Genetic Variability of *Phyllosticta ampellicida*, the Agent of Black Rot Disease of Grapevine

Pietro A. Rinaldi, Donatella Paffetti,[†] Cecilia Comparini, Giovanni A. L. Broggin, Cesare Gessler, and Laura Mugnai

First, third, and sixth authors: Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente–Sezione Patologia Vegetale ed Entomologia, P. le delle Cascine 28, 50144 Florence, Italy; second author: Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente–Sezione Genetica Agraria, via Maragliano, 75-77, 50144 Florence, Italy; and fourth and fifth authors: Plant Pathology Institute of Integrative Biology, Swiss Federal Institute of Technology (IBZ) ETH Zurich Universitätstrasse, 2 8092, LFW–Zurich. Current address of G. A. L. Broggin: Molecular Plant Breeding, Institute of Agricultural Sciences, ETH Zurich, CH-8092 Zurich. Accepted for publication 31 May 2017.

ABSTRACT

Phyllosticta ampellicida causes black rot disease of *Vitis* spp. Genetic homogeneity of pathogen populations was investigated by analyzing the number of haplotypes present in infected samples from Europe and America. The fungus was identified from an analysis of the internal transcribed spacer (ITS)1-ITS2 region, and partial sequences of β -tubulin and calmodulin genes. The analysis of nuclear microsatellites applied to strains from *Vitis* spp. confirmed the existence of a high degree of genetic variability in the fungal populations, revealed four subpopulations, and showed that strains from

America are distinct from the European ones. Furthermore, the results obtained by landscape genetics showed that there were different introductions of the pathogen in the main vine areas of Europe, confirming what was observed in the first reports of the disease. The genetic variability of the fungus revealed by this study confirms the ability to generate new haplotypes by sexual reproduction. The difference found between the European populations and the American one confirms that the pathogen originated from America.

Phyllosticta ampellicida (Engelm.) Aa (syn. *Guignardia bidwellii*, following the recommendations of the International Commission on the Taxonomy of Fungi, Rossman et al. 2015) is the causal agent of black rot of grapevine, a devastating disease known throughout most of the grape-growing regions of the world. Disease symptoms can develop on all green tissues, forming cankers on the tendrils, leaf petioles, shoots, and rachis of the grapevine and round necrotic spots on the leaves (Molitor and Berkemann-Loehnertz 2011; Molitor and Beyer 2014; Wilcox and Hoffman 2015). Infected berries can mummify within 1 to 2 weeks or more after infection and their surfaces soon become covered by pycnidia and, later, pseudothecia of the fungus. Damage on the clusters can be limited to single berries or can lead to complete mummification of the whole bunch. The mummified berries typically remain firmly attached to the cluster. Grapevines are most susceptible from 1 to 5 weeks after flowering, depending on the weather conditions (Hoffman and Wilcox 2002; Molitor and Berkemann-Loehnertz 2011; Molitor and Beyer 2014).

The first reports of the disease were from Kentucky in the United States in 1805, when Michaux, during his travels, noticed symptoms of the disease on a farm near Lexington. In Europe, the pathogen was reported for the first time in France in 1885, in the upper Valley of Hérault, where vines from America had been introduced just 6 years before (Galet 1977). After this first introduction, the disease spread throughout Europe. The first report of the disease in Italy dates back to 1891 (Martelli 1891), when symptoms were reported only on the leaves. In the vine areas of the northeastern regions in Italy, it was assumed that the pathogen came from the neighboring Eastern European countries, probably from the former ex-Yugoslavia area, where the pathogen had been known since 1935 (Rui 1935). In 2002, black rot was recorded in Germany (Harms et al. 2005; Holz 2003), and the increasing spread worried the growers of Hungary (Mikulás et al. 1999) and, more recently, Romania (Tomoioaga and

Comsa 2010), Portugal, and Italy (Rinaldi et al. 2013). The concern raised by the increasing incidence of the disease recently led European researchers to set up disease support systems to better control it (Molitor et al. 2016; Onesti et al. 2016).

The pathogen is responsible for black rot disease of species in several genera of the Vitaceae family such as *Vitis*, *Ampelopsis*, *Cissus*, and *Parthenocissus* (Wikee et al. 2011). The fungus was hypothesized to be indigenous to America, where the native vine species underwent a selective process for resistance (Reddick 1911). Luttrell (1946, 1948) demonstrated the existence of different forms of “*G. bidwellii*” based on morphological differences and differences in pathogenicity on ‘Muscadine’ grape (*Vitis rotundifolia* subgenus *Muscadinia*), *V. vinifera*, *Parthenocissus* spp., and American native grape. The form *muscadinii*, which mainly infects *V. rotundifolia*, causes primarily a disease of the vegetative parts, appearing as superficial scabs or lesions on the berries, and it can infect *V. vinifera* as well. The other two forms detected were *P. ampellicida* f. *euveitis*, pathogenic to *V. vinifera* and American species, and *P. ampellicida* f. *parthenocissi*, a pathogen of *Parthenocissus* spp. A study carried out by Zhang et al. (2013) demonstrated different species in the *P. ampellicida* complex, proposing *P. parthenocissi* as a distinct species based on phylogenetic and morphological data.

Several reasons could be hypothesized for the recent outbreaks of the disease. It is possible that strains resistant to the commonly used fungicides are naturally selected; transport of infected material from one vine-growing region to another could favor the introduction of strains more suited to the new environment; and multiplication of the fungus by both sexual and asexual means makes it particularly able to adapt to various environmental conditions. Another aspect possibly related to the recent increase in the spread of the disease is the recent changes in the management of powdery and downy mildew chemical control. Some of the chemicals used are also effective against black rot but, with the increased sensitivity toward the environment and the increasingly restrictive legislation of the European Union toward the use of pesticides, the application strategies have changed. Some broad-spectrum pesticides such as dithiocarbamates, which are very effective against *P. ampellicida*, are less used today, and application is

[†]Corresponding author: D. Paffetti; E-mail: donatella.paffetti@unifi.it

carried out more and more specifically on powdery and downy mildew risk stages. These intervention strategies may have little or no effect on black rot. Therefore, conditions suitable for spread of the fungus in the most sensitive phenological stages of the vine can have drastic effects. Lack of knowledge of the typical symptoms and wrong diagnosis will increase the effect, as suggested by Gessler et al. (2006, 2008), and was ascertained in the recent outbreak in Italy (Rinaldi and Mugnai 2012).

Therefore, we investigated the genetic homogeneity of pathogen populations by analyzing the number of haplotypes present in infected samples from Europe and America. Analysis of the internal transcribed spacer (ITS)1-ITS2 region, β -tubulin, and calmodulin sequences was

used to identify the pathogen (Wicht et al. 2012). Finally, microsatellites (nuclear simple-sequence repeat [nSSR]) were used to assess the genetic variability of the fungus and to understand the migration flows of the pathogen and determine the genetic distance between the populations from different vine areas. Spatial analysis was also applied to assess genetic diversity of a *P. ampellicida* population in a single vineyard.

MATERIALS AND METHODS

Sampling, fungal isolation, and DNA extraction. Isolations were made from leaf necrotic spots, cane cankers, mummified

TABLE 1. *Phyllosticta ampellicida* strains used in pathogen identification analysis

ID	Provenance	Host (cultivar)	GenBank accession number		
			ITS ^a	β -Tubulin	Calmodulin
G1.2	Mercatale (Tuscany, Italy)	<i>Vitis vinifera</i> L. (Sangiovese)	KF015253	KF831472	...
G1.6	Mercatale (Tuscany, Italy)	<i>V. vinifera</i> L. (Sangiovese)	KF015254	KF831473	KF831428
G1.7	Mercatale (Tuscany, Italy)	<i>V. vinifera</i> L. (Sangiovese)	KF015255	KF831474	KF831429
G1.8	Grassina (Tuscany, Italy)	<i>V. vinifera</i> L. (Sangiovese)	KF015256	KF831475	KF831430
G1.10	Grassina (Tuscany, Italy)	<i>V. vinifera</i> L. (Sangiovese)	KF015257	KF831476	KF831431
G1.12	San Casciano (Tuscany, Italy)	<i>V. vinifera</i> L. (Sangiovese)	KF015258	KF831477	KF831432
G1.13	San Gimignano (Tuscany, Italy)	<i>V. vinifera</i> L. (Sangiovese)	KF015259	KF831478	KF831433
G1.14	San Gimignano (Tuscany, Italy)	<i>V. vinifera</i> L. (Vernaccia)	KF015260	KF831479	KF831434
G1.15	Quarrata (Tuscany, Italy)	<i>V. vinifera</i> L. (Merlot)	KF015261	KF831480	KF831435
G1.16	Quarrata (Tuscany, Italy)	<i>V. vinifera</i> L. (Colorino)	KF015262	KF831481	KF831436
G1.17	Florence (Tuscany, Italy)	<i>V. vinifera</i> L. (Sangiovese)	KF015263	KF831482	KF831437
G1.21	Florence (Tuscany, Italy)	<i>V. vinifera</i> L. (Colorino)	KF015264	KF831483	KF831438
G1.29	Rauscedo (Friuli-Venezia Giulia, Italy)	<i>V. vinifera</i> L. (Merlot)	KF015265	KF831484	KF831439
G1.30	Rauscedo (Friuli-Venezia Giulia, Italy)	<i>V. vinifera</i> L. (Merlot)	KF015266	KF831485	KF831440
G1.31	Rauscedo (Friuli-Venezia Giulia, Italy)	<i>V. vinifera</i> L. (Merlot)	KF015267	KF831486	...
G1.34	Vezzano (Liguria, Italy)	<i>V. vinifera</i> L.	KF015268	KF831487	KF831441
G1.35	Vezzano (Liguria, Italy)	<i>V. vinifera</i> L.	KF851288	KF831488	KF831442
SS01	Oliena (Sardinia, Italy)	<i>V. vinifera</i> L.	KF851289	KF831489	KF831443
SS02	Sorso (Sardinia, Italy)	<i>V. vinifera</i> L.	KF851290	KF831490	KF831444
Piem7/12	Unknown (Piedmont, Italy)	<i>V. vinifera</i> L.	KF851291	KF831491	KF831445
TVac1	Treviso (Veneto, Italy)	<i>V. vinifera</i> L. (Merlot)	KF851292	KF831492	KF831446
TVac2	Treviso (Veneto, Italy)	<i>V. vinifera</i> L. (Merlot)	KF851293	KF831493	KF831447
Lomb5	Traona (Lombardia, Italy)	<i>V. vinifera</i> L.	KF851294	KF831494	KF831448
Lomb7	Traona (Lombardia, Italy)	<i>V. vinifera</i> L.	KF851295	KF831495	KF831449
Lomb8	Traona (Lombardia, Italy)	<i>V. vinifera</i> L.	KF851296	KF831496	KF831450
POR2	Lisboa (Lisboa, Portugal)	<i>V. vinifera</i> L.	KF851297	KF831497	KF831451
POR11	Lisboa (Lisboa, Portugal)	<i>V. vinifera</i> L.	KF851298	KF831498	KF831452
POR12	Lisboa (Lisboa, Portugal)	<i>V. vinifera</i> L.	KF851299	KF831499	KF831453
POR19	Lisboa (Lisboa, Portugal)	<i>V. vinifera</i> L.	KF851300	KF831500	KF831454
HUNGI10.2	Dula (Eger, Hungary)	<i>V. vinifera</i> L. (Kékfrancos)	KF851301	KF831501	KF831455
HUNGI15.3	Dula (Eger, Hungary)	<i>V. vinifera</i> L. (Kékfrancos)	KF851302	KF831502	KF831456
HUNGI17.3	Dula (Eger, Hungary)	<i>V. vinifera</i> L. (Kékfrancos)	KF851303	KF831503	KF831457
GM05	Kindel (Mosel, Germany)	<i>V. vinifera</i> L. (Muller-Thurgau)	KF851304	KF831504	KF831458
12Li6	Wolf (Mosel, Germany)	<i>V. vinifera</i> L.	KF851309	KF831509	KF831463
FRA 6.2	Rosieres (Midi-Pirenei, France)	<i>V. vinifera</i> L. (Gamay)	KF851305	KF831505	KF831459
FRA10.1	Rosieres (Midi-Pirenei, France)	<i>V. vinifera</i> L. (Gamay)	KF851306	KF831506	KF831460
LUX3EC	Ehnen (Luxemburg)	<i>V. vinifera</i> L.	KF851308	KF831508	KF831462
LUX3ES	Ehnen (Luxemburg)	<i>V. vinifera</i> L.	KF851307	KF831507	KF831461
VirginiaG1A1	Upperville (Virginia)	<i>V. vinifera</i> L. (Cabernet Sauvignon)	KF851310	KF831510	KF831464
VirginiaG8A1	Upperville (Virginia)	<i>V. vinifera</i> L. (Cabernet Sauvignon)	KF851311	KF831511	KF831465
VirginiaG8A2	Upperville (Virginia)	<i>V. vinifera</i> L. (Cabernet Sauvignon)	KF851312	KF831512	KF831466
Pensyl 1	Northeast (Pennsylvania)	<i>V. labrusca</i> L. (Concord)	KF851313	KF831513	KF831467
Pensyl 2	Northeast (Pennsylvania)	<i>V. labrusca</i> L. (Concord)	KF851314	KF831514	KF831468
Pensyl 6	Northeast (Pennsylvania)	<i>V. labrusca</i> L. (Concord)	KF851315	KF831515	KF831469
Pensyl 8	Northeast (Pennsylvania)	<i>V. labrusca</i> L. (Concord)	KF851316	KF831516	KF831470
Pensyl 13	Northeast (Pennsylvania)	<i>V. labrusca</i> L. (Concord)	KF851317	KF831517	KF831471
Sb9.5	Salleboeuf (Aquitaine, France)	<i>V. vinifera</i> L. (Merlot)	...	...	GU991587
Sb22.6	Salleboeuf (Aquitaine, France)	<i>V. vinifera</i> L. (Merlot)	...	GU991580	...
Sb25.7	Salleboeuf (Aquitaine, France)	<i>V. vinifera</i> L. (Merlot)	...	GU991581	GU991586
Sb28.3	Salleboeuf (Aquitaine, France)	<i>V. vinifera</i> L. (Merlot)	...	HM065512	...
Ti18.5.1	Cugnasco (Ticino, Switzerland)	<i>V. vinifera</i> L. (Chamboucin)	...	GU991579	...
Ti34.1.8	Cugnasco (Ticino, Switzerland)	<i>V. vinifera</i> L. (Isabella)	...	GU991582	...
ATCC200578	Unknown (New York)	<i>V. riparia</i> Michx.	KC193586	...	...
CBS111645	Unknown (United States)	<i>Parthenocissus quinquefolia</i> (L.) Planch., 1887	FJ824766	FJ824777	...
MUCC0019	Unknown (Aichi, Japan)	<i>P. tricuspidata</i> (Siebold & Zucc.) Planch.	AB454268	...	...
MUCC0120	Unknown (Aichi, Japan)	<i>P. tricuspidata</i> (Siebold & Zucc.) Planch.	AB454313	...	...
MUCC0037	Unknown (Tokyo, Japan)	<i>P. tricuspidata</i> (Siebold & Zucc.) Planch.	AB454276	...	...

^a Internal transcribed spacer.

Fig. 1. Aerial view of the vineyard in Florence divided into 90 plots. From each plot, 1 infected cluster was collected and 25 of them (those whose number is enclosed in a circle) were analyzed with nuclear microsatellites. Map obtained from Google Earth.

berries, and shriveled berries. The samples were collected in 2011 and 2012 in all of the central and northern Italian grapevine areas in which the disease was reported, in the main European countries where the disease is usually present, and in two U.S. states (Virginia and Pennsylvania) (Table 1). Only the samples from Pennsylvania are from *V. labrusca*; all of the others are from *V. vinifera* of international and local varieties. Samples were disinfected by immersion in 10% hydrogen peroxide for few seconds, followed by two washes with distilled water. The tissues were sectioned and placed on malt agar (MA) and left for 15 to 20 days at room temperature. Monohyphal isolates were obtained from actively growing colonies in the following way. A small sample of the pathogen mycelium was scraped from the surface of a colony grown on MA and placed in a sterilized 2-ml Eppendorf tube, and 1.5 ml of sterile distilled water added. The tube was vortexed for 2 min and 30 µl of the suspension was spread over a plate of water agar. A single-hyphal portion was selected and marked. When the colony was big enough to be visible, it was transferred to another MA dish. DNA was extracted from the mycelium with DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

A second sampling was carried out in a 35-year-old vineyard (5,000 m²) growing on the hill of Montici, close to Pian De' Giullari, in the surroundings of Florence, Italy and planted with 'Sangiovese' and 'Trebiano' grape. Heavy damage by *P. ampellicida* had been recorded in 2011, with 100% of the clusters in both cultivars completely dried up. The vineyard was divided into 90 rectangular plots (11 by 3 m each). Each plot included three rows (Fig. 1). In each plot, a mummified cluster, chosen at random, was collected from the middle row and stored at 4°C. In all, 25 plots were selected at random although taking care to cover the entire surface of the vineyard, and

TABLE 2. *Phyllosticta ampellicida* strains used in nuclear microsatellites analysis

ID	Provenance	Host (cultivar)	Number of samples
A	Florence (Tuscany, Italy)	<i>Vitis vinifera</i> L. (Sangiovese)	148
G	Florence (Tuscany, Italy)	<i>V. vinifera</i> L. (Trebiano)	4
B	San Gimignano (Tuscany, Italy)	<i>V. vinifera</i> L. (Sangiovese)	9
C	San Gimignano (Tuscany, Italy)	<i>V. vinifera</i> L. (Vernaccia)	9
D	Grassano (Tuscany, Italy)	<i>V. vinifera</i> L.	11
E	San Casciano (Tuscany, Italy)	<i>V. vinifera</i> L. (Sangiovese)	6
F	Strada in Chianti (Tuscany, Italy)	<i>V. vinifera</i> L. (Trebiano)	7
H	Mercatello (Tuscany, Italy)	<i>V. vinifera</i> L. (Sangiovese)	5
I	Vinci (Tuscany, Italy)	<i>V. vinifera</i> L.	2
J	Rauscedo (Friuli-Venezia Giulia, Italy)	<i>V. vinifera</i> L. (Merlot)	7
K	Farra d'Isonzo (Friuli-Venezia Giulia, Italy)	<i>V. vinifera</i> L. (Pinot Grigio)	4
L	Riomaggiore (Liguria, Italy)	<i>V. vinifera</i> L.	11
M	Vezzano (Liguria, Italy)	<i>V. vinifera</i> L.	25
N	Sarzana (Liguria, Italy)	<i>V. vinifera</i> L.	15
O	Calizzano (Liguria, Italy)	<i>V. vinifera</i> L.	3
P	Teglio (Lombardia, Italy)	<i>V. vinifera</i> L.	4
Q	Traona (Lombardia, Italy)	<i>V. vinifera</i> L.	4
R	Balzarro (Lombardia, Italy)	<i>V. vinifera</i> L.	3
S	Lugnacco (Piedmont, Italy)	<i>V. vinifera</i> L. (Erbaluce)	4
T	Mazzè (Piedmont, Italy)	<i>V. vinifera</i> L. (Erbaluce)	4
U	Romano Canavese (Piedmont, Italy)	<i>V. vinifera</i> L. (Erbaluce)	4
V	Domodossola (Piedmont, Italy)	<i>V. vinifera</i> L. (Erbaluce)	4
W	Treviso (Veneto, Italy)	<i>V. vinifera</i> L. (Merlot)	3
X	Oliena (Sardinia, Italy)	<i>V. vinifera</i> L.	1
Y	Sorso (Sardinia, Italy)	<i>V. vinifera</i> L.	1
Z	Rosieres (Midi-Pyrénées, France)	<i>V. vinifera</i> L. (Gamay)	23
AA	Ehnen (Luxembourg)	<i>V. vinifera</i> L.	5
AB	Lobão da Beira (Aveiro, Portugal)	<i>V. vinifera</i> L.	14
AC	Lisboa (Lisboa, Portugal)	<i>V. vinifera</i> L.	2
AD	Sabugosa (Tondela, Portugal)	<i>V. vinifera</i> L. (Touriga Nacional)	17
AE	Pècs (Baranya, Hungary)	<i>V. vinifera</i> L.	6
AF	Dula (Eger, Hungary)	<i>V. vinifera</i> L. (Blauburger)	4
AG	Dula (Eger, Hungary)	<i>V. vinifera</i> L. (Kékfrancos)	4
AH	Demjen (Eger, Hungary)	<i>V. vinifera</i> L. (Leányka)	4
AI	Demjen (Eger, Hungary)	<i>V. vinifera</i> L. (Zenit)	3
AJ	Unknown (Mosel, Germany)	<i>V. vinifera</i> L.	7
AK	Upperville (Virginia)	<i>V. vinifera</i> L. (Cabernet Sauvignon)	34

the corresponding 25 clusters were used for further analysis. Isolations and DNA extraction were as reported above.

Polymerase chain reaction amplification and sequencing. The ITS1-ITS2 region, fragment of the β -tubulin gene, and fragment of the calmodulin gene were amplified with the primers ITS4 and ITS5 (White et al. 1990), Bt2a and Bt2b (Glass and Donaldson 1995), and CL1 and CL2A (O'Donnell et al. 2000), respectively. Reaction conditions consisted of 0.5 μ l of 10 μ M dNTP, 0.25 μ l of 1 μ M each primer, 2.5 μ l of 10 $\times$ DreamTaq Green Buffer (Thermo Fisher Scientific Inc.), 0.25 μ l of DreamTaq DNA Polymerase (5 U/ μ l) (Thermo Fisher Scientific Inc.), 3 μ l of DNA, and water to a final volume of 25 μ l. Polymerase chain reaction (PCR) cycling parameters for the ITS1-ITS2 region and fragment of the β -tubulin gene were 3 min of initial denaturation at 94°C; 35 cycles of 95°C for

30 s and annealing for 30 s at 50°C, extended at 72°C for 30 s; and a final 7-min extension at 72°C. PCR cycling parameters for the calmodulin gene were 5 min of initial denaturation at 94°C; 35 cycles of 94°C for 50 s and annealing for 50 s at 55°C, extended at 72°C for 1 min; and a final 7-min extension at 72°C. Amplification products were detected on agarose gels (1% w/vol) stained with ethidium bromide in 1 $\times$ Tris-acetate EDTA buffer and purified using ExoSAP-IT (USB Corporation), according to the manufacturer's protocol.

Amplification products were sequenced at the Centre for Biotechnological Services (CIBIACI) of the University of Florence with an ABI Prism 310 CE. Direct sequencing of amplified DNA was done in both directions from independent amplification reactions as above, with the necessary modifications for direct PCR sequencing.

Fig. 2. A, Internal transcribed spacer (ITS) maximum-likelihood tree; **B**, β -tubulin maximum-likelihood tree; and **C**, calmodulin maximum-likelihood tree of *Phyllosticta ampellicida* strains from different hosts of the family Vitaceae. Bootstrap values at the nodes. **D**, Maximum-likelihood tree resulting from the concatenated ITS, β -tubulin, and calmodulin dataset.

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Pathogen identification. Nucleotide sequences of *P. ampellicida* isolates from *V. vinifera*, *V. lambrusca*, *Parthenocissus quinquefolia*, and *P. tricuspidata*, as well as *Neofusicoccum parvum* (used as outgroup), were retrieved from GenBank. BLAST probing of the

DNA databases was performed with the BLASTN option of the BLAST program (Altschul et al. 1997). Sequences were aligned with CLUSTAL W (Thompson et al. 1994). Each alignment was checked and corrected manually when necessary. The dendrograms

Fig. 2. Continued from previous page.

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Fig. 2. Continued from previous page.

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were constructed by the maximum-likelihood method (Felsenstein and Churchill 1996) using PHYLIP software (version 3.695) by Joseph Felsenstein.

Microsatellite analyses. The samples used for *n*SSR analysis are listed in Table 2 and primer pairs are as reported by Narduzzi-Wicht et al. (2014). PCR amplification was performed in 5- μ l reactions containing 2.5 μ l of 2 $\times$ Qiagen Multiplex, 0.5 μ l of both forward and reverse primers, 0.5 μ l of 5 $\times$ Q-Sol, 0.5 μ l of double-distilled H₂O, and 1 μ l of DNA. PCR cycle parameters were 15 min of initial denaturation at 95°C; 37 cycles of 94°C for 40 s and annealing for 90 s at 55°C, extended at 72°C for 90 s; and a final 30-min extension at 60°C. Amplicons for the SSR analysis were eluted at a rate of 1:50 and sequenced with a 3730 Genetic Analyzer (Life Technologies, Applied Biosystems); genotyping was performed with Gene Mapper (v. 4.0; Life Technologies, Applied Biosystems).

Microsatellite data analyses. General estimates of genetic diversity from *n*SSR data were calculated using SPAGeDi 1.3a (Hardy and Vekemans 2002): number of alleles, allele frequency, and genetic diversity, according to Nei (1987). Statistical significance was determined by jack-knifed estimators (Sokal and Rohlf 1995) after 20,000 permutations. Wright's fixation index, averaged over all loci, was calculated, and deviations from Hardy-Weinberg expectations were determined using GENEPOP 3.3 (Raymond and Rousset 1995). *F* statistics and null alleles were calculated according to Weir

and Cockerham (1984). Pairwise comparisons of fixation index (F_{ST}) values were tested for significance, and critical values were adjusted for multiple tests with the Bonferroni correction. To examine the relationship between the genetic distance and the geographic distance, a Mantel test on the matrix of F_{ST} values and that of the geographic distances was performed (50,000 permutations) using GENEPOP 3.3.

Population structure was inferred using a model-based clustering algorithm implemented in STRUCTURE v.2.3 (Falush et al. 2003, 2007; Pritchard et al. 2000) and the empirical statistic *K* (Earl and vonHoldt 2012; Evanno et al. 2005) to determine the number of subpopulations (*K*). The default model parameters and varying *K* from 1 to 10 were used for both plots to run STRUCTURE. Each run consisted of 250,000 burn-in iterations and 1,000,000 data collection iterations, and was replicated 20 times.

Spatial subpopulation structure was inferred by a Bayesian Monte Carlo Markov Chain method implemented in Geneland package (version 3.0) (Guillot et al. 2009) under the R Language and Environment for Statistical Computing software, as described by Guillot et al. (2005 a,b, 2008). Ten independent Monte Carlo Markov Chain runs were performed with the following settings: 1,000,000 iterations with 100 thinning intervals and a burn-in period of 250,000, using the correlated allele frequencies model. The maximum number of populations was set to 20. A map of posterior probabilities (membership) was obtained by Post-Process

Fig. 2. Continued from previous page.

Chain and Post Tessellation functions into Geneland by tesselling the landscape at a resolution of 1 m. Null alleles for *n*SSR were calculated by Geneland (version 3.0) (Guillot et al. 2008).

RESULTS

Pathogen identification. Maximum-likelihood trees were constructed for the ITS region, β -tubulin, and calmodulin sequences with sequence lengths after alignment of 628, 333, and 592 bp, respectively. A fourth tree was prepared from concatenated sequences with total length of 1,524 bp.

In the ITS tree, all strains of *Phyllosticta ampellicida* from *Vitis* spp. clustered in a single cluster, whereas the strains from other hosts (*Parthenocissus quinquefolia* and *P. tricuspidata*) formed a separate cluster (Fig. 2A). In the tree, the samples studied all grouped in a single cluster in all dendrograms, confirming that all strains belong to *Phyllosticta ampellicida* (Fig. 2B, C, and D).

nSSR analysis. Among 421 isolates analyzed, the *n*SSR analysis allowed the detection of 56 haplotypes. Five haplotypes comprised 59.38% of the whole population. Haplotype 9 (H9) was found in 111 samples (26.37%), H38 was found in 58 samples (frequency

13.78%), and H14 was present in 35 samples (frequency 8.31%). Both H29 and H55 were found in 23 samples (frequency = 5.46%). Of the remaining haplotypes, only nine (H5, H10, H13, H19, H23, H30, H39, H45, and H46) reached frequencies greater than 1%, found in 5 to 9 samples. The remaining 42 haplotypes (75% of the total) resulted in a frequency lower than 1%, meaning that almost half of the haplotypes were found from one to four times. All American haplotypes were unique and no haplotype shared with European populations was found (Table 3). H9 and H38 are the haplotypes most widespread in Europe and both were found in 7 of the 14 localities sampled. Despite the high number of samples collected in Tuscany, H38 was not found there but it did occur in Friuli, Veneto, Piedmont, France, Germany, Portugal, and Hungary; whereas H9 was found in Italy in all Tuscan localities, and in Liguria, Lombardy, Sardinia, France, and Portugal. A relevant percentage of unique haplotypes (42.42%) was found in the Tuscan population, which comprised 152 samples from Florence and 49 from the rest of the region (total = 201). High numbers of unique haplotypes were found among the Liguria and the Luxembourg populations, where four of nine (Liguria), and all five (Luxemburg) samples were unique.

Spatial structure of genetic diversity. The spatial structure analysis of genetic diversity in *P. ampellicida* populations identified four subpopulations. The log-likelihoods from runs of STRUCTURE with K of 1 to 20 had a clear peak at K = 4 (Fig. 3) and were spatially explicit in clustering analyses using GENELAND (Fig. 4, European distribution). The U.S. subpopulation (named cluster A) was spatially isolated from each subpopulation, as demonstrated by maps of posterior probabilities (Fig. 4). Moreover, it was genetically isolated, as demonstrated by high values of F_{ST} and D_s , but it presents the highest level of genetic diversity among strains (Table 4). The European subpopulations were not spatially isolated and presented a significantly similar genetic diversity among strains (Table 4). The subpopulation named cluster A appears to be the most genetically isolated, as shown by F_{ST} and D_s . In contrast, subpopulations D and C were more similar (Table 4).

Microspatial structure of genetic diversity in a vineyard. The spatial structure analysis of genetic diversity in a 35-year-old vineyard (5,000 m²) growing in the surroundings of Florence, Italy (Sangiovese and Trebbiano grape) identified three subpopulations. The log-likelihoods from runs of STRUCTURE with K of 1 to 20 had a clear peak at K = 3 (data not shown) and were spatially explicit in clustering analyses using GENELAND (Fig. 5). No gene flow was

TABLE 3. Number of unique and shared haplotypes and vine regions in which they were found

Locality	Samples	Haplotypes		
		Total	Unique	Shared
Italy				
Tuscany	201	28	16	12
Friuli	11	1	0	1
Liguria	54	9	5	4
Lombardy	11	3	0	3
Piedmont	16	2	0	2
Sardinia	2	2	1	1
Veneto	3	1	0	1
France	23	10	2	8
Germany	7	4	0	4
Luxembourg	5	5	4	1
Portugal	33	6	1	5
Hungary	21	1	0	1
United States				
Virginia	34	13	13	0

Fig. 3. Population substructure detected using the STRUCTURE. Left side indicates average log-likelihoods from 20 program runs (see Materials and Methods) for each assumed number of subpopulations (K), and error bars correspond to 1 standard deviation. Right side indicates values of the ad hoc statistic ΔK , which is based on the rate of change of the log-likelihood as K is increased (Evanno et al. 2005). ΔK tends to peak at the value of K that corresponds to the highest level of hierarchical substructure.

detected among strains belonging to the three different clusters but there was gene flow within each cluster. Actually, the F_{ST} and genetic diversity values among clusters were very high (Table 5). In contrast, genetic analyses of the population of the pathogen present in a single small vineyard confirmed the very high genetic variability of the pathogen, with the presence of up to 24 different haplotypes within the selected plots.

DISCUSSION

The present study, although it was not focused on the detection of different species within the *P. ampellicida* complex, confirms the genetic variability of the fungus and assigns the pathogen isolated from infected samples showing typical black rot symptoms to *P. ampellicida*.

The use of microsatellites (*n*SSR) confirmed the presence of a high degree of genetic variability in this fungus. Among 421 berries analyzed from a worldwide set of infected samples, 56 haplotypes were found. This number and the low value of their frequencies indicate that sexual reproduction of the fungus is important in the diffusion of the disease and its evolution. The main part of the population investigated belonged to the same five haplotypes, whereas 40 of the 56 were unique and only 16 were shared. Among the latter, H55, found in Florence and shared with the rest of the region, could be considered typical of this area. H9 and H38, as well as being the most frequent, were also the two most shared, with the first one, H9, strongly present in Tuscany, Sardinia, and Portugal but not in the northeastern Italian regions; while the second one, H38, is present in the other European countries and in the northeast of Italy. Considering that the DNA analyzed in this study was extracted from mummified berries and considering such a great spread of different

Fig. 4. Results of Geneland analysis by nuclear microsatellite data of the *Phyllosticta ampellicida* European populations showing the spatial organization into three subpopulations (B, C, and D) and maps of posterior probabilities for each subpopulation (black indicates low gene flow and white indicates high gene flow). Map obtained from Google Earth.

TABLE 4. Genetic diversity (*D*) within four subpopulations, and matrix of pairwise F_{ST} among the subpopulations (above diagonal) and genetic divergence (D_s) among subpopulation (below diagonal) in the *Phyllosticta ampellicida* population

Subpopulations	A (<i>D</i> = 0.480)	B (<i>D</i> = 0.250)	C (<i>D</i> = 0.268)	D (<i>D</i> = 0.257)
A	...	0.618	0.547	0.618
B	1.654	...	0.241	0.258
C	1.332	0.114	...	0.137
D	1.420	0.126	0.057	...

haplotypes, we can deduce that infection of berries by ascospores is far more probable than by conidia. The wide variety of haplotypes found on berries suggests that infections by sexual spores is an important stage in the beginning of the epidemic and we can speculate that secondary infections derived from pycnidia following the development of leaf symptoms do not play an important role in the disease on bunches, as suggested by Jermini and Gessler (1996) on the basis of field observations.

The microsatellite analysis demonstrates that all haplotypes are divided into four main subpopulations (Figs. 3 and 4): one comprising the American haplotypes; a second group comprising a Portuguese subpopulation; another group comprising the Italian and French subpopulations; and the last one made up of the rest of the European populations, part of the Portuguese haplotypes, and those from northeastern Italy. This geographical grouping is in agreement with what was previously stated. The distinction of the American strains is confirmed, while the haplotypes nearer to them are from Italy (Tuscany and Sardinia). Within these two groups (American and European), the high “mean diversity population” value shows that variability is highest within the investigated population. This close relationship could even suggest the hypothesis that the recent outbreak of the disease in Sardinia could be caused by a recent introduction of strains of the pathogen from America, whereas the high variability recorded in Tuscany suggests that either there were multiple introductions or the pathogen was in the area for quite a long time.

The presence of some American haplotypes (A) in France is in accordance with the first observations of the disease in Europe. The disease was first seen in France following the introduction of American vines, confirming the hypothesis that the disease was introduced from North America. The other three subpopulations indicate that three different introductions of the pathogen occurred in the different European vine areas. Subpopulation B, present in France and Tuscany, confirms the observations by Martelli (1891) of the disease in Tuscany 6 years after the first records in France, suggesting that the disease arrived in Italy from France. The

presence in Portugal of two subpopulations (B and C) suggests two separate introductions of the pathogen, one from France or Italy (subpopulation B) and a second from America. Population D, found in a wide vine area comprising Central Europe, Eastern Europe, and northeast Italy, is in accordance with the observations of the disease in Germany (Lüstner 1935) and ex-Yugoslavia (Rui 1935), for the first time, during the 1930s. It is known that the introduction of the disease in the northeast of Italy was from ex-Yugoslavia (Rui et al. 1987) at the end of the 1980s. In Hungary, the disease could have been introduced in 1999 from Germany or ex-Yugoslavia (Mikulás et al. 1999).

The presence of haplotypes shared between Italy and the rest of Europe confirms that the pathogen has been present in Italy for a long time, whereas the large number of haplotypes (38) found in the northeast of Italy and in Hungary confirms the introduction of the pathogen from Eastern Europe to Italy. The high rate of different haplotypes in the total samples obtained from France (52.17%) strongly suggests that the fungus arrived in Europe for the first time in France. The American origin of the pathogen is underlined by the same high rate (38.23%) of haplotype variability found in Virginia, as expected in the land of origin.

Analysis of the microspatial structure of genetic diversity within a vineyard suggests that sexual reproduction of the pathogen is an important stage in disease progress in the vineyard, with a numerically dominant haplotype that can cross with a wide range of individuals, generating new strains, possibly with a different or greater virulence. Further studies are necessary to understand why different clusters in which gene flow is only within the cluster are present, and their significance in disease development.

This study demonstrates the importance of sexual reproduction in the spread of the pathogen and in the outbreak of the disease. It confirms that the origin of the pathogen is North America and that the first introduction into Europe was in France. It suggests that the pathogen was introduced to northeastern Italy from Eastern Europe. The proximity of the haplotypes found in Europe with those from the northeast of Italy, as well as some from Tuscany, suggests that, in these Italian vine areas, the disease spread from northern Europe and France, and that these strains may be more ancient than the ones that caused devastating outbreaks of the disease in the recent years in some Italian vine areas.

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Fig. 5. Results of Geneland analysis by nuclear microsatellite data of the *Phyllosticta ampelicida* population in the vineyard in Florence showing the spatial organization into three subpopulations. Map obtained from Google Earth.

TABLE 5. Genetic diversity (D) within three subpopulations, and matrix of pairwise F_{ST} among subpopulations (above diagonal) and genetic divergence (D_s) among subpopulation (below diagonal) in the *Phyllosticta ampelicida* population in the vineyard in Florence

Subpopulations	1 (D = 0.224)	2 (D = 0.161)	3 (D = 0.099)
1	...	0.668	0.313
2	0.574	...	0.782
3	0.061	0.731	...

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