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Alnus glutinosa and *Orientus ishidae* (Matsumura, 1902) share phytoplasma genotypes linked to the 'Flavescence dorée' epidemics

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Abstract

Flavescence dorée (FD) is a grapevine disease caused by associated phytoplasmas (FDp) which are epidemically spread by their main vector *Scaphoideus titanus*. The possible roles of alternative and secondary FDp plant hosts and vectors have gained interest in terms of better understanding of the FDp ecology and epidemiology. The findings of a survey conducted in the surroundings of three vineyards in the southern Swiss Alps aimed at studying the possible epidemiological role of the FDp secondary vector *Orientus ishidae* and the FDp host plant *Alnus glutinosa* are reported. This work demonstrates that *O. ishidae* is able to complete its biological cycle on *A. glutinosa* and to acquire FDp and 16SrV phytoplasmas very efficiently with an infection rate of 69% for the nymphal instars and 85% for the imagoes. A high prevalence of the *map* genotype M50 (*map* type FD1), which is included in the *S. titanus*—grapevine epidemiological cycle, was found in *O. ishidae* and *A. glutinosa*. Additionally, M12 (*map* type FD3), M44 and M47 were also sporadically detected. Surprisingly, the grapevines tested during this work were all infected by M54 (*map* type FD2) only, while the few *S. titanus* caught in the vineyard canopy were all FDp free. In conclusion, the occurrence of infected common alder stands and *O. ishidae* nearby vineyards do not seem to play a prominent role in FD epidemics in southern Switzerland. Nevertheless, wild vegetation acts as a reservoir of the FDp inoculum, which may locally trigger a FD emergence if *S. titanus* populations are established inside vineyards.

KEYWORDS

16SrV phytoplasma, Grapevine yellows, insect vectors, *map* gene, Switzerland, *Vitis vinifera*

1 | INTRODUCTION

'Flavescence dorée' (FD), a quarantine grapevine disease caused by FD phytoplasmas (FDp), was first reported in the 1950s in south-western France (Caudwell, 1957). The spread of FD to an epidemic level within vineyards is caused by the Nearctic leafhopper *Scaphoideus titanus* (Ball, 1932), which, in Europe, usually accomplishes its whole life cycle on plants of the genus *Vitis* (Chuche & Thiéry, 2014; Schvester et al., 1961). Despite the quarantine status of the disease, and the systematic mandatory control applied

in FD-infected areas, mainly consisting of insecticide applications against the FDp vector *S. titanus*, in the removal of infected grapevines, and the introduction of certified propagation material for the replacements, FD is currently present in many European wine-growing areas (EFSA et al., 2020; Jeger et al., 2016).

FDp belong to the taxonomic ribosomal group 16SrV (subgroups 16SrV-C and 16SrV-D) (Davis & Dally, 2001; Lee et al., 2004). The subgroups can be further subdivided into FD1 and FD3 types, which include strains from subgroup 16SrV-C, and FD2 type, which includes strains from both subgroups based on the *map* genetic locus (Arnaud et al., 2007; Malembic-Maher et al., 2011).

Meanwhile, other Auchenorrhyncha species have been found to be infected by different FDp strains, and among these, some are even able to transmit FDp to healthy grapevines. In particular, *Dictyophara europaea* (Linnaeus, 1767) may transfer some strains of 16SrV-C from *Clematis vitalba* L. to grapevines (Filippin et al., 2009), while the ability of *Orientus ishidae* (Matsumura, 1902) to transmit 16SrV phytoplasmas to grapevines was demonstrated after experimentally forced acquisition of FDp on grapevine and broad bean (Lessio et al., 2016). Moreover, naturally infected *O. ishidae* specimens collected on alder were also found able to transmit the *map* genotype M38 to broad bean. M38 later resulted transmissible by *S. titanus* (Malembic-Maher et al., 2020). Furthermore, *O. ishidae* has been found to be able to lay eggs on grapevine, especially in abandoned vineyards and/or wild rootstocks close to the forest edges (Lessio et al., 2019). Due to the lower FDp transmission capability of these species with respect to *S. titanus* and the fact that they ideally sustain primary infections only, *D. europaea* and *O. ishidae* are usually indicated as alternative and secondary FDp vectors (Lessio et al., 2016; Malembic-Maher et al., 2020). Moreover, wild woody species such as *Alnus glutinosa* (L.) Gaertn., *A. incana* (L.) Moench. (Arnaud et al., 2007; Radonjić et al., 2013), *Corylus avellana* L., *Salix* spp. (Casati et al., 2017) and *Ailanthus altissima* (Mill.) Swingle (Filippin et al., 2011) have been found infected with FDp and are thus suspected to act as potential, although mostly asymptomatic, phytoplasma reservoirs.

FDp epidemics and ecology are not limited to the 'grapevine–*S. titanus*' interactions within the vineyard, but may represent a complex system at a broader landscape scale (Casati et al., 2017; Lessio et al., 2016). In particular, the presence of woody species and forest stands in the surroundings of vineyards could play an important role in preserving and increasing the pressure of the FDp inoculum on grapevines. In this respect, Malembic-Maher et al. (2020) not only confirmed that FDp are endemic to European alders, but also demonstrated the ability of *O. ishidae* to acquire and transmit *map*-FD2 and *map*-FD1 genotypes compatible with both *S. titanus* and grapevine.

To test this hypothesis, three vineyards surrounded by common alder (*A. glutinosa*) stands in Canton Ticino (southern Switzerland) were monitored in 2019. The specific aims of the study were to: (i) demonstrate that *O. ishidae* is able to carry out most of its biological cycle (i.e. from juvenile to adult forms) on common alder; (ii) verify the FDp infection rate of the *O. ishidae* population; (iii) verify the identity of FDp genotypes found in *O. ishidae*, common alder, and in FDp-infected grapevines; and (iv) discuss the possible role of the common alder—*O. ishidae*–grapevine system in FD epidemics.

2 | MATERIALS AND METHODS

2.1 | Study area and experimental design

The study area is represented by the wine-growing area of Canton Ticino (southern Switzerland), where FD epidemics emerged in 2004 and now affect almost the entire region, despite the systematic control measures applied since the first symptoms were detected (Jermini et al., 2014; Schaerer et al., 2007; Sezione Agricoltura Cantone Ticino, 2019).

The experimental design included three FDp-infected vineyards (Origlio, Montalbano and Camorino) with occurrence of *A. glutinosa* in the immediate surroundings (i.e. in the first 100 m from the vineyard's edge, see Table 1 and Figure 1).

2.2 | Field survey

The field survey consisted of four separate tasks. The first task focused on leafhopper nymphal instars living on the common alders around the three vineyards, with the aim of assessing presence and abundance of nymphal instars of *O. ishidae*. Sampling took place weekly between weeks 23 and 32 in 2019 and consisted of shaking a crown and an epicormic branch of each selected common alder for ~20 s and collecting the fallen insects into a sweeping net. Collected insects were then put into labelled plastic bags and conserved in a cooling container for transport to the laboratory facilities where they were transferred at −20°C for conservation before further processing.

The second task aimed at assessing abundance and distribution of adult specimens of both *O. ishidae* and the main FDp vector *S. titanus* inside the vineyards by means of yellow sticky traps (Rebell Giallo, Andermatt Biocontrol AG, Switzerland, hereinafter referred to as YST). YST were homogeneously distributed inside vineyards (see Figure 1b for the example of Origlio) on the highest wire of the training system based on the plot's surface and shape, and workload, and were changed weekly from week 26 through week 41. Collected YST were frozen (−20°C) upon arrival to the laboratory facilities until insect determination and counting.

The third task consisted in systematically surveying all grapevines in the three vineyards under investigation to detect plants with grapevine yellows (GY) symptoms according to EPPO/CABI (1997). This activity took place twice (weeks 28 and 39) during the 2019 season. Leaf samples were then collected from all symptomatic grapevines and transported to the laboratory facilities inside a cooling container (5°C) for further processing.

The fourth task assessed the presence of FDp-related strains in common alders. To this purpose, in January 2020 (week 2), three-year-old wood from crown branches of all common alders was collected and transported to the laboratory for further processing.

TABLE 1 Characteristics of the monitored vineyards, including the number of sampled common alders and the number of yellow sticky traps (YST) placed in the canopy

Plot	Cultivar	Rootstock	Area (m^2)	Coordinates[a]	Elevation (m a.s.l.)	N_{Alders}	N_{YST}
Camorino	'Merlot'	3309 C[b]	2,138	46.165, 9.014	370	4	6
Montalbano	'Chardonnay'	3309 C[b]	3,224	45.856, 8.927	420	5	9
Origlio	'Chardonnay', 'Sauvignon Blanc'	3309 C[b]	13,394	46.043, 8.941	434	13	15

[a]Coordinates WGS84.

[b]*Vitis riparia x Vitis rupestris* 3309 Couderc

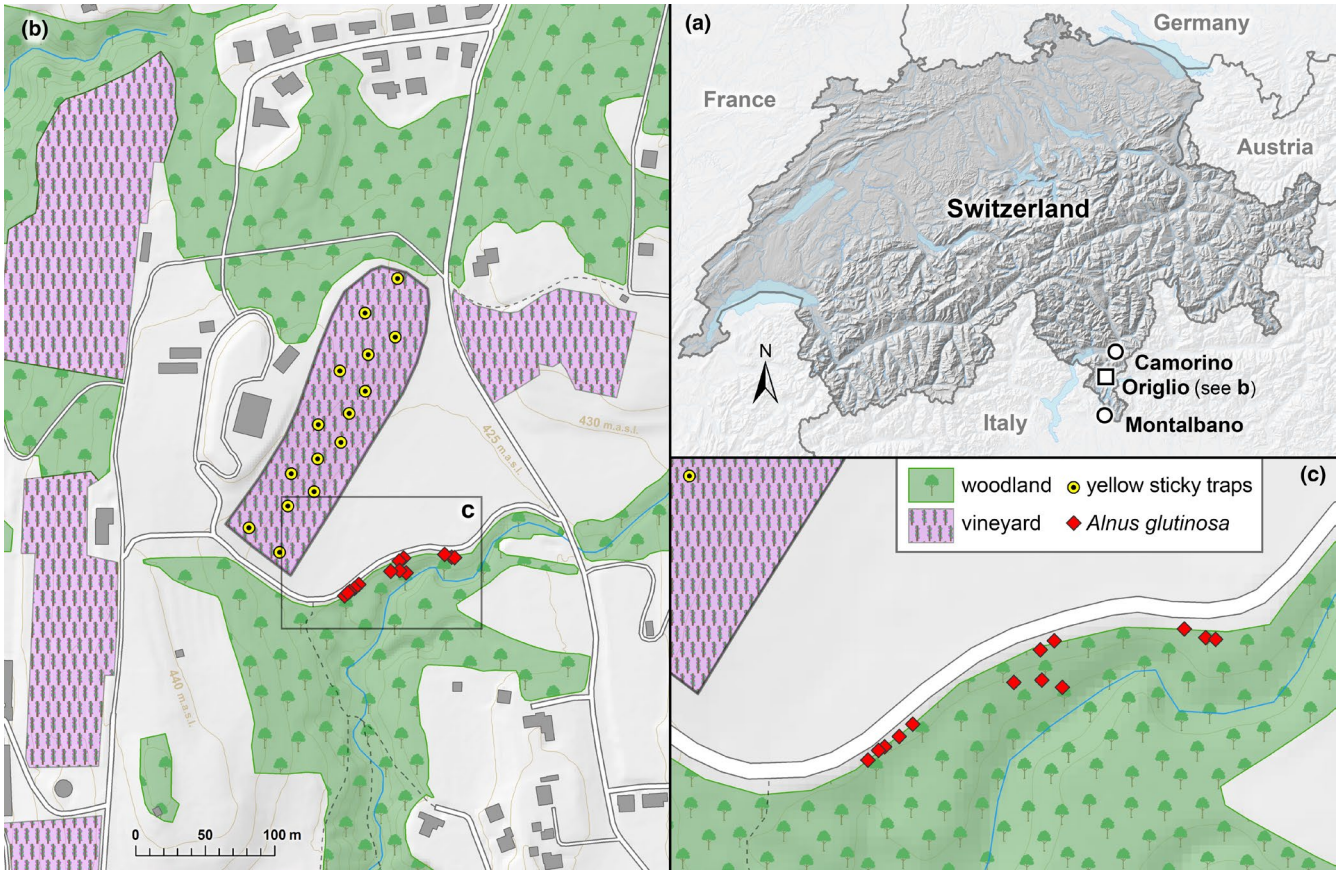

FIGURE 1 Study area and sampling design. (a) Location of the three investigated vineyards; (b) sampling design in the vineyard of Origlio; (c) detailed position of the sampled common alder trees at the Origlio site. Pink areas = vineyards; area with bold line: reference vineyard in Origlio; yellow circles = yellow sticky traps; red diamonds = common alder trees.

2.3 | Sample pre-processing

Insects were determined and counted using a stereo microscope (Olympus SZX16 with SDF PLAPO 1XPF objective lenses, made in Japan) and by following the keys of Della Giustina et al. (1992) and Günthart and Mühlethaler (2002) for *S. titanus* and *O. ishidae*, respectively. Specimens of *O. ishidae* were further subdivided into early nymphal instars L1-2 (wing pads absent), nymphal instars L3-5 (wing pads present) and imagoes. All determined insects were then transferred into tubes with 99% ethanol (v/v) and stored at −20°C.

The petioles and the major veins of leaves of symptomatic grapevines were excised with scissors and frozen at −20°C until further processing. Cortical scrapings were obtained from the collected common alder branches before proceeding to the nucleic acid extraction.

2.4 | Molecular analysis scheme

A set of preliminary molecular analyses was conducted for all three plots, whereas, for cost reasons, the subsequent detailed molecular analyses were carried out for Origlio only, that is, for the most promising plot in terms of insect captures and common alder abundance (Figure 1 and Table 2).

TABLE 2 Molecular analyses scheme

Analysis type	Task/result	Site	Alders (N)	*O. ishidae*				*S. titanus*		
				Sampled on alder		Sampled in vineyard		Sampled in vineyard	Grapevines (N)	Subtotal
				Nymphs (N)	Imagoes (N)	Nymphs (N)	Imagoes (N)	Imagoes (N)		
Preliminary analyses 16SrV	TNAs extraction +qPCR	All sites	22	146	44	7	7	10^a	–	236
	16SrV-positive		22	105	36	0	1	0	–	164
Detailed molecular analysis and sequencing	TNAs extraction +qPCR	Origlio	13^a	73^a	27^a	5^a	4^a	8^a	32	162
	16SrV-positive		13^a	51^a	24^a	0^a	1^a	0^a	29	118
	16SrXII-positive		–	–	–	–	–	–	3	3
	Nested PCR +RFLP		13^b	20^b	0	0	1^b	0	10^b	44
	16SrV-C		13	20	0	0	1	0	0	34
	FD-D		0	0	0	0	0	0	10	10
	Genotype sequencing		13^c	20^c	0	0	1^c	0	5^c	39

Note: Preliminary analyses were conducted on common alders and insects in all sites. The detailed molecular analyses were conducted in Origlio only. Number of analysed samples and results gathered at each stage. *Scaphoideus titanus* specimens caught in Camorino were not analysed. TNA: total nucleic acid. a, b, and c: selected from previous step. Refer to Tables 3–5 for the intermediate results.

2.5 | Nucleic acid extraction

Damaged or badly preserved insect specimens were discarded (e.g. broken abdomen). Similarly, early nymphal instars (L1-2) were discarded from the molecular analyses assuming that post-acquisition phytoplasma multiplication inside their tissues was not sufficient for detection.

From all other individual insects, total nucleic acids (TNAs) were extracted using the protocol described in Gatineau et al. (2001) with minor modifications. Each specimen was ground using a sterile micro-pestle in a 1.5-ml Eppendorf tube containing 400 µl of cetyltrimethyl-ammonium bromide buffer (2% w/v CTAB, 1 M Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 4% PVP, pH 8.0), with 2% g/ml of metabisulphite added as an antioxidant. The suspension was incubated for 20 min at 65°C and then extracted twice with chloroform–isoamyl alcohol (24:1). TNAs were precipitated by adding 1 vol of cold isopropanol and recovered by centrifugation, washed with 70% ethanol (v/v), dried and resuspended in 50 µl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6).

TNAs were extracted from *A. glutinosa* and symptomatic grapevines following the CTAB method described in Angelini et al. (2001).

2.6 | PCR amplification and RFLP

TaqMan real-time PCR analysis on ribosomal genes of grapevine phytoplasmas was carried out to detect the presence of the 16SrV group phytoplasmas in plants and insects, and 16SrXII group phytoplasmas in grapevines, based on the method described in Angelini et al. (2007). The 16SrXII group of phytoplasmas is associated with 'Bois noir' (BN), another GY disease that causes the same external symptoms as FDp on grapevines. The differentiation between the two GYs is thus possible only through molecular analysis. The assays were performed in 96-well plates on a CFX96 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA).

The detection of phytoplasmas was confirmed by nested conventional PCR targeting the 16S-23S ribosomal RNA gene on all positive common alders ($N = 13$) and some *O. ishidae* specimens ($N = 21$) in Origlio (Table 2) and on selected symptomatic grapevines with the lowest Cq values found on the vineyard margin facing the common alder trees in Origlio ($N = 10$; Table S1). The first PCR was performed using the phytoplasma universal primer pair P1/P7 (Deng & Hiruki, 1991; Smart et al., 1996), while the nested PCR with 16r758f/M23Sr primers (Gibb et al., 1995; Padovan et al., 1995), following the reagent and thermal conditions described in Angelini et al. (2001).

Amplicons were analysed by 1% agarose gel electrophoresis in TBE buffer 0.5X (Tris-Borate 90 mM, EDTA 1 mM), stained with GelRed (Biotium, Inc., Fremont, CA, USA) and visualized under a GelDoc XR UV transilluminator (Bio-Rad Laboratories, Hercules, CA, USA). Amplicons from nested PCR were then digested with the endonuclease *Taq*I (MBI, Fermentas, Vilnius, Lithuania) to identify the 16SrV subgroup phytoplasmas based on RFLP (restriction fragment length polymorphism) profile (Angelini et al., 2001). Restriction products were separated by

10% polyacrylamide gel electrophoresis in TBE buffer 1X, stained with GelRed and visualized under the UV transilluminator.

2.7 | Sequencing

After consulting the existing literature (Arnaud et al., 2007; Knezović et al., 2019; Lee et al., 2004; Malembic-Maher et al., 2020; Martini et al., 2002; Renaudin et al., 2015; Rossi et al., 2019) and databases (i.e. DDJB/EMBL/GenBank databases), the *secY-map* genetic *locus* was selected for the fine molecular characterization and sequencing in view of its known polymorphism and the wide availability of nucleotide sequences from infected *A. glutinosa* and *O. ishidae* gathered from different sites all over Europe.

All 16SrV-C-infected samples collected in Origlio, together with five FD-D-infected grapevines, were subjected to the amplification of the *secY-map* genetic locus with FD9f5/MAPr1 primer pair followed by FD9f6/MAPr2 primer pair, using the conditions described in Arnaud et al. (2007). In addition, the *secY-map* fragment of the reference strain FD92, coming from *Vicia faba* L. in the INRA collection and stored at CREA as a DNA extract, was amplified and sequenced. *SecY-map* gene PCR fragments were purified using the PCR Clean-Up System (Promega, Madison, WI, USA) and quantified for nucleotide sequence analysis. The positive amplicons were sequenced with the primers MAPr2 and MAPr3 (5′-CGCCGAAGCCTTGATAACCTTT-3′) by a commercial service (BMR Service, Padua, Italy). The raw sequence chromatograms were assembled and edited using the Staden Package software (Bonfield et al., 1995).

2.8 | Data analysis

Each type of obtained nucleotide sequence was entered into the DDJB/EMBL/GenBank databases (see Table 3 for the corresponding accession numbers). The most similar nucleotide reference sequences from GenBank were selected by running a BLAST query. Alignment and comparison with phytoplasma sequences from GenBank were carried out using CLUSTAL OMEGA (Madeira et al., 2019). Phylogenetic analyses were conducted using the MEGA4 software package using the neighbour-joining (NJ) and the maximum parsimony (MP) methods (Tamura et al., 2007). To check reliability, the cladograms were subjected to bootstrap tests with 1,000 and 500 replicates, respectively. All other analyses were performed using R statistical software (version 3.6.3; R Core Team, 2020). All geographic mapping renditions were generated with ArcGIS (release 10.6.1; ESRI, 2011).

3 | RESULTS

3.1 | *O. ishidae* population on common alder trees

O. ishidae specimens (nymphs and imagoes) were caught on common alders in all surveyed sites (Figure 2 and Table S2). Early nymphal instars (L1-2) were collected from the beginning of the field sampling up to week 27, while later nymphal instars (L3-5) were collected from week 26 up to week 31. The emergence of imagoes was observed during weeks 27 and 28. Captures were not significantly different among vineyards (Wilcoxon signed-rank test, $p > 0.05$). Among the 22 sampled common alders, instars L1-2 were not caught on three common alder trees only, which, however, subsequently hosted nymphal instars L3-5. Only a single tree did not host any L3-5, while imagoes were not found on ten of the surveyed trees.

3.2 | Insect captures on yellow sticky traps

Table 4 reports the captures of *O. ishidae* (nymphal instars and imagoes) and *S. titanus* adults on YST placed in vineyards. The *S. titanus* and *O. ishidae* average captures per vineyard and per YST during the whole vegetative season were fewer than or equal to 1.17 and 0.73, respectively.

3.3 | Molecular analyses

3.3.1 | Insects

The infection rate of *O. ishidae* collected on common alder trees was 0.69 ± 0.09 and 0.85 ± 0.17 (*mean* ± *standard deviation*) for L3-5 nymphal instars and imagoes, respectively. Among the *O. ishidae* specimens caught on YST placed in the vineyard canopy, only one imago (out of the four analysed in Origlio) was infected. All the analysed *S. titanus* specimens caught inside the vineyards were found to be negative. Table 5 reports the results per single vineyard and sampling unit.

3.3.2 | Grapevines

The incidence of GY symptomatic grapevines was 1.51%, 1.78% and 1.37% in Camorino, Montalbano, and Origlio, respectively. Table S1 reports the results of the molecular analyses on a selection of the symptomatic grapevines collected in Origlio (32 out of 104 symptomatic grapevines). Among the 32 analysed samples, 29 were infected by FDp and three by BN phytoplasma (BNp), respectively. All ten FDp-infected grapevines selected for the molecular characterization were found to be infected by 16SrV-D, and therefore related to the *map*-FD2 type.

3.3.3 | Common alders

All analysed common alders were infected by 16SrV phytoplasma. All common alders sampled in Origlio and tested with nested PCR and RFLP were found to be infected by 16SrV-C phytoplasma (Table S3).

TABLE 3 Features of the phytoplasma isolates analysed in this work and reference strains, together with relative GenBank accession numbers and host

Sample ID	GenBank accession no.	Host	Genotype	Reference
Vv1002	MW660819	*Vitis vinifera*	M54	This paper
Vv1003	as MW660819	*Vitis vinifera*	M54	This paper
Vv1005	as MW660819	*Vitis vinifera*	M54	This paper
Vv1010	as MW660819	*Vitis vinifera*	M54	This paper
Vv1022	as MW660819	*Vitis vinifera*	M54	This paper
Ag1006	YY MW660815	*Alnus glutinosa*	M50	This paper
Ag1007	as MW660815	*Alnus glutinosa*	M50	This paper
Ag1008	as MW660815	*Alnus glutinosa*	M50	This paper
Ag1010	as MW660815	*Alnus glutinosa*	M50	This paper
Ag1012	MW660816	*Alnus glutinosa*	M44	This paper
Ag1013	as MW660815	*Alnus glutinosa*	M50	This paper
Ag1014	as MW660815	*Alnus glutinosa*	M50	This paper
Ag1016	as MW660815	*Alnus glutinosa*	M50	This paper
Ag1017	MW660817	*Alnus glutinosa*	M47	This paper
Oi1001	MW660818	*Orientus ishidae*	M12	This paper
Oi1004	as MW660815	*Orientus ishidae*	M50	This paper
Oi1006	as MW660815	*Orientus ishidae*	M50	This paper
Oi1007	as MW660815	*Orientus ishidae*	M50	This paper
Oi1011	as MW660815	*Orientus ishidae*	M50	This paper
Oi1013	as MW660815	*Orientus ishidae*	M50	This paper
Oi1014	as MW660815	*Orientus ishidae*	M50	This paper
Oi1015	as MW660815	*Orientus ishidae*	M50	This paper
Oi1020	as MW660815	*Orientus ishidae*	M50	This paper
Oi1022	as MW660815	*Orientus ishidae*	M50	This paper
Oi1027	as MW660815	*Orientus ishidae*	M50	This paper
Oi1029	as MW660815	*Orientus ishidae*	M50	This paper
Oi1031	as MW660815	*Orientus ishidae*	M50	This paper
Oi1036	as MW660815	*Orientus ishidae*	M50	This paper
Oi1040	as MW660815	*Orientus ishidae*	M50	This paper
Oi1042	as MW660815	*Orientus ishidae*	M50	This paper
Oi1043	as MW660815	*Orientus ishidae*	M50	This paper
Oi1061	as MW660815	*Orientus ishidae*	M50	This paper
Oi1062	as MW660818	*Orientus ishidae*	M12	This paper
Oi1066	as MW660815	*Orientus ishidae*	M50	This paper
Oi1201	as MW660815	*Orientus ishidae*	M50	This paper
FD92	MW660819	*Vicia faba*	M54	This paper
FD70	AM238512	*Catharanthus roseus*	M50	Arnaud et al., 2007
VIO4-188-04	LT221907	*Vitis vinifera*	M12	Malembic-Maher et al., 2020
VS-Loza37	LT221946	*Vitis vinifera*	M51	Malembic-Maher et al., 2020
VF06-30-18	LT221949	*Vitis vinifera*	M54	Malembic-Maher et al., 2020
VF-09-876	LT222007	*Vitis vinifera*	M112	Malembic-Maher et al., 2020
AF-09-27-3	LT221939	*Alnus glutinosa*	M44	Malembic-Maher et al., 2020
AF-07-22-32	LT221942	*Alnus glutinosa*	M47	Malembic-Maher et al., 2020
AI-352-07	LT221959	*Alnus glutinosa*	M64	Malembic-Maher et al., 2020

(Continues)

TABLE 3 (Continued)

Sample ID	GenBank accession no.	Host	Genotype	Reference
AI-365-07	LT221967	*Alnus glutinosa*	M72	Malembic-Maher et al., 2020
AI-014-08	LT221995	*Alnus glutinosa*	M100	Malembic-Maher et al., 2020
AI-AL4	LT222008	*Alnus glutinosa*	M113	Malembic-Maher et al., 2020
Ag-5201	LT221948	*Alnus glutinosa*	M53	Malembic-Maher et al., 2020
AF-06-30-27	LT221943	*Alnus glutinosa*	M48	Malembic-Maher et al., 2020
AI-031-08	LT221933	*Alnus glutinosa*	M38	Malembic-Maher et al., 2020
AF07-25-7	LT221941	*Alnus glutinosa*	M46	Malembic-Maher et al., 2020
Ag30_MAC	KJ605451	*Alnus glutinosa*	-	Atanasova et al., 2014
Ag31_MAC	KJ605452	*Alnus glutinosa*	M38	Atanasova et al., 2014
74-08-MNE	KC188998	*Alnus glutinosa*	-	Radonjić et al., 2013

3.4 | Sequencing of infected samples and phylogenetic tree

The *secY-map* amplicons from grapevines, common alders and insects were sequenced, obtaining a total of 40 sequences with 2X coverage. Four samples from *A. glutinosa* showed the presence of double peaks on the chromatograms (mixed infection with more than one phytoplasma strain) and were therefore not included in the construction of the phylogenetic tree.

The resulting NJ and MP phylogenetic trees encompassed 675 nt positions and showed almost identical topologies (Figure 3 and Figure S1). The main cluster comprised the majority of the phytoplasma identified in *A. glutinosa* (7 out of 9) and *O. ishidae* (19 out of 21), including the imago caught in the vineyard. Both *A. glutinosa* and *O. ishidae* showed to be infected with the M50 *map* genotype, which is present in the FD70 reference strain, belonging to the *map*-FD1 group (Malembic-Maher et al., 2020). One strain from Montenegrin (GenBank acc. No. KJ605451), one from Macedonian alders (GenBank acc. No. KC188998) and M113 *map* genotype from Italian alders grouped closely, however, with low bootstrap (BT) support. In the second cluster, robustly supported by high BT values, all the five grapevine isolates sharing the same nucleotide sequence were grouped together and found to be identical to the FD92 reference strain in the *secY-map* gene, classified as M54 *map* genotype and belonging to the *map*-FD2 group. A sister group included the M38 reference *map* genotype, also present in alders in Montenegro (GenBank acc. No. KJ605452). The other four samples were distributed into three different clusters supported by lower BT values: two *O. ishidae* nymphs (Oi1001 and Oi1062) were infected with the M12 *map* genotype, belonging to the *map*-FD3 group, while the two remaining common alders (Ag1012 and Ag1017) were infected with the M44 and M47 *map* genotypes and clustered with the reference strain PGY-C. Finally, PGY-A and PGY-B *map* genotypes were different from all the samples analysed in this study and were grouped separately.

4 | DISCUSSION

4.1 | The ecology and 16SrV phytoplasma infection rates of *O. ishidae*

The present study focused on the possible role of common alder trees surrounding vineyards as FDp reservoirs and hosts of the alternative vector *O. ishidae*. The obtained results show that in southern Switzerland, common alders are capable of hosting all the main developmental stages of *O. ishidae* (nymphal instars and imagoes). The particular abundance of L1-2 nymphal instars and their widespread presence on the surveyed *A. glutinosa* trees suggests that *O. ishidae* females may also lay eggs in the bark of common alders, as already demonstrated for other plant species including grapevine (Lessio et al., 2019). This is further corroborated by the fact that L1-2 nymphal instars were found on common alder branches that were not in the proximity of any other known host plant species of *O. ishidae*, such as hazelnut or willow (Hamilton, 1985; Mehle et al., 2019). Instead, insect captures in vineyards were rather low. In the case of *O. ishidae* (14 captured specimens, one infected imago), the result is in line with previous findings from the same study area (Jermini et al., 2017, 2019) and in other European wine-growing regions (Lessio et al., 2016, 2019; Malembic-Maher et al., 2020).

Concerning the main vector *S. titanus* (only 17 specimens captured over the whole season, none of the ten analysed specimens FDp-infected), the reduced number of captures confirmed the success of the mandatory control measures implemented in the study area, which consists of two insecticide applications per season, along with the systematic removal of FDp-infected vines (Jermini et al., 2014). Given the few sources of FDp inoculum in the vineyards and the low number of *S. titanus* caught by the traps, it is not surprising that all the analysed *S. titanus* specimens were found to be negative for FDp.

All analysed alders (22 out of 22) were infected by 16SrV-p, confirming the high phytoplasma infection rate found in alders in Europe, as highlighted by several studies (Arnaud et al., 2007; Atanasova et al., 2014; Cvrković et al., 2008; Desqué et al., 2019;

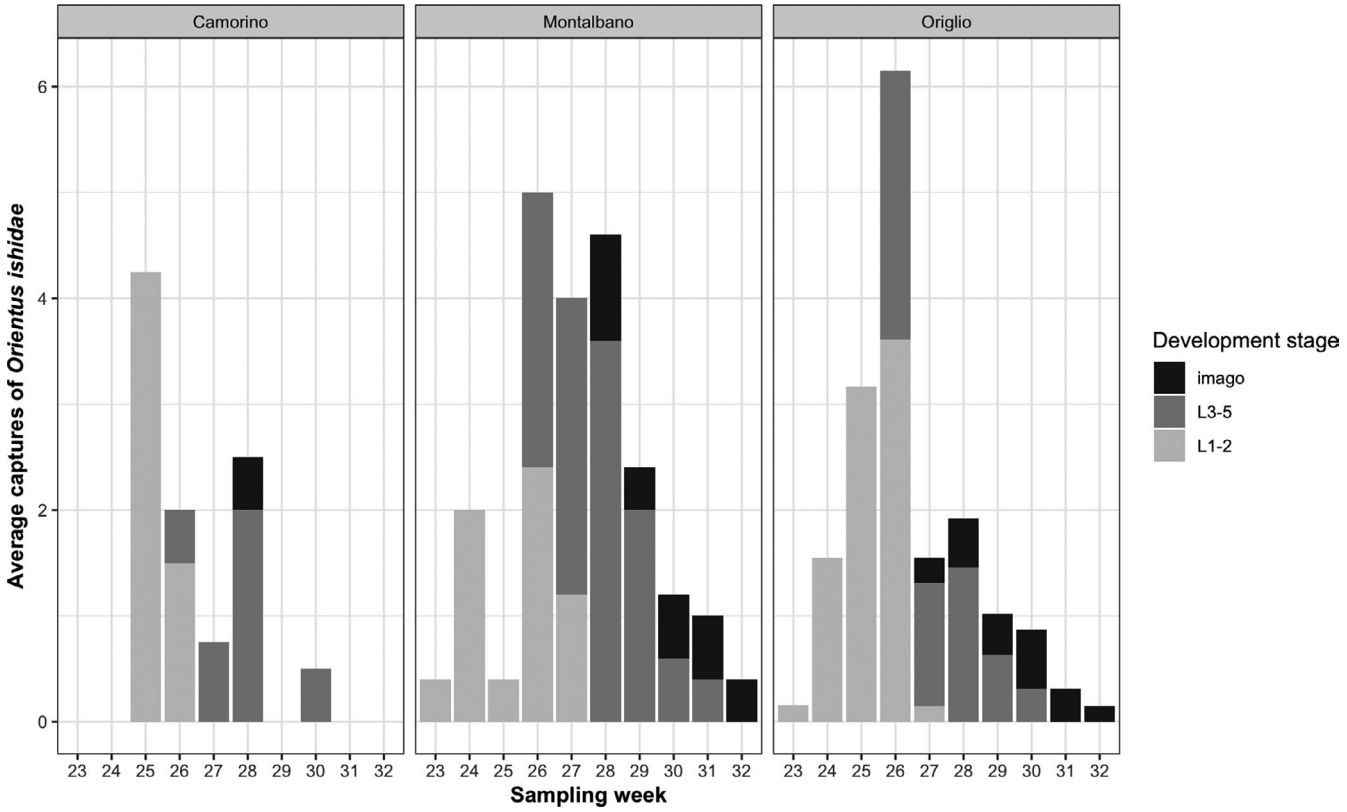

FIGURE 2 Weekly average captures of *Orientus ishidae* specimens per common alder caught with shaking and sweeping net. Development stages L1-2: light grey bars; L3-5: dark grey bars; imago: black bars. Number of sampled common alders per site: Camorino, $N = 4$; Montalbano, $N = 5$; Origlio, $N = 13$

TABLE 4 Leafhoppers captured on yellow sticky traps (YST) placed in vineyards and sampled for molecular analyses

		O. ishidae (total)		*O. ishidae* (nymphs)		*O. ishidae* (imago)		*S. titanus* (imago)	
Plot	N_{YST}	Captured	Sampled	Captured	Sampled	Captured	Sampled	Captured	Sampled
Camorino	6	1	1	0	0	1	1	7	7
Montalbano	9	6	5	3	2	3	3	2	2
Origlio	15	11	10	6	5	5	5	8	8
Total	30	18	16	9	7	9	9	17	17

Notes: Counts may differ whether the quality of the individual specimens was deemed as insufficient for the molecular analysis. Specimens of *Scaphoideus titanus* caught in Camorino were sampled but not analysed.

Holz et al., 2016; Jarausch et al., 2019; Maixner et al., 2000; Malembic-Maher et al., 2020; Radonjić et al., 2013).

The very high 16SrV-p infection rate of *O. ishidae* caught on alders (i.e. 69% for L3-5 nymphal instars and 85% for imagoes, respectively) is in line with recent studies from the same area (50% to 87% in pooled samples, depending on the study site; Jermini et al., 2019), and other regions such as France and Germany (50% and 61%, respectively; Malembic-Maher et al., 2020). In contrast, the infection rates of *O. ishidae* populations collected on other host plants such as hazelnut, or generically in the landscape using YST, are usually lower not only in the study area (e.g. 26% in both Trivellone et al., 2016 and Casati et al., 2017), but also in other regions such as Piedmont (Italy) (7.4% and 12.8%, based on the monitoring year; Lessio et al., 2016).

4.2 | Possible epidemiological role of the *Alnus glutinosa*-*Orientus ishidae* system in FD epidemics

The sequencing of the *secY-map locus* from *A. glutinosa* and *O. ishidae* provided evidence of the presence of genotypes belonging to the *map*-FD1 and FD3 groups, while the only *map* genotype found in the nearby grapevine samples was M54 (*map*-FD2 group), which is the dominant genotype in FDp-infected grapevines in southern Switzerland (Casati et al., 2017). Consequently, the present case study does not reveal any role of the landscape nor, in particular, of common alder as an infection reservoir for the vineyard, despite the high phytoplasma infection rate of common alders and *O. ishidae* vectors. A different situation was instead suggested by the results of

TABLE 5 Number of analysed and 16SrV-infected specimens collected on common alder trees and on yellow sticky traps (YST) placed inside the vineyard and corresponding infection rate

Plot	Species/Stage	Sampling unit	$N_{Analysed}$	$N_{Infected}$	Infection rate
Camorino	*O. ishidae* (nymphs)	Alder (*N* = 4)	15	9	0.60
		YST (*N* = 6)	0	*NA*	*NA*
	O. ishidae (imagoes)	Alder (*N* = 4)	2	2	1.00
		YST (*N* = 6)	0	*NA*	*NA*
	S. titanus	Alder (*N* = 4)	0	*NA*	*NA*
		YST (*N* = 6)	0	*NA*	*NA*
Montalbano	*O. ishidae* (nymphs)	Alder (*N* = 5)	58	45	0.78
		YST (*N* = 9)	2	0	0.00
	O. ishidae (imagoes)	Alder (*N* = 5)	15	10	0.67
		YST (*N* = 9)	3	0	0.00
	S. titanus	Alder (*N* = 5)	0	*NA*	*NA*
		YST (*N* = 9)	2	0	0.00
Origlio	*O. ishidae* (nymphs)	Alder (*N* = 13)	73	51	0.70
		YST (*N* = 15)	5	0	0.00
	O. ishidae (imago)	Alder (*N* = 13)	27	24	0.89
		YST (*N* = 15)	4	1	0.25
	S. titanus	Alder (*N* = 13)	0	*NA*	*NA*
		YST (*N* = 15)	8	0	0.00

Casati et al. (2017), who found *map*-FD1 and FD2 genotypes in host plants such as grapevine, hazelnut and willow, as well as in insect vectors such as *S. titanus* and *O. ishidae* in a closed site.

Among the 16SrV-C phytoplasmas, some are classified as FDp sensu *stricto*, as they can be transmitted by *S. titanus* and consequently trigger epidemics in European wine-growing areas. Others are transferred to grapevines by alternative vectors, although they do not cause FD epidemics as they are not transmissible by *S. titanus*. Others still have never been found in grapevine to date. Clearly, host ecology and FD epidemiology are strictly linked and can determine the outburst of FD epidemics, depending on environmental conditions. Concerning alders, they seem to host almost all diverse strains belonging to the 16SrV-C phytoplasma group, as identified all over Europe. Some of these strains can be transmitted by *Oncopsis alni* (Schrank, 1801) only, others by *O. ishidae* and/or *Allygus* spp., some also by *S. titanus* (Arnaud et al., 2007; Atanasova et al., 2014; Cvrković et al., 2008; Holz et al., 2016; Jurga & Zwolińska, 2020; Maixner et al., 2000; Malembic-Maher et al., 2020; Plavec et al., 2019; Radonjić et al., 2013). In particular, diverse molecular features of phytoplasma *vmp* gene sequences and VmpA protein binding properties appear to be associated with transmissibility by *S. titanus* and/or other vectors (Malembic-Maher et al., 2020).

The most common *map* genotype found in the present study on common alder and *O. ishidae* samples was M50, which is typical of the reference strain FD70 (*map*-FD1 group). Other surveys carried out on alder elsewhere, in particular in Montenegro, Macedonia and Germany, have sporadically identified phytoplasmas belonging to the *map*-FD1 group (Atanasova et al., 2014; Holz et al., 2016; Radonjić et al., 2013). The broad survey of Malembic-Maher et al. (2020) points out that the M50 *map* genotype is frequently found in Italy, Hungary and France not only in *A. glutinosa* and *O. ishidae*, but also in *V. vinifera* and *S. titanus* in presence of FD outbreaks, and sometimes even in *C. vitalba* and *O. alni*. Recent studies by Desqué et al. (2019) and Jarausch et al. (2019) have also reported common alder as an infection source of M50 and M38 for *O. ishidae*. In particular, Jarausch et al. (2019) demonstrated that *O. ishidae* (and *Allygus* spp.) was able to transmit M50 and M38 to *V. faba* and back to *A. glutinosa*. In the present study, one imago of *O. ishidae* caught in the vineyard of Origlio was found to be infected by the M50 *map* genotype (sample Oi1201 in Figure 3), which was also found in most of the common alder samples. Even though the nucleotide identity of a single genetic *locus* cannot unambiguously prove the identity of the phytoplasma strains found in host plants and insect vectors, it is intriguing to speculate that the specimen Oi1201 indeed acquired the M50 genotype on common alder and subsequently made its way to the vineyard.

In addition to M50, M12 (*map*-FD3 group; Figure 3) was found to infect two *O. ishidae* specimens in Origlio (Oi1001 and Oi1062). M12 is also compatible with *S. titanus* and *V. vinifera* as it has been found in grapevines in FD epidemic areas in north-western Italy (Malembic-Maher et al., 2020). Likewise, Casati et al. (2017) caught infected insects carrying *map*-FD3 group genotypes in southern Switzerland, in particular several specimens of *O. ishidae* in the landscape surrounding the studied vineyard. It is worth noting that *map*-FD3 genotypes had never been found in alders in countries other than Italy (Malembic-Maher et al., 2020).

In the present work, the *map* genotypes M44 and M47 were also found in common alder trees. M44 and M47 have already been

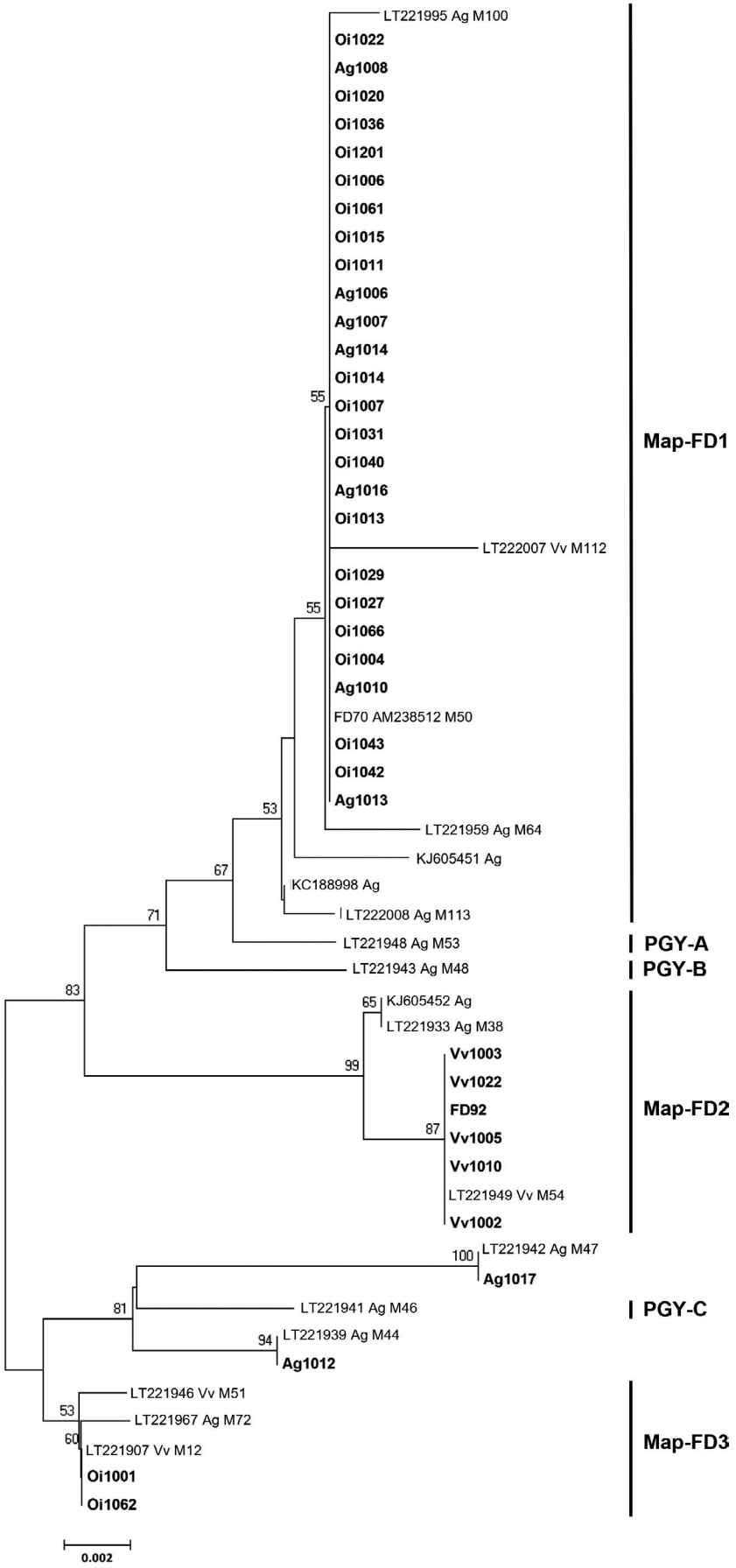

FIGURE 3 Unrooted phylogenetic tree based on 675 nt sites of the *secY-map* gene sequences obtained from analysed *Orientus ishidae* (Oi), *Vitis vinifera* (Vv) and *Alnus glutinosa* (Ag) samples (in bold), together with reference phytoplasma sequences from GenBank. The tree was constructed using the Neighbour-Joining clustering method using the MEGA4 software. Numbers on the branches are confidence bootstrap values obtained for 1,000 replicates. Phytoplasma strains and their GenBank accession numbers are reported in Table 3. The bar represents the genetic distance (number of substitutions per site), computed using the maximum composite likelihood method

described as infecting *A. glutinosa* and *O. alni* in France, Germany and Hungary, and, in particular, M47 has also been sporadically found in *Allygus* spp. and in *V. vinifera* in Germany, but not linked to FD epidemics (Malembic-Maher et al., 2020). However, both M44 and M47 have never been found in *O. ishidae* (Malembic-Maher et al., 2020), which is in line with the results obtained in the present study.

So far, most of the phytoplasma strains identified in *Alnus* spp. are not relevant to FD epidemics in Europe. In Germany, they are instead associated with palatinate grapevine yellows (PGY) phytoplasmas (clustered in three groups PGY-A, PGY-B and PGY-C), which are transmitted from common alder to grapevine by *O. alni*, but cannot be transmitted by *S. titanus* as a vector of secondary infections within vineyards (Maixner et al., 2000; Malembic-Maher et al., 2020). Other phytoplasmas phylogenetically similar to PGYs have been found all over Europe in alder, but rarely or never in grapevine (Arnaud et al., 2007; Atanasova et al., 2014; Cvrković et al., 2008; Holz et al., 2016; Malembic-Maher et al., 2020; Plavec et al., 2019; Radonjić et al., 2013). In contrast, although based on few alder specimens, data from Switzerland, Macedonia and Montenegro suggest the high prevalence of genotypes similar to FDp in *A. glutinosa* that are potentially transmissible even by *S. titanus* (Figure 3; Atanasova et al., 2014; Radonjić et al., 2013).

5 | CONCLUDING REMARKS AND RESEARCH OUTLOOK

Except for the role of American grapevine in the wild compartment (Lessio et al., 2007; Ripamonti et al., 2020), there is lively debate about the alternative components of the FDp epidemiological system. A particular challenge in this context remains to properly assess the possible role of the landscape surrounding vineyards in hosting alternative FDp host plant species and vectors able to induce a flow of FDp genotypes from the wild compartment to the vineyard that may eventually increase the risk of FD outbreaks (Casati et al., 2017).

The results of the present study confirm the existence of an alternative FDp epidemiological cycle represented by *A. glutinosa* and *O. ishidae*. However, they do not indicate any role in the FDp epidemics in the vineyards of southern Switzerland. Nevertheless, considering the compatibility of several *map* genotypes shared by *S. titanus*, *V. vinifera*, alternative vectors such as *O. ishidae*, and alternative FDp host plant species such as *A. glutinosa*, primary phytoplasma flow may occasionally take place from the forest into vineyards. If compatible with *S. titanus*, such FDp genotypes may even trigger local FD outbreaks and secondary infections in vineyards with previously undetected FDp genotypes.

Primary FDp infections in vineyards caused by the association between *A. glutinosa* and *Allygus* spp. have already been observed by Jarausch et al. (2021) in Germany. The absence of *S. titanus* spared the concerned vineyard from secondary infections. In Sicily (Italy), the alternative FDp host plant *Spartium junceum* was found to be infected by FDp, without, however, posing a threat to vineyards since no infections to grapevine have been confirmed to date. Indeed, *S. titanus* is currently not present in the area (Rizza et al., 2021). In contrast, for the alternative system *C. vitalba*–*D. europaea*, FD outbreaks have already been documented in Italian and Serbian vineyards, where *S. titanus* has demonstrated its ability to cause subsequent secondary infections after acquiring *map*-FD3 genotypes (Filippin et al., 2009).

The future role of *S. titanus* as a vector of secondary infections inside vineyards may not depend on natural and human-assisted expansion only, but also on the effects of climate change (Chuche & Thiéry, 2014; Ge & Wen, 2006; Sneiders et al., 2019; Steffek et al., 2007). Indeed, in a scenario in which *S. titanus* is able to colonize new areas where common alder and alternative secondary vectors such as *O. ishidae* already occur, the conditions for an epidemic spread of FDp inside vineyards could be met (Chuche & Thiéry, 2014; Rigamonti et al., 2018).

Finally, the employment of the *secY-map* genetic *locus*, although not combined with other markers, allowed the comparison of the genotypes found in this and other studies and provided the first insight into the potential contribution of *A. glutinosa* to the FD epidemics in southern Switzerland. The inclusion of a greater number of genetic markers such as *malG*, *dnaK* and *vmpA* may further improve the understanding of phytoplasma flow between landscape compartments, as already proposed by Rossi et al. (2019) among others.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

AR contributed to experimental design, field data collection, insect determination, data analysis and manuscript writing and editing. EB contributed to field data collection, insect determination, molecular analyses and writing. MJ contributed to experimental design, manuscript editing and funding acquisition. MC contributed to experimental design, manuscript editing, funding acquisition and project coordination. LF contributed to conceptualization, molecular and bioinformatic analyses, and manuscript editing. EA contributed to conceptualization, manuscript editing and project coordination. All authors contributed to the writing of this manuscript and agreed on its content. AR and EB contributed equally.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available at https://www.doi.org/10.16904/envidat.221.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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