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Cryptic diversity of *Plasmopara viticola* (Oomycota, Peronosporaceae) in North America

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Abstract *Plasmopara viticola* is the causal agent of grapevine downy mildew and is among the most important diseases in viticulture. It originates from North America, where it coevolved with wild *Vitis* species. Beginning in the 1870s it turned into a global epidemic that has been causing severe yield losses. It is generally believed that a single species is causing downy mildew on a large variety of economically important cultivars. Here we report, based on one nuclear and two mitochondrial markers, that isolates from vineyards in the United States fall into three highly distinct phylogenetic lineages. One of these contains European strains and affects *Vitis vinifera* cultivars, while the other two lineages affect also other species of *Vitis*. The divergence between these lineages is high, and, judging from the genetic variation in other *Plasmopara* lineages, might reflect distinct species. Due to the potentially significant implications for quarantine regulations and resistance breeding, detailed studies will be necessary to clarify whether these genetically distinct lineages occur outside of North America or are still confined there.

Keywords Cryptic species · Grape downy mildew · Herbarium specimens · *cox*2 · nrLSU · *ypt*1

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Introduction

Plasmopara viticola is one of the most important pathogens of grapevine. It originates from North America, where it infects wild and cultivated *Vitis* hosts. A comparative study (Jürges et al. 2009) has revealed that these North American host species are endowed with a high degree of resistance, whereas cultivated grapevine are colonised extensively. *Plasmopara viticola* was introduced into France in 1878 with contaminated rootstocks of wild American *Vitis* species used for *Phylloxera* control, and rapidly spread to other parts of Europe, e.g. to Germany by 1880 (Müller and Sleumer 1934). From Europe it subsequently invaded vineyards all over the world, and has become one of the most important pathogens of grape.

Although some specialised forms of the species have been described as parasites of Vitaceae (Golovina 1955; Săvulescu 1941; Săvulescu and Săvulescu 1952), it was generally assumed that *P. viticola* is the sole *Plasmopara* species occurring on the genus *Vitis* and other genera in the Vitaceae. This was based on the notion that morphological characteristics are too variable to allow species delimitation (Rafailă et al. 1968). As a consequence, the East Asian *P. amurensis* (Protsenko 1946) was not widely recognised as a species but is usually treated as a synonym of *P. viticola*. It should be noted, however, that *Vitis amurensis*, a wild grape species sympatric with *P. amurensis*, similarly to some North American *Vitis* species, is able to arrest *P. viticola* at an early stage (Jürges et al. 2009). This might be indicative of coevolution with a *Plasmopara* that was present before the more recent introduction of *P. viticola* from North America, in

which case *P. amurensis* might be valid after all as a geographically separated species.

It has been reported that there is a significant degree of genetic variability in *Plasmopara* isolated from cultivated *Vitis* hosts (Gobbin et al. 2006). However, as no study has yet investigated several strains of *P. viticola* with molecular phylogenetic tools, it was the aim of the present work to clarify, based on a limited but geographically diverse sampling, whether *Plasmopara viticola* represents a single homogenous species, or whether there might be cryptic speciation among *Plasmopara* lineages on *Vitis*.

Material and methods

The collection details for the strains used in this study are given in Table 1. DNA extraction was carried out using UltraClean™ Soil DNA Kit (MO BIO Laboratories Inc., 2746 Loker Ave West, Carlsbad, CA 92010, USA), according to the manufacturer's instructions, for all *Plasmopara* samples except the herbarium specimens, for which the best protocol identified by Telle and Thines (2008) was used instead. The mitochondrial *cox*2 region was amplified by PCR according to the protocol of Hudspeth et al. (2000). Partial nrLSU was amplified according to the protocol given

Table 1 Oomycete specimens sequenced in this study

Pathogen	Host	Strain number	Year	Country, state, locality	GenBank accession numbers		
					*cox*2	*ypt*1	nrLSU
Plasmopara nivea	*Aegopodium podagraria*	HOH HUH 586	2004	Germany	HM628761	n.a.	n.a.
Plasmopara nivea	*Aegopodium podagraria*	HV 2233	2006	Austria	HM628760	n.a.	n.a.
Plasmopara pimpinellae	*Pimpinella* sp.	HV660	2000	Austria	HM628740	n.a.	n.a.
Plasmopara halstedii	*Helianthus annuus*	Ph 110[a]	Laboratory strain	Germany	HM628743	n.a.	n.a.
Plasmopara halstedii	*Helianthus annuus*	Ph GG[a]	Laboratory strain	Germany	HM628739	n.a.	n.a.
Plasmopara angustiterminalis	*Xanthium strumarium*	HOH HUH 671	2004	Hungary	HM628738	n.a.	n.a.
Plasmopara angustiterminalis	*Xanthium strumarium*	HOH HUH 674	1982	Hungary	HM628742	n.a.	n.a.
Plasmopara angustiterminalis	*Xanthium strumarium*	HOH HUH 676	2002	Hungary	HM628747	n.a.	n.a.
Plasmopara angustiterminalis	*Xanthium strumarium*	X03[a]	Laboratory strain	Hungary	HM628741	n.a.	n.a.
Plasmopara viticola	*Vitis vinifera*	WBI 1[a]	2009	Germany,	HM628745	HM628736	HM628765
Plasmopara viticola	*Vitis vinifera*	Syngenta[a]	2009	Switzerland	HM628744	HM628737	HM628769
Plasmopara viticola	*Vitis vinifera*	JI[a]	2006	USA, NY, Seneca County	HM628751	HM628732	HM628763
Plasmopara viticola	*Vitis vinifera*	KI[a]	2006	USA, MD, Keedysville	HM628752	HM628729	HM628766
Plasmopara viticola	*Vitis vinifera*	TxI[a]	2007	USA, TX, Cat Spring	HM628748	HM628734	HM628762
Plasmopara viticola	*Vitis vinifera*	TxII[a]	2007	USA, TX, Cypress	HM628746	HM628733	HM628767
Plasmopara viticola	*Vitis vinifera*	WBI 2[a]	2009	Germany	HM628750	HM628730	HM628768
Plasmopara viticola	*Vitis vinifera*	PvI[a]	2006	USA, NY, Geneva	HM628749	n.a.	HM628764
Plasmopara viticola	*Vitis aestivalis*	U360 ex FH No. 6389)	1937	USA, MA, Medford	HM628753	n.a.	n.a.
Plasmopara viticola s.l.	*Vitis vinifera*	TxIII[a]	2007	USA, TX, Hill County	HM628754	HM628728	HM628771
Plasmopara viticola s.l.	*Vitis* hybrid	TxIV[a]	2007	USA, TX, Hill County	HM628756	HM628735	HM628770
Plasmopara viticola s.l.	*Vitis riparia*	MI[a]	2006	USA, NY, Marathon	HM628757	HM628731	HM628772
Plasmopara viticola s.l.	*Vitis* cf. *cordifolia*	U366, ex Coll. Farlow in FH)	1874	United Kingdom	HM628758	n.a.	n.a.
Plasmopara viticola s.l.	*Vitis riparia*	U375, ex Cryptogams. Plants of Iowa No 28 in FH)	1896	USA, IA, Boone	HM628755	n.a.	n.a.
Plasmopara majewskii	*Arctotis* sp.	DAR 69721	1993	Australia, NSW, Glenorie	HM628759	n.a.	n.a.

[a] DNA extracts only; requests for DNA should be addressed to the corresponding author

in Riethmüller et al. (2002). For amplification of *ypt*1 the primers Ypt1F (described in Chen and Roxby 1996) and Ypt4R (Moorman et al. 2002) were used. PCR amplicons were sequenced by a commercial provider (GATC biotech, Germany). GenBank accession numbers for the sequences obtained are listed in Table 1. Sequences were aligned using mafft (Katoh et al. 2002), version 6, using the Q-INS-i algorithm (Katoh and Toh 2008). To ensure reproducibility, the alignments were used without manual editing. Phylogenetic reconstruction was performed with two methods: for minimum-evolution inference with MEGA 4.0 (Tamura et al. 2007), with all parameters set to default values, except for using the Tamura-Nei substitution model; and for maximum-likelihood inference with RAxML (Stamatakis 2006) in the

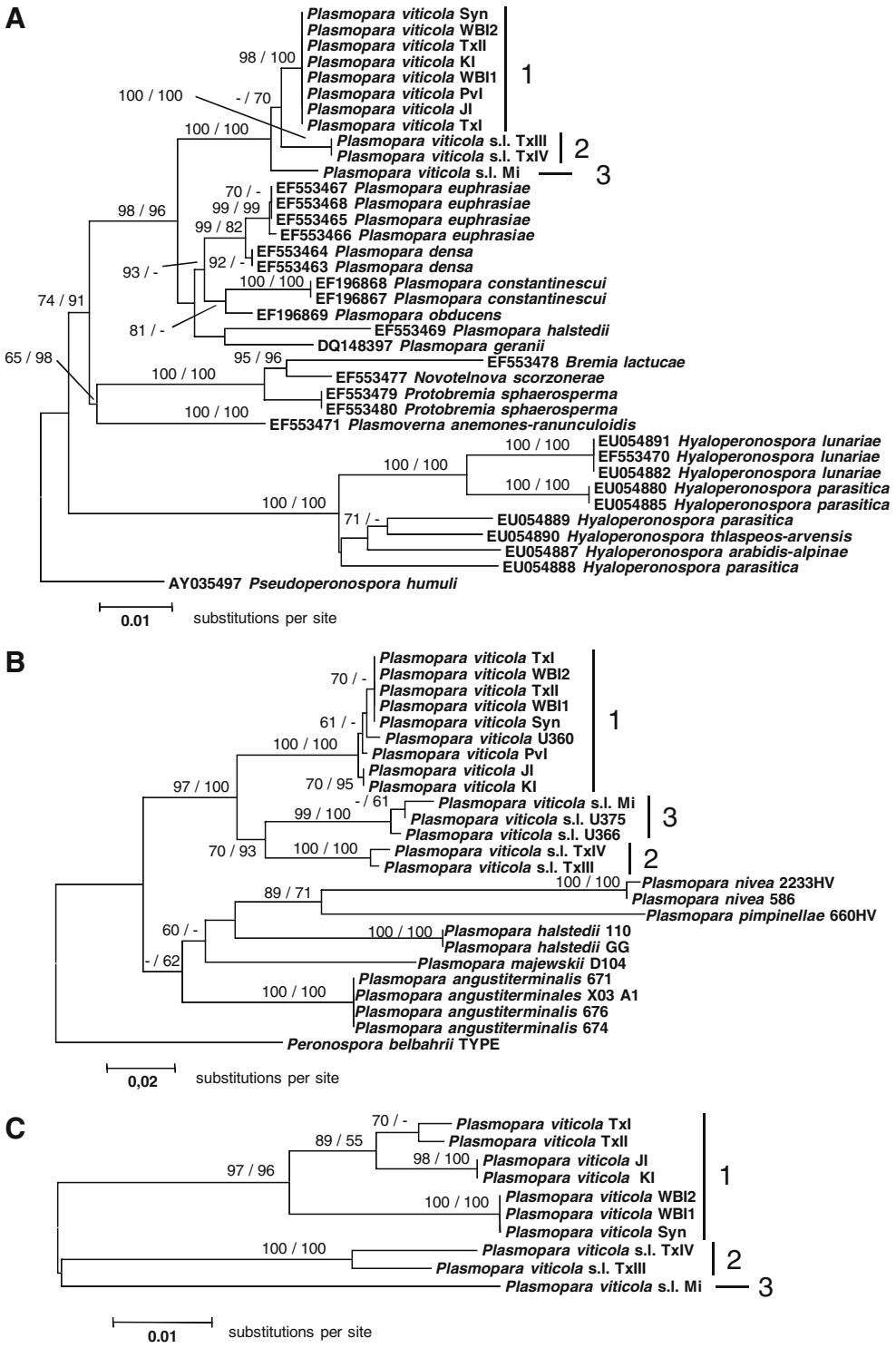

Fig. 1 Minimum-evolution phylogenetic reconstruction using partial nrLSU (**a**), *cox*2 (**b**), and *ypt*1 (**c**) sequences; where bootstrap support values in minimum-evolution or maximum-likelihood analyses were above 50%, these are shown on the branches in that order of analyses, while "–" labels denote lack of such support

webserver version (Stamatakis et al. 2008), estimating the proportion of invariable sites and with all parameters set to default values. In both cases, 500 bootstrap replicates were carried out to test tree robustness. Alignments and the trees shown in Fig. 1 have been deposited in TreeBASE under Accession number 10992.

Results

Tree topology for the *Plasmopara* strains infecting *Vitis* was similar among phylogenetic reconstructions based on partial nrLSU (nuclear; Fig. 1a), *cox*2 (mitochondrial; Fig. 1b), and *ypt*1 (mitochondrial; Fig. 1c) sequences. In the different analyses for each gene, no conflicts between nodes, each of which displays a high support value for conflicting topologies, were observed in any of the analyses. All three genes revealed three distinct phylogenetic lineages within *Plasmopara* from *Vitis*, in both minimum-evolution and maximum-likelihood inference. One of these clades (clade 1) contained samples from both North America and Europe, while the other two lineages (clades 2 and 3) contained only American isolates. In *ypt*1, which offers the highest phylogenetic resolution of the genes investigated, clade 1 was divided in two subclades, one containing the European isolates, which were all identical in sequence, and one containing the North American isolates, which showed some genetic variation between the samples from Texas and those from the North-East. Clade 1 contained the samples from *Vitis vinifera* and cultivated hybrids; clade 2 contained samples from *Vitis vinifera* (TxIII from the grapevine variety 'Barbera') and a cultivated hybrid (TxIV from variety 'C1613' (*Vitis solonis* x *V. vinifera* var. 'Othello 1613')); clade 3 contained two samples from *Vitis riparia*, and one from a specimen labelled as *V. cordifolia*.

Discussion

Several varieties of *P. viticola* and the rare East Asian species *P. amurensis* have been described from members of the genus *Vitis* (Golovina 1955; Săvulescu 1941; Săvulescu and Săvulescu 1952), but the investigations of Rafailă et al. (1968) did not reveal clear-cut morphological differences for *Plasmopara* accessions from the Vitaceae. Thus it seems possible that the minor differences observed by Săvulescu (1941), Săvulescu and Săvulescu (1952), and Golovina (1955) are the result of modifications caused either by environmental conditions (Dudka et al. 2007) or by the host matrix, which can have a major impact on downy mildew morphology (Runge and Thines 2010). However, the lack of morphological divergence does not necessarily indicate species identity—as the present study corroborates.

The three distinct lineages found show significant genetic divergence (1.0–1.4%). This is lower than the divergence between the genera *Novotelnova* and *Protobremia* (1.9%), comparable to that known from some other species of *Plasmopara*, e.g. between *P. obducens* and *P. constantinescui* (1.5%, Voglmayr and Thines 2007), and significantly higher than between closely related species such as *Plasmopara densa* and *P. euphrasiae* (0.4–0.5%, Voglmayr and Constantinescu 2008). The cryptic, undescribed species found as parasites of species in the genus *Vitis* are morphologically similar. Thus, thorough morphological and physiological investigations will be necessary to determine whether statistically significant differences exist that could be used for morphological species delimitation. The host ranges of the three lineages remains unclear, but Jürges et al. (2009) have shown that European isolates of *P. viticola* do not readily infect *Vitis* spp. from North America and East Asia. In the former case, strong defence reactions were observed; in the latter, surface mycelium without successful entry was produced; both results suggest a high degree of specialisation. Based on the limited sampling available for the present study, two of the three lineages might be absent from Europe, but it cannot be ruled out that they were introduced to Europe together with cultivated hybrids of *Vitis vinifera* and the corresponding native hosts.

It will be important to determine in future studies, how widespread the cryptic species parasitic to *Vitis* are, and whether they pose a potential threat to cultivated grape. The latter might be the case especially for the cryptic species constituting clade 2, which was found on a cultivated species hybrid as well as on *Vitis vinifera* var. *Barbera*. If detailed phylogeographic studies indicate absence from Europe, strict quarantine regulations might be needed to confine the two new cryptic species to their native range.

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