origin for several fungal plant diseases, including rice blast caused by *Magnaporthae oryzae* (Bonman *et al.*, 1989), septoria tritici blotch caused by *Mycospaerella graminicola* (Ahmed *et al.*, 1996; Stukenbrock *et al.*, 2011), potato late blight caused by *Phytophthora infestans* (Andrivon *et al.*, 2007), apple scab caused by *Venturia inaequalis* (Lê Van *et al.*, 2012) and wheat leaf rust caused by *Puccinia triticina* (Pariaud *et al.*, 2009).

Implications for viticulture

Beyond the necessary revision of the taxonomic status of *P. viticola*, the results presented here have important implications for breeding for resistance to grapevine downy mildew, disease management and quarantine regulations.

First, our work highlights the importance of taking into account pathogen variability when screening germplasms for resistance to grapevine downy mildew. The differences reported by Cadle-Davidson (2008) in the identification of resistant sources between screens might result from an unrecognized structure of the pathogen, such as the cryptic species described here, or the use of virulent strains (Peressotti *et al.*, 2010; Calonnec *et al.*, 2012).

Second, the results obtained when confronting *V. vinifera* with *P. viticola* isolates derived from *V. riparia* should be taken into account for the construction of new resistant varieties. Indeed, although *V. vinifera* is highly susceptible to downy mildew, inoculation with *V. riparia*-derived isolates results in a strong necrosis, suggesting a resistance response from the plant. The *V. vinifera* genes responsible for this response should potentially be retained in the process of breeding for new varieties in order to make sure that the new varieties are resistant to *V. riparia*-derived isolates.

The unmasking of cryptic species means that some previous research on *P. viticola* will need to be revisited, because it was unclear which species were under study. Although challenging, it is also necessary to determine the agricultural importance of these newly revealed cryptic species of *P. viticola* by better assessing their spatio-temporal distribution during the course of the epidemics in vineyards. The accurate identification of plant pathogens is of particular importance to national biosecurity agencies, which have a mandate to prevent the introduction of exotic pests and pathogens. In the case of grapevine downy mildew, phylogeographical studies are now required to compare native and introduced populations and to reconstruct the routes of invasion of the pathogen into Europe (Gobbin *et al.*, 2006; Rouxel *et al.*, 2012). An understanding of the invasive pathways and a determination of how many of the *P. viticola* cryptic species have established in Europe are crucial to assess the potential threat to viticulture represented by the introduction of new species and to help define new quarantine regulations.

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Supporting Information

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

Fig. S1 Maximum likelihood trees of *Plasmopara viticola* isolates.

Fig. S2 Aggressiveness of *Plasmopara viticola* isolates assessed from the cross-inoculation experiment.

Table S1 Detailed information on the *Plasmopara viticola* isolates used for phylogenetic analysis

Table S2 Detailed information on the *Plasmopara viticola* isolates used in cross-inoculation experiments

Table S3 Primers for the four genomic regions sequenced in *Plasmopara viticola*

Table S4 Divergence between *Plasmopara viticola* phylogenetic lineages

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