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Geographic Distribution of Cryptic Species of *Plasmopara viticola* Causing Downy Mildew on Wild and Cultivated Grape in Eastern North America

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ABSTRACT

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The putative center of origin of *Plasmopara viticola*, the causal agent of grape downy mildew, is eastern North America, where it has been described on several members of the family Vitaceae (e.g., *Vitis* spp., *Parthenocissus* spp., and *Ampelopsis* spp.). We have completed the first large-scale sampling of *P. viticola* isolates across a range of wild and cultivated host species distributed throughout the above region. Sequencing results of four partial genes indicated the presence of a new *P. viticola* species on *Vitis vulpina* in Virginia, adding to the four cryptic species of *P. viticola* recently recorded. The phylogenetic analysis also indicated that the *P. viticola* species found on *Parthenocissus quinquefolia* in North America is identical to *Plasmopara muralis* in Europe. The geographic distribution and host range of five pathogen species was determined through analysis of the internal transcribed spacer polymorphism of 896 isolates of *P. viticola*. Among three *P. viticola* species found on cultivated grape, one was restricted to *Vitis* interspecific hybrids within the northern part of eastern North America. A second species was recovered from *V. vinifera* and *V. labrusca*, and was distributed across most of the sampled region. A third species, although less abundant, was distributed across a larger geographical range, including the southern part of eastern North America. *P. viticola* clade *aestivalis* predominated (83% of isolates) in vineyards of the European winegrape *V. vinifera* within the sampled area, indicating that a single pathogen species may represent the primary threat to the European host species within eastern North America.

Additional keywords: plant pathogen.

The European winegrape species *Vitis vinifera* is cultivated worldwide. Grapevine downy mildew is considered one of the most important grapevine diseases in temperate climates. *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni, the causal agent of downy mildew, is a heterothallic oomycete endemic to eastern North America, where it has been described on many members of the family Vitaceae (1), including *Vitis vinifera (*European wine grape*)*, *V. labrusca* L. (fox grape), *V. riparia* Michx. (riverbank grape), *V. aestivalis* Michx. (summer grape), *V. cinerea* (graybark grape), and *V. vulpina* (frost grape), as well as *Parthenocissus quinquefolia* L. Planch (Virginia creeper).

Plasmopara viticola was accidentally introduced into Europe in the 1870s with the importation of American wild *Vitis* spp. and phylloxera-resistant hybrids (29). In the last decade, several population genetics studies have been carried out in Europe to assess the genetic diversity of *P. viticola* using microsatellite markers (1,8–12,21,22). Most of these studies were conducted on a small spatial scale (i.e., within vineyards) and indicated that *P. viticola* populations were mainly panmictic, although footprints of clonal propagation were also found in some cases (8). Recently, Fontaine et al. (5) reported a genetic signature indicative of range expansion and a leap-frog event during the introduction and spread of *P. viticola* in Europe: a significant continent-wide population structure, with two geographically and genetically distinct clusters of *P. viticola* in western and eastern European vineyards.

Comparatively little information has been published regarding the genetic structure of *P. viticola* populations within its native range (eastern North America). North American populations of *P. viticola* were reported to be much more diverse than European populations, suggesting the existence of a founder effect at the introduction of this pathogen in Europe (21). A phylogenetic study on 14 isolates of *P. viticola* reported significant diversity among North American *P. viticola* isolates, suggesting the possibility of species boundaries in *P. viticola* (24). To assess cryptic speciation in *P. viticola*, Rouxel et al. (20) combined

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genetic, morphological, and virulence data on a large set of American *P. viticola* isolates and provided evidence that *P. viticola* is, in fact, a complex of four cryptic species, each with a unique degree of pathogenic specialization within the family Vitaceae. The study also indicated that two cryptic *P. viticola* species exhibited complete host plant specialization toward *Parthenocissus quinquefolia* and *V. riparia*, whereas two other cryptic species discovered on *V. aestivalis*, *V. labrusca*, and *V. vinifera* could infect a wider range of hosts in controlled experiments. Although the taxa evidenced by Rouxel et al. (20) should represent distinct species according to their genetic distances and their host ranges, the cryptic species were described as different formae speciales of *Plasmopara viticola*. This is because the pathogen species cannot be named until we examine the type specimen of *P. viticola* to determine the species to which the epithet *viticola* should be applied. In addition, a formal species description requires a thorough morphological investigation. For purposes of clarity, we employ a provisional nomenclature of *P. viticola* species and use the term clade in place of forma specialis.

More generally, these results on *P. viticola* confirm the finding that many plant diseases formerly believed to be caused by a single species are indeed associated with a complex of multiple cryptic species (3), such as Septoria tritici blotch of wheat. For *P. viticola*, ecological divergence through host adaptation is probably favored by its biotrophic lifestyle (6), a hypothesis that is supported by the growing number of genetic studies reporting cryptic speciation in biotrophic plant pathogens such as powdery and downy mildews (6,16,17,23,26,27). The discovery of sibling species causing grapevine downy mildew naturally raises questions about their epidemiological implications. A better understanding of the spatial-temporal distribution of pathogen species may have important implications for disease management in vineyards. Investigating the association of *P. viticola* species with wild and cultivated hosts will also provide critical information for grapevine breeding, because this often involves interspecific hybridization and introgression of resistance from distantly related members of the host family. Rouxel et al. (20) provided valuable information on the level of specialization of *P. viticola* species; however, sampling was limited to the Great Lakes region of the United States, and did not include all the potential host species of *P. viticola*.

The present study represents the first large-scale sampling of *P. viticola* isolates across a range of wild and cultivated host species distributed throughout the putative center of origin of the pathogen: eastern North America. Here, we first used multiple-gene genealogies to describe the phylogenetic relationships of the phylogenetic *P. viticola* species identified across the sampling. We then developed and used a rapid diagnostic tool based on internal transcribed spacer (ITS) polymorphism to infer the geographical range and the degree of pathogenic specialization within *P. viticola* across wild grape and cultivated vineyards.

MATERIALS AND METHODS

***P. viticola* sampling.** Between 2007 and 2010, a large-scale survey of *P. viticola* was conducted on cultivated (*V. labrusca*, *V. vinifera*, and interspecific hybrids) and wild (*V. aestivalis*, *V. vulpina*, *V. cinerea, V. riparia*, and *Parthenocissus quinquefolia*) (Fig. 1) grapevines in Canada (Québec) and in the United States (Florida, Michigan, North Carolina, New York, Ohio, Pennsylvania, Virginia, and West Virginia). In total, 890 isolates from 89 geographical sites on 5 wild *Vitis* spp. and 54 cultivated varieties were sampled (Table 1; Fig. 2). The cultivated varieties included 24 *V. vinifera* cultivars, 4 *V. labrusca* cultivars, and 27 interspecific hybrids (Table 2). In addition, three isolates of *Plasmopara muralis* collected on *Parthenocissus tricuspidata* in Bordeaux were used to assess the genetic relatedness with *Plasmopara viticola* clade *quinquefolia* described by Rouxel et al. (20) from North America isolates.

DNA extraction. Isolates correspond to sporulation present on 1 cm^2 of grapevine leaf infected with downy mildew. Fragments obtained from infected leaves were lyophilized overnight, and the DNA of each isolate was extracted according to the standard method (cetyl-trimethyl-ammonium-bromide, phenol-chloroform), followed by precipitation with isopropanol, as explained by Delmotte et al. (4).

Determination of ITS1 sequence polymorphism. A subsample of 254 isolates, corresponding to one to three isolates per host plant for each sampling site, was used for further sequencing of *ITS1* (Table 1). Polymerase chain reaction (PCR) was performed using primers ITS1-O (CGGAAGGATCATTACC) and ITS2 (GCTGCGTTCTTCATCGATGC), as described by Rouxel et al. (20). Sequencing of PCR amplicons was outsourced to the Genomic and Sequencing Center of Bordeaux (Pierroton, France).

Cleaved amplified polymorphism sequence. In order to design a rapid diagnostic tool for inferring *P. viticola* species on a large number of isolates, we used the described single-nucleotide polymorphisms of the *ITS1* and β-*tubulin* genes (20) to develop cleaved amplified polymorphic sequence (CAPS) markers. Four restriction enzymes (*AseI*, *XmnI*, *TfiI*, and *HpyCH4V*) were used that allow recognition of the different haplotypes of *P. viticola*. First, *ITS1* PCR products were digested separately with three enzymes (*AseI*, *XmnI*, and *TfiI*) in parallel at the appropriate temperature in a final volume of 10 µl containing 0.1 µl of enzyme, 1 µl of PCR product, 1× buffer, and 0.1 µl of bovine serum albumen when necessary (*XmnI* digestion). Restriction fragments were visualized on 2% agarose gels. Altogether, this allowed us to distinguish four restriction profiles corresponding to five *P. viticola* species (Table 2). In order to discriminate the species that have the same *ITS1* restriction profile, the β-tubulin PCR products were digested with *HpyCH4V* using the same conditions as described for the other enzymes (Table 3).

Sequencing, alignments, and phylogenetic inference. In order to reconstruct the phylogenetic relationships among *P. viticola* species, 19 isolates of *P. viticola*, including the five *P. viticola* species and 3 isolates of *P. muralis*, were sequenced on four genomic regions: *ITS1* and fragments of actin (*act*), β-tubulin (*tub*), and cytochrome b (*cytb*) (Supplementary Table 1). PCR amplifications were performed using primers and PCR conditions described by Rouxel et al. (20) for ITS1, *act*, and *tub* genes and by Chen et al. (2) and Giresse et al. (7) for the cyt*b* gene.

Sequences of the four partial genes were aligned using Muscle implemented in Seaview (14). The best-fit models of nucleotide substitution were selected using MODELTEST v. 3.06 (18) based on likelihood scores for 88 different models and the Akaike information criterion. Phylogenetic relationships among isolates were inferred using maximum likelihood methods implemented in PhyML (15). Genealogies were constructed using the heuristic search function, with 1,000 random addition replicates, and tree bisection and reconstruction using a branch-swapping algorithm. Gaps were treated as unknown characters. To estimate branch support, bootstrap values were determined using 1,000 bootstrap replicates.

A species tree inference approach was used to establish 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 (15). A Yule speciation process was specified because it is the most appropriate when comparing relationships between species. We ran Monte Carlo Markov chains for 10 million generations, sampling trees every 1,000 generations and using a strict molecular clock. We used Tracer v1.5 (Rambaut and Drummond [19]) to assess likelihood stabilization and convergence for BEAST analyses, and discarded the first 10% of trees as burn-in.

RESULTS

ITS1 polymorphism. The *ITS1* region (238 bp) presents 16 polymorphic sites determining five haplotypes. Among the 254 isolates sequenced for *ITS1*, 247 presented one of the four haplotypes described by Rouxel et al. (20) that correspond each to a cryptic species of *P. viticola* (Tables 1 and 2). A new haplotype was found for seven isolates collected on *V. vulpina* in Virginia, revealing the detection of a new species of *P. viticola* in North America.

Species inference by CAPS. The 254 isolates sequenced for *ITS1* were used to validate the detection of grapevine downy mildew species by the CAPS method (Table 3). Results obtained by CAPS of ITS1 and β-tubulin and ITS1 sequencing were

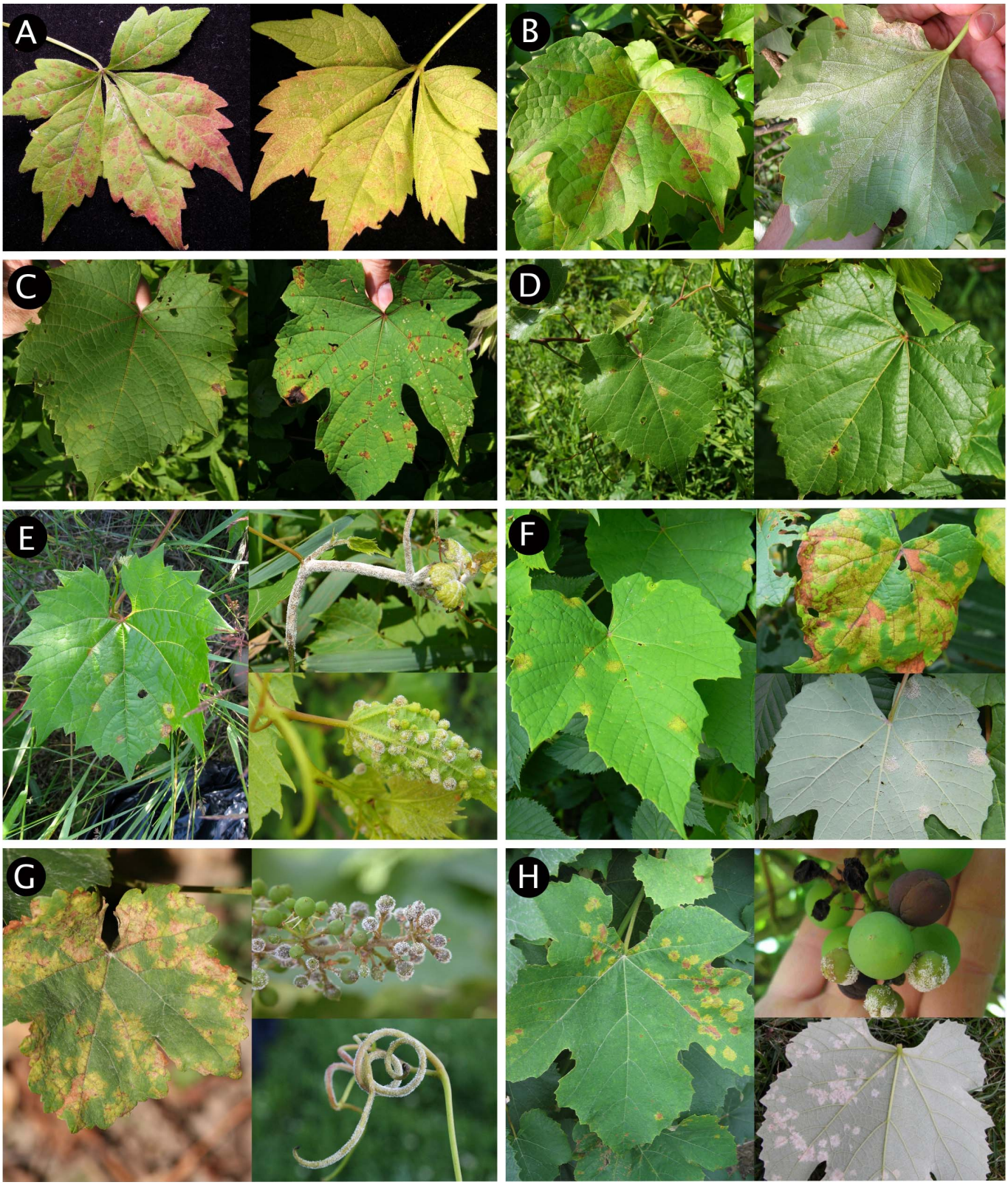

Fig. 1. Wild and cultivated *Vitis* spp. infected with grapevine downy mildew: **A,** *Parthenocissus quinquefolia*; **B,** *P. tricuspidata*; **C,** *Vitis cinerea*; **D,** *V. vulpina*; **E,** *V. riparia*; **F,** *V. aestivalis*; **G,** *V. vinifera*; and **H,** *V. labrusca*.

entirely congruent. Amplification of the *ITS1* region and β-tubulin was successfully obtained for 764 of 890 isolates analyzed. CAPS analysis allowed us to infer 290 isolates to *P. viticola* clade *riparia*, 304 isolates to *P. viticola* clade *aestivalis*, 126 isolates to *P. viticola* clade *vinifera*, 37 isolates to *P. viticola* clade *quinquefolia*, and 7 isolates to *P. viticola* clade *vulpina* (Table 2).

Distribution of *P. viticola* species. The geographical range of the different *P. viticola* species on their host plants is presented in Figure 3 and Table 3. *P. viticola* clade *riparia* was restricted to Canada and the Great Lakes region, where it was present on a unique wild species, *V. riparia*. We found that this species can also infect many cultivated interspecific hybrids in Canada,

TABLE 1. Number of samples of downy mildew collected on wild and cultivated grapevine

	Sampling area (sequenced isolates)[z]									
Samples	Canada	Michigan	Ohio	New York	Pennsylvania	West Virginia	Virginia	North Carolina	Florida	Total
Number of locations	20	16	3	19	2	5	16	5	3	89
Wild species										
Parthenocissus quinquefolia	0	38 (2)	0	6 (0)	0	0	0	0	0	44 (2)
Vitis aestivalis	0	22 (2)	8 (5)	0	11 (2)	18 (9)	16 (3)	0	10 (1)	85 (22)
V. cinerea	0	0	0	0	0	7 (5)	0	0	0	7 (5)
V. riparia	0	84 (10)	6 (2)	69 (4)	0	0	0	0	0	159 (16)
V. vulpina	0	0	0	0	7 (5)	10 (10)	28 (17)	0	0	45 (32)
Total	0	144 (14)	14 (7)	75 (4)	18 (7)	35 (24)	44 (20)	0	10 (1)	340 (77)
Cultivated species										
V. vinifera	0	64 (10)	10 (2)	46 (11)	0	0	42 (12)	26 (8)	0	188 (43)
V. labrusca	0	48 (7)	10 (4)	16 (4)	0	0	13 (2)	0	0	87 (17)
Interspecific hybrids	114 (79)	93 (21)		18 (3)	0	0	25 (4)	0	25 (10)	275 (117)
Total	114 (79)	205 (38)	20 (6)	80 (18)	0	0	80 (18)	26 (8)	25 (10)	550 (177)
Grand total	…	…	…	…	…	…	…	…	…	890 (254)

[z] Number of isolates for which the internal transcribed spacer 1 region has been sequenced is indicated in parentheses.

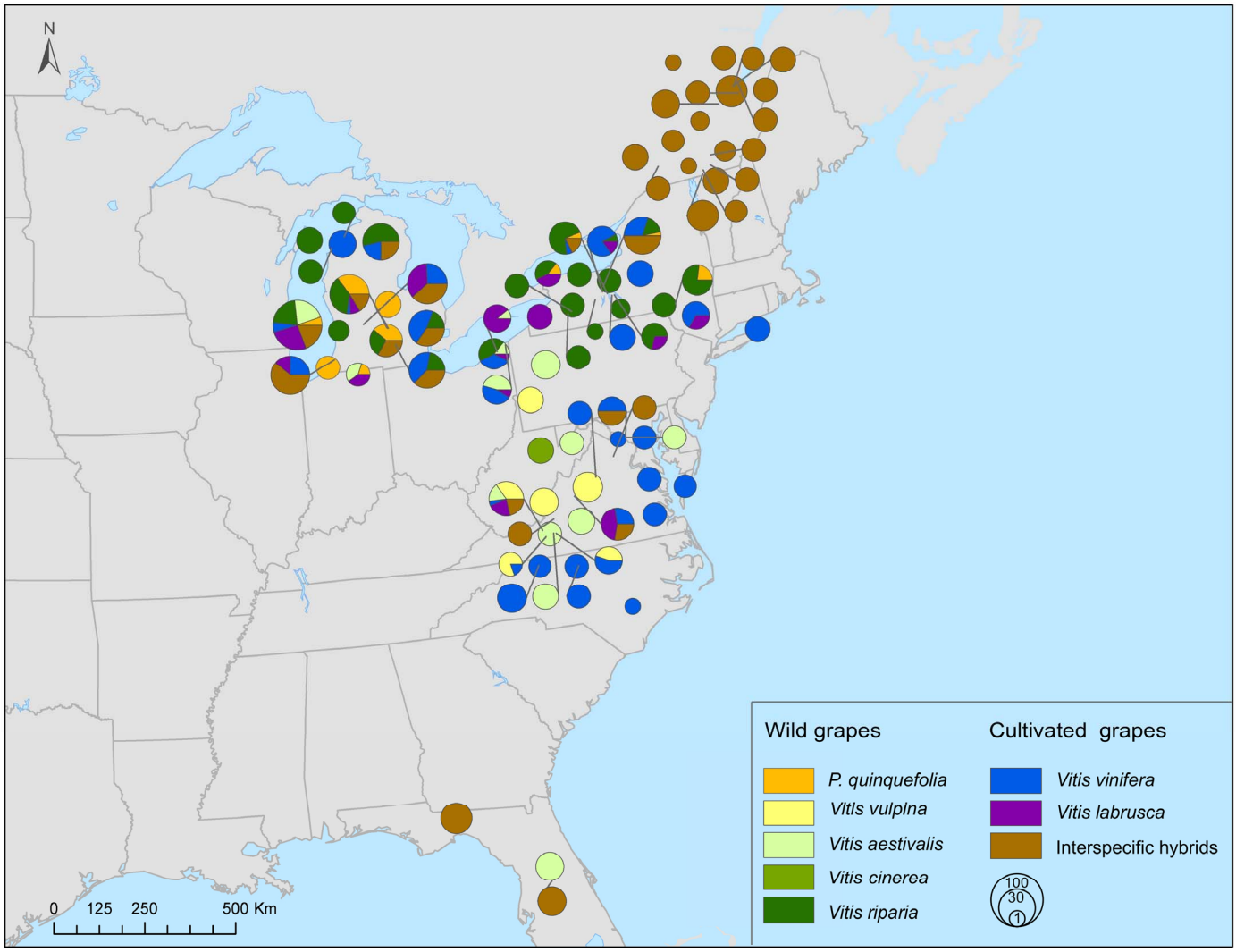

Fig. 2. Sampling of *Plasmopara viticola* on wild and cultivated species of the family Vitaceae in northeastern America. Circle sizes are proportional to number of isolates.

Michigan, and New York (Table 3). *P. viticola* clade *aestivalis* is widespread in the eastern United States, from Canada to North Carolina. This species was present on a single wild species, *V. aestivalis.* It was also abundant on *V. vinifera*, *V. labrusca* cultivars, and some interspecific hybrids (Table 3). *P. viticola* clade *vinifera* was widely distributed across northeastern America. It was the only species of grapevine downy mildew present in the southern United States (Florida). This species was present on three wild species (*V. vulpina*, *V. cinerea*, and *V. aestivalis*) but, for *V. aestivalis*, it was detected only in Florida. This species was also found on *V. vinifera* cultivars in Michigan and on cultivated interspecific hybrids having *V. aestivalis* genetic background in Florida. *P. viticola* clade *quinquefolia* was restricted to the northern United States on the wild species *Parthenocissus*

TABLE 2. Detailed information of the distribution of *Plasmopara viticola* species found on cultivated grape

Species, cultivar	Sampling locations (*n*)[y]	Samples of *P. viticola* species[z]		
		Clade *riparia*	Clade *aestivalis*	Clade *vinifera*
Vitis vinifera				
Aglianico	Virginia (1)	0	4	0
Cabernet Franc	New York (3), North Carolina (1), Virginia (1)	0	9	0
Cabernet Sauvignon	North Carolina (2), Ohio (1), Virginia (1)	0	5	0
Chambourcin	Virginia (1)	0	2	0
Chardonnay	Michigan (4), New York (3), North Carolina (3), Virginia (5)	0	46	6
Frankentahaler	New York (1)	0	1	0
Frontenac	Michigan (1)	0	0	1
Gamay	Michigan (1)	0	0	1
Gewurtztraminer	Michigan (1), New York (1)	0	1	5
Lakemond	New York (1), Virginia (1)	0	9	0
Merlot	New York (1), North Carolina (1)	0	5	0
Muscat	Ohio (1)	0	4	0
Nebbiolo	North Carolina (1)	0	1	0
Petit Verdot	North Carolina (1), Virginia (1)	0	2	0
Pinot noir	Michigan (3), New York (1)	0	9	6
Pinotage	New York (1)	0	1	0
Riesling	Michigan (2), New York (3), Ohio (1)	0	8	11
Sangiovese	New York (1), North Carolina (2), Virginia (1)	0	5	0
Syrah	Michigan (1)	0	0	4
Tannat	North Carolina (1), Virginia (2)	0	8	0
Traminette	Michigan (1), North Carolina (1)	0	2	3
Unknown	Michigan (3), Virginia (1)	0	4	3
Viognier	North Carolina (1)	0	1	0
Riesling	New York (1)	0	0	4
Total	…	0	127	44
V. labrusca				
Catawba	New York (1)	0	2	0
Concord	Michigan (4), New York (4), Ohio (3), Virginia (1)	0	29	0
Fredonia	New York (1)	0	1	0
Niagara	Michigan (3)	0	0	38
Total	…	0	32	38
Interspecific hybrids				
Mars	Michigan (1)	2	3	0
Blanc du bois	Florida (2)	0	0	11
Chancellor	Canada (1), Michigan (4), New York (1)	37	0	0
Chardonel	Michigan (1)	0	3	0
Cliche	Canada (3)	10	1	0
E-4-7	Canada (1)	1	1	0
Freedom	New York (1)	0	1	0
Hybrid 30-5-1	Florida (1)	0	0	4
Hybrid 30-7-1	Florida (1)	0	0	3
Lake Emerald	Florida (1)	0	0	4
Landol	Michigan (1)	0	0	1
Lucie Kuhlmann	Canada (1)	7	0	0
Marechal Foch	Canada (3)	8	2	0
Marquis	Michigan (3)	0	7	4
Muscat de Swenson	Canada (1)	4	0	0
NY-73.136	Michigan (1)	0	0	2
Ortega	Michigan (1)	2	0	0
Rkatsiteli	Michigan (1)	4	0	0
Sainte Croix	Canada (4)	11	7	0
Seyval blanc	Canada (1)	0	2	0
Seyval noir	Canada (1)	0	1	0
Stover	Florida (1)	0	0	3
Table grape	Michigan (1)	0	4	0
Unknown	Canada (1), Michigan (2)	1	0	6
Vandal-Cliche	Canada (6)	29	1	0
Vidal	Canada (2), Michigan (1), Virginia (3)	10	15	5
Vignoles	Michigan (3), New York (1)	18	3	0
Total	…	144	51	43
Grand total	…	144	210	125

[y] Number of locations per region (*n*).
[z] Total number of samples showing positive results is indicated for each *P. viticola* species.

quinquefolia. *Plasmopara viticola* clade *vulpina* has been found on *V. vulpina* at Blacksburg and Pilot (Virginia).

Phylogenetic analyses. Sequences for *ITS1*, *tub*, and *act* were obtained from 19 *P. viticola* and three *P. muralis* isolates. Gene *cytb* was sequenced for 13 *P. viticola* isolates but it could not be amplified from *P. muralis* isolates and *P. viticola* clade *quinquefolia* isolates. Among the 22 isolates analyzed, we obtained five haplotypes for *ITS1*, 16 for *act*, 18 for *tub*, and 11 for *cytb*. Variable sites identified in coding regions resulted only from synonymous substitutions, except for *cytb*, which presented 14 nonsynonymous substitutions. Globally, the most polymorphic region was *cytb* (nucleotide diversity [pi] = 0.05209) and the least polymorphic was *ITS1* (pi = 0.02255). The numbers of parsimony informative sites (excluding gaps) were 15, 47, 75, and 93 for *ITS1*, *act*, *tub*, and *cytb*, respectively.

MODELTEST indicated that the best model for the data was the General Time Reversible model for each of the four gene regions. The phylogenies obtained from the sequence data of the gene regions were first determined separately. Individual phylogenetic trees (*ITS1*, *act*, *tub*, and *cytb*) supported five monophyletic groups of isolates delimiting five *P. viticola* species (Fig. 4). Individual trees were congruent (i.e., for each gene tree, the five monophyletic lineages supported included the same isolates). The reconstruction of the phylogenetic relationships among the five downy mildew species were well resolved by *tub* whereas *ITS*1, *act*, and *cytb* individual trees did not resolve the among taxa relationships. The species tree obtained with *BEAST indicated that *P. viticola* clade *vulpina* is closely related to *P. viticola* clade *aestivalis* (Fig. 5).

DISCUSSION

Rouxel et al. (20) previously described that *P. viticola* includes four independent evolutionary lineages corresponding to different host-specialized cryptic species. Using the phylogenetic concordance of multiple unlinked genes (25), this study discriminates five cryptic species of *P. viticola* in eastern North America. The sampling of a larger geographical area and host plant diversity allowed the detection of a new phylogenetic lineage on *V. vulpina,* hereafter named *P. viticola* clade *vulpina*, evolving without gene flow and showing significant genetic divergence from the already described *P. viticola* species. This species is represented by seven isolates that have been found on *V. vulpina* at Blacksburg and Pilot, VA. More samples of *P. viticola* clade *vulpina* together with virulence results from cross-pathogenicity tests would be necessary before conclusions can be drawn about the specialization of this species on *V. vulpina*. It is worth noting that our data already indicate that another species of *P. viticola* (clade v*inifera*) is able to infect *V. vulpina*. Altogether, our data confirm that grapevine downy mildew is caused by a complex of at least five cryptic species that have radiated on the Vitaceae family.

A recent phylogenetic and morphological study indicates that outbreaks of downy mildew on *Parthenocissus tricuspidata* are caused by a new species described as *Plasmopara muralis* (28). Our data reveal that *P. viticola* species specialized on Virginia creeper (*Parthenocissus quinquefolia*) in North America (20) is identical to the one found on Japanese ivy (*P. tricuspidata*) in Europe (28). The genus *Parthenocissus* contains different species grown for ornamental use worldwide; some originated in North America, such as *P. quinquefolia*, others in Asia, such as *P. tricuspidata*. Therefore, several scenarios could explain the current distribution of the downy mildew species on this genus: in one of them, the pathogen is native to North America, where it infects *P. quinquefolia* and is invasive in Europe on *P. tricuspidata*; alternatively, an undetected species of downy mildew present in Asia on *Parthenocissus* spp. has spread on *P. tricuspidata* in Europe and on *P. quinquefolia* in North America. It is worth noting that different varieties of *Plasmopara viticola* have been described on endemic Asian *Vitis* spp. (13). However, in the absence of clear-cut distinguishing features, these taxa are not widely recognized and remain to be confirmed by molecular phylogenetic investigations. Whatever the scenario, it remains to be explained why the species has not been described yet on *Parthenocissus quinquefolia* in Europe and *P. tricuspidata* in America. However, it is possible that the mild symptoms caused by *Plasmopara* spp. on *Parthenocissus* spp. have led to the pathogen being overlooked on these species.

Beyond the identification of these species of *Plasmopara viticola*, this study brings new insights into the geographical and host plant ranges of the five *P. viticola* species that are now described. In the north- and central-eastern United States, several grapevine downy mildew species coexist at different spatial scales (i.e., within a plant and at the vineyard scale). The same individual host plant can be infected by two different *P. viticola* species, as seen for 'Marechal Foch' at Saint Hilaire and Knowlton in Canada or for the table grape 'Marquis' at Benton-Harbor, MI. We have also found several *P. viticola* species coexisting at the vineyard scale: in Fennville, MI, a *P. viticola* species infected *V. riparia*, a second species infected *V. aestivalis* and *V. vinifera*, and a third one was found on *Parthenocissus quinquefolia*.

In contrast to the northeast, only one species was detected on grapevines in Florida (*Plasmopara viticola* clade *vinifera*). Compared with other *P. viticola* species that are confined to a restricted area, this species is, indeed, the most widely distributed geographically in North America, present from Canada to Florida. However, one should not lose sight of the fact that the geographical range of grapevine downy mildew species is strongly dependent on the host range of the pathogen and host plant distribution. The wide host range of *P. viticola* clade v*inifera,* with hosts widely distributed, may therefore explain why it has been able to spread over the whole of eastern North America.

The present study confirms previous results on the host range of grapevine downy mildew species (20) and describes their

TABLE 3. Cleaved amplified polymorphic sequence description for the deduction of *Plasmopara viticola* species (clades) based on the restriction profiles of internal transcribed spacer (*ITS*)*1* and tubulin sequences

															Restriction profile			
		ITS1 polymorphic sites													*ITS1*			β-Tubulin
Clade	Accession[v]	2	25	37	38	53	56	70	75	93	94	102	172	173	TfiI[w]	AseI[x]	XmnI[y]	HpyCH4V[z]
riparia	JF897779	T	A	T	A	C	T	T	C	C	T	T	T	G	a1	a1	a1	…
aestivalis	JF897780	T	A	C	T	T	C	G	C	T	T	T	C	G	a1	a1	a2	b1
vinifera	JF897781	A	C	C	T	T	C	T	C	C	A	A	C	G	a1	a2	a2	…
quinquefolia	JF897782	T	C	C	T	T	C	T	T	T	T	T	C	G	a2	a1	a2	…
vulpina	KF652198	T	A	C	T	T	C	T	C	T	T	T	C	A	a1	a1	a2	b2

[v] *ITS1* GenBank accession.
[w] Digestion fragments sizes: a1 = 71, 169; a2 = 240.
[x] Digestion fragments sizes: a1 = 240; a2 = 100, 140.
[y] Digestion fragments sizes: a1 = 101, 139; a2 = 66, 73, 101.
[z] Digestion fragments sizes: b1 = 75, 215, 229; b2 = 75, 101, 103, 229.

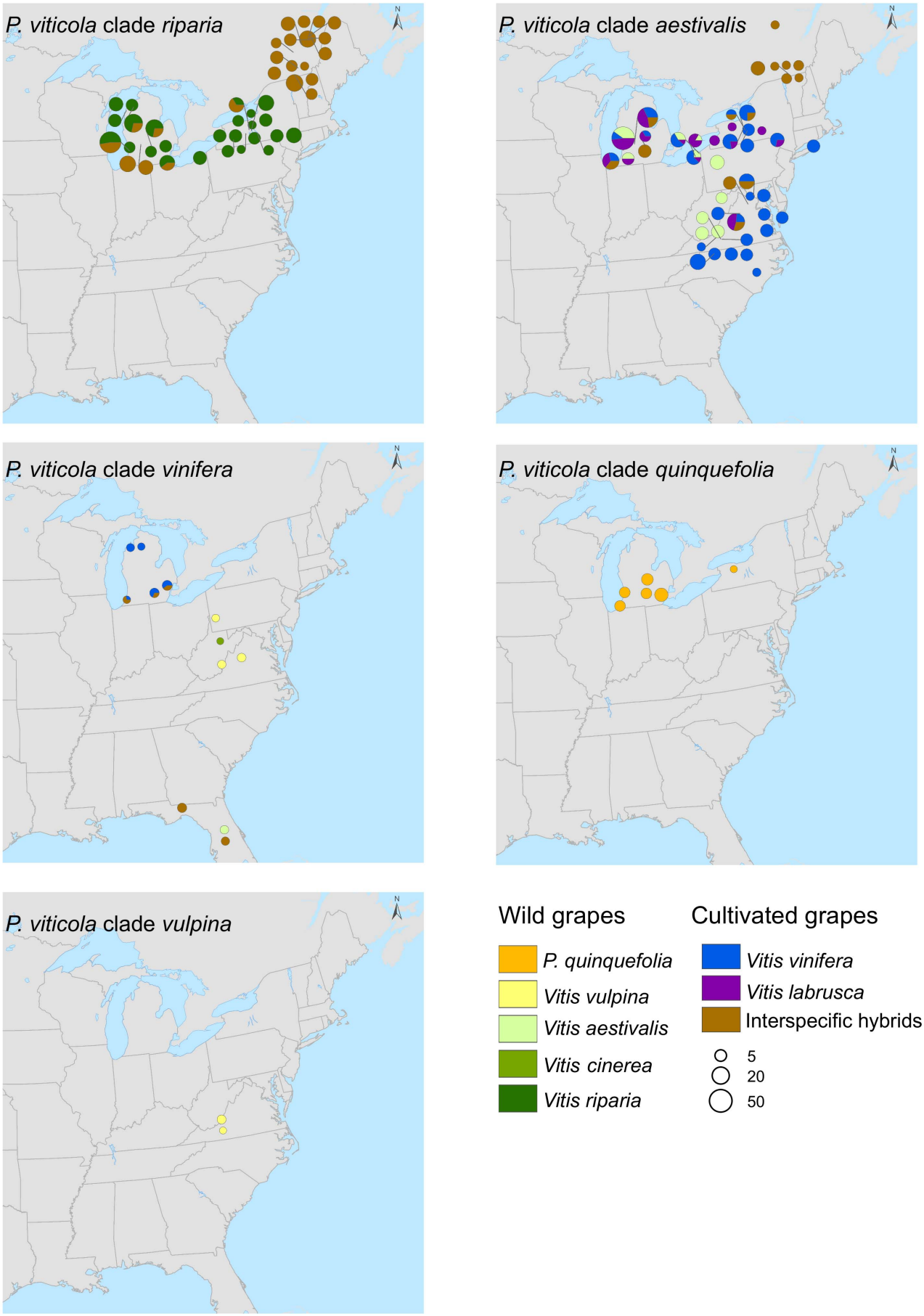

Fig. 3. Geographical range of the *Plasmopara viticola* species on their host-plants in northeastern North America. Circle sizes are proportional to number of isolates.

distribution on wild and cultivated grapevines at a larger geographical scale. We found a complete host plant specialization of the pathogen toward *Parthenocissus quinquefolia* and *V. riparia*, whereas *Plasmopara viticola* species found on *V. aestivalis*, *V. cinerea*, *V. vulpina, V. labrusca*, and *V. vinifera* exhibited a comparatively broader host range. Three different species of *P. viticola* were found to attack cultivated grapevines: the first one (*P. viticola* clade *riparia*) is only able to infect interspecific hybrids such as 'Chancellor', Marechal Foch, 'Vandal-Cliché', and 'Vignoles'. The second one (*P. viticola* clade *vinifera*) was found on *V. vinifera* cultivars and on hybrids. The third one (*P. viticola* clade *aestivalis*) has the widest host range, being able to infect *V. vinifera* cultivars, *V. labrusca* cultivars, and hybrids. It is worth noting that, although *V. vinifera* is susceptible to two *P. viticola* species, most of the *V. vinifera* vineyards (83%) were specifically attacked by *P. viticola* clade *aestivalis*. Therefore, this downy mildew species is likely to be the most important when the European (and therefore exotic in North America) species *V. vinifera* is planted into the center of origin for *P. viticola*.

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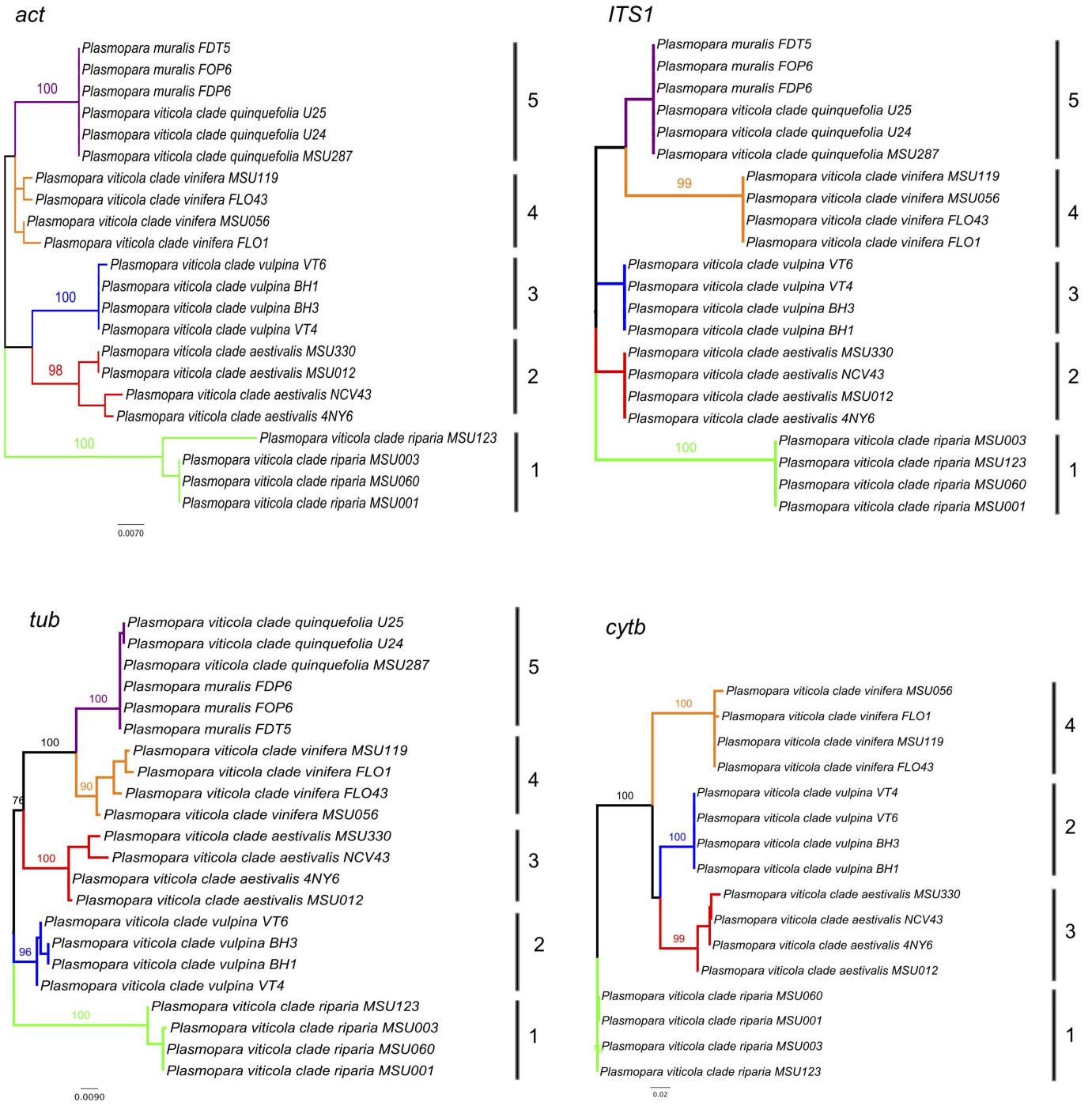

Fig. 4. Phylogenetic reconstruction maximum likelihood (ML) based on partial sequence data of internal transcribed spacer (*ITS)1*, actin (*act*), β-tubulin (*tub*), and cytochrome b (*cytb*). Numbers at branches indicate bootstrap support >70% in ML analysis. 1: *Plasmopara viticola* clade *riparia*; 2: *P. viticola* clade *aestivalis*; 3: *P. viticola* clade *vulpina*; 4: *P. viticola* clade *vinifera*; 5: *Plasmopara viticola* clade *quinquefolia* and *P. muralis*.

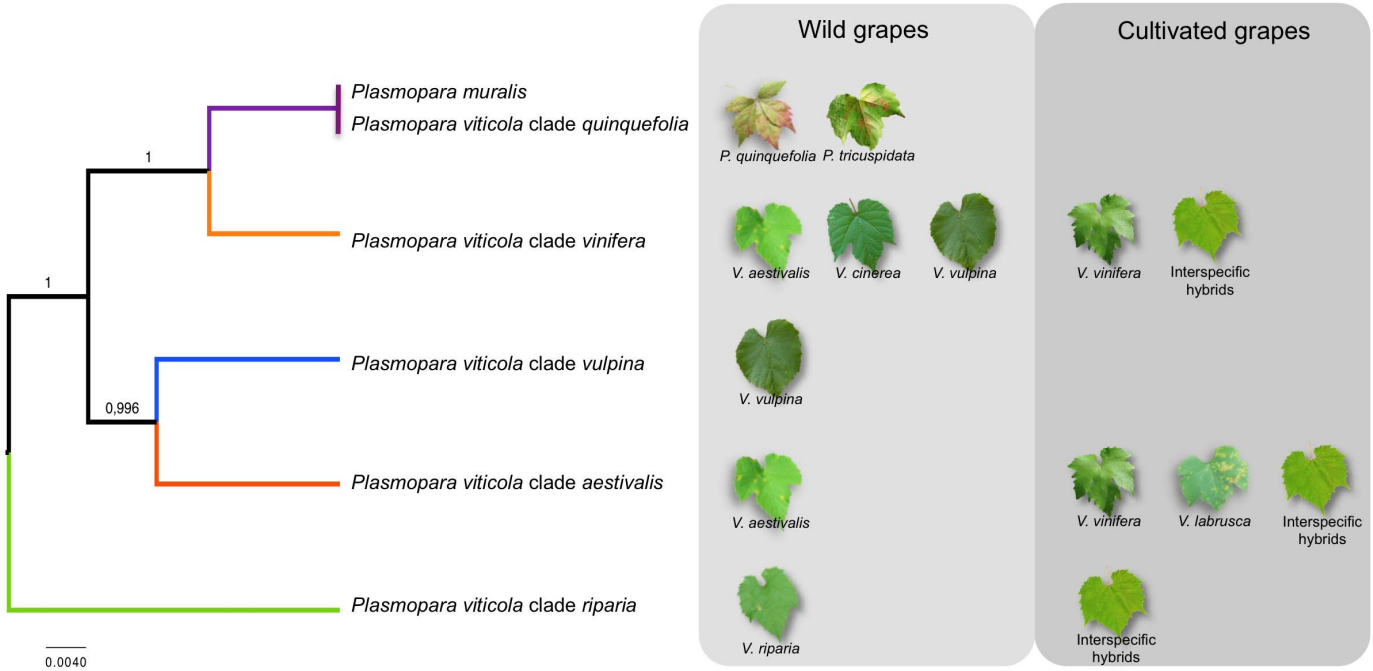

Fig. 5. Species tree of *Plasmopara viticola* species constructed using *BEAST based on the four partial sequences (internal transcribed spacer 1, β-tubulin, actin, and cytochrome b) from *P. viticola* and *P. muralis* isolates. Posterior probabilities of the branches are given in the tree. Leaf pictures indicate the source host plants for each *P. viticola* clade.

LITERATURE CITED

1. Bush & Son, and Meissner. 1883. Illustrated Descriptive Catalogue of American Grape Vines; A Grape Grower's Manual. R. P. Studley & Co., St. Louis.
2. Chen, W. J., Delmotte, F., Richard-Cervera, S., Douence, L., Greif, C., and Corio-Costet, M. F. 2007. At least two origins of fungicide resistance in grapevine downy mildew populations. Appl. Environ. Microbiol. 73:5162-5172.
3. Crous, P. W., and Groenewald, J. Z. 2005. Hosts, species and genotypes: opinions versus data. Australas. Plant Pathol. 34:463-470.
4. Delmotte, F., Chen, W. J., Richard-Cervera, S., Greif, C., Papura, D., Giresse, X., Mondor-Genson, G., and Corio-Costet, M. F. 2006. Microsatellite DNA markers for Plasmopara viticola, the causal agent of downy mildew of grapes. Mol. Ecol. Notes 6:379-381.
5. Fontaine, M., Austerlitz, F. T., Giraud, T., Labbé, F., Papura, D., Richard-Cervera, S., and Delmotte, F. 2013. Genetic signature of a range expansion and leap-frog event after the recent invasion of Europe by the grapevine downy mildew pathogen *Plasmopara viticola*. Mol. Ecol. 22:2771-2786.
6. Giraud, T., Gladieux, P., and Gavrilets, S. 2010. Linking the emergence of fungal plant diseases with ecological speciation. Trends Ecol. Evol. 25:387-395.
7. Giresse, X., Ahmed, S., Richard-Cervera, S., and Delmotte, F. 2010. Development of new oomycete taxon-specific mitochondrial cytochrome b region primers for use in phylogenetic and phylogeographic studies. J. Phytopathol. 158:321-327.
8. Gobbin, D., Bleyer, G., Keil, S., Kassemeyer, H. H., and Gessler, C. 2007. Evidence for sporangial dispersal leading to a single infection event and a sudden high incidence of grapevine downy mildew. Plant Pathol. 56:843-847.
9. Gobbin, D., Jermini, M., Loskill, B., Pertot, I., Raynal, M., and Gessler, C. 2005. Importance of secondary inoculum of *Plasmopara viticola* to epidemics of grapevine downy mildew. Plant Pathol. 54:522-534.
10. Gobbin, D., Pertot, I., and Gessler, C. 2003. Identification of microsatellite markers for *Plasmopara viticola* and establishment of high throughput method for SSR analysis. Eur. J. Plant Pathol. 109:153-164.
11. Gobbin, D., Pertot, I., and Gessler, C. 2003. Genetic structure of a *Plasmopara viticola* population in an isolated Italian mountain vineyard. J. Phytopathol. 151:636-646.
12. Gobbin, D., Rumbou, A., Linde, C. C., and Gessler, C. 2006. Population genetic structure of *Plasmopara viticola* after 125 years of colonization in European vineyards. Mol. Plant Pathol. 7:519-531.
13. Golovina, N. P. 1955. Sravnitelnaya karakteristika obrazov *Plasmopara viticola* Berl. et de Toni iz raznih stran. Botanicheskie materialy otdela sporovih rastenii, Botanicheskovo Instituta im. V. L. Komarova, izd. A. N. SSR 10:138-144.
14. Gouy, M., Guindon, S., and Gascuel, O. 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27:221-224.
15. Guindon, S., and Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52:696-704.
16. Kiss, L., Khosla, K., Jankovics, T., Niinomi, S., Braun, U., and Takamatsu, S. 2006. A morphologically ill-founded powdery mildew species, *Pleochaeta indica*, is recognized as a phylogenetic species based on the analysis of the nuclear ribosomal DNA sequences. Mycol. Res. 110:1301-1308.
17. Mougou, A., Dutech, C., and Desprez-Loustau, M. L. 2008. New insights into the identity and origin of the causal agent of oak powdery mildew in Europe. For. Pathol. 38:275-287.
18. Posada, D., and Crandall, K. A. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817-818.
19. Rambaut, A., and Drummond, A. J. 2007. Tracer v1.4: MCMC trace analyses tool. http://beast.bio.ed.ac.uk/Tracer
20. Rouxel, M., Mestre, P., Comont, G., Lehman, B. L., Schilder, A., and Delmotte, F. 2013. Phylogenetic and experimental evidence for host-specialized cryptic species in a biotrophic oomycete. New Phytol. 197:251-263.
21. Rouxel, M., Papura, D., Nogueira, M., Machefer, V., Dezette, D., Richard-Cervera, S., Carrere, S., Mestre, P., and Delmotte, F. 2012. Microsatellite markers for characterization of native and introduced populations of *Plasmopara viticola*, the causal agent of grapevine downy mildew. Appl. Environ. Microbiol. 78:6337-6340.
22. Rumbou, A., and Gessler, C. 2006. Particular structure of *Plasmopara viticola* populations evolved under Greek island conditions. Phytopathology 96:501-509.
23. Runge, F., Choi, Y.-J., and Thines, M. 2012. Phylogenetic investigations in the genus *Pseudoperonospora* reveal overlooked species and cryptic diversity in the *P. cubensis* species cluster. Eur. J. Plant Pathol. 129:135-146.
24. Schroder, S., Telle, S., Nick, P., and Thines, M. 2011. Cryptic diversity of *Plasmopara viticola* (Oomycota, Peronosporaceae) in North America. Org. Div. Evol. 11:3-7.
25. Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S., and Fisher, M. C. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet. Biol. 31:21-32.
26. Telle, S., Shivas, R. G., Ryley, M. J., and Thines, M. 2011. Molecular phylogenetic analysis of *Peronosclerospora* (Oomycetes) reveals cryptic

species and genetically distinct species parasitic to maize. Eur. J. Plant Pathol. 130:521-528.
27. Thines, M., Runge, F., Telle, S., and Voglmayr, H. 2010. Phylogenetic investigations in the downy mildew genus *Bremia* reveal several distinct lineages and a species with a presumably exceptional wide host range. Eur. J. Plant Pathol. 128:81-89.
28. Thines, M., Runge, F., Telle, S., and Voglmayr, H. 2011. Recent outbreaks of downy mildew on grape ivy (*Parthenocissus tricuspidata*, Vitaceae) in Germany are caused by a new species of *Plasmopara*. Mycol. Progr. 10:415-422.
29. Viennot-Bourgin, G. 1949. Les champignons parasites des plantes cultivées. Masson & Cie, Librairies de l'Académie de Médecine, Paris.