New insights on Flavescence dorée phytoplasma ecology in the vineyard agro-ecosystem in southern Switzerland

Journal:	*Annals of Applied Biology*
Manuscript ID	AAB-2016-0324.R2
Manuscript Type:	Research paper
Date Submitted by the Author:	n/a
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Key Words:	Grapevine yellows, Phytoplasma, Insect vectors, map gene, Orientus ishidae, Hazel, Willow

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New insights on Flavescence dorée phytoplasma ecology in the vineyard agro-ecosystem in southern Switzerland

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Running title: Insights on Flavescence dorée ecology

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SUMMARY

Phytoplasmas associated with Flavescence dorée (FDp) grapevine disease are quarantine pathogens controlled through mandatory measures including the prompt eradication and destruction of diseased plants, and the insecticide treatments against the insect vector, the ampelophagous leafhopper *Scaphoideus titanus*. In the present study, a multidisciplinary approach has been applied to investigate the FDp ecological cycle in a test vineyard agro-ecosystem in Canton Ticino, south Switzerland. Despite the scarce population density of *S. titanus*, a regular trend of new infections (3.4% of the total vines) through the years was observed. The leafhopper *Orientus ishidae* was found as the most abundant among the captured insect species known as phytoplasma vectors (245 out of 315 specimens). The population of *O. ishidae* was evidenced prevalently (167 specimens) in the south-western side of the vineyard and within the neighboring forest constituted mainly by hazel (*Corylus avellana*) and willow (*Salix* spp.). These plant species were found infected by FDp related strains (30% of analyzed trees) for the first time in this study. Interestingly, *O. ishidae* was found to harbor FDp related strains in high percentage (26% of the analyzed pools). In addition, 16SrV phytoplasma group was detected for the first time in the insect *Hyalesthes obsoletus* and a FDp related strain in *Thamnotettix dilutior*, present in low populations within the test vineyard. Molecular characterization and phylogenetic analyses of *map* gene sequences of FDp and related strains, here identified, revealed the great prevalence of the *map*-type FD2 in grapevines (97%) and in *O. ishidae* pools (72%). Such a *map*-type was found also in hazel and in *T. dilutior*, but not in *S. titanus*. Moreover, *map*-types FD1 and FD3 were identified for the first time in Switzerland in several host plants and phytoplasma vectors, including grapevine (FD1), *S. titanus* (FD1) and *O. ishidae* (FD1 and FD3). Based on the data obtained in this study, it is reasonable to hypothesize that the ecological cycle of FDp could be related not exclusively to the grapevine-specific feeding diet of *S. titanus*, but it could include other insect vector(s) and/or plant host(s). Further studies will be

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needed to prove the role of *O. ishidae* as vector able to transmit FDp from wild plants (e.g. hazel) to grapevine.

Keywords: Grapevine yellows; Phytoplasma; Insect vectors; *map* gene; *Orientus ishidae*; Hazel; Willow

INTRODUCTION

Flavescence dorée (FD) is the most epidemic disease within the grapevine yellows (GY) complex, and causes strong economic damages to viticulture in Europe, both in terms of quality and quantity (Belli *et al*., 2010). Typical FD symptoms, indistinguishable from those associated with other diseases of the GY complex, include berry shrivel, desiccation of inflorescences, color alterations and curling of the leaves, reduction of growth, and irregular ripening of wood (Belli *et al*., 2010).

Based on nucleotide sequence analysis of the gene *16S rRNA* and the intergenic spacer between *16S* and *23S rRNA* genes, FD phytoplasmas (FDp) have been classified in two taxonomic subgroups belonging to the 16SrV group (Elm yellows group), 16SrV-C and -D (Lee *et al*., 1998; Angelini *et al*., 2001; Davis & Dally, 2001; Lee *et al*., 2004). As reported for other phytoplasmas belonging to taxonomic group 16SrV (Martini *et al*., 2002; Navrátil *et al*., 2009; Malembic-Maher *et al*., 2011), the variability of the *16S rRNA* gene is insufficient to account for the emerging ecological differences in FDp biological cycles. Thus, further studies focus on finer differentiation of phytoplasmas associated with FD. Molecular and phylogenetic analyses on the genetic locus *map* demonstrate the presence of three *map*-types: FD1 and FD3 (including strains from subgroup 16SrV-C), and FD2 (including strains from subgroup 16SrV-D) (Arnaud *et al.*, 2007). Geographic distribution of *map*-types is different: (i) FD1 and FD2 have been identified, respectively, in 17% and 83% of symptomatic grapevines in France, where FD3 is not present (Arnaud *et al*., 2007); (ii)

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the three *map*-types have been identified in Italy, with a prevalence of FD1 and FD3 in north-western and north-eastern regions, respectively (Arnaud *et al*., 2007; Foissac & Maixner, 2013); (iii) FD2 and FD3 have been detected in Slovenia and Croatia, with a prevalence of FD3 (Mehle *et al*., 2011; Plavec *et al*., 2015); (iv) only FD3 has been identified in Serbia (Filippin *et al*., 2009); (v) in northern Spain, Portugal and Switzerland only FD2 has been reported (Foissac & Maixner, 2013).

Moreover, nucleotide sequences and phylogenetic analyses from multiple genes indicate that FDp, Palatinate grapevine yellows phytoplasmas (PGYp) and Alder yellows phytoplasma (AldYp) are clustered together, supporting their monophyletic origin (Arnaud *et al*., 2007; Holz *et al*., 2015). AldYp, widespread in Europe and never detected in America, is transmitted by the insect *Oncopsis alni* (Schrank) (Maixner & Reinert, 1999). Such highlights suggest that FDp could have originated in Europe. As a matter of fact, it has been hypothesized that (i) AldYp strains, genetically related to FD1, FD2 and FD3 *map*-types of FDp, are occasionally transmitted to grapevine by *O. alni* (Maixner *et al.*, 2000); (ii) after the entrance of *Scaphoideus titanus* Ball in Europe, such AldYp strains have been transmitted exclusively to grapevine, generating the FD epidemics (Arnaud *et al*., 2007).

Due to the host plant range and feeding preferences of each phytoplasma vector, the majority of GY (e.g. Bois Noir and Palatinate grapevine yellows) spreads only slowly and, apparently, not from vine to vine. On the other hand, FD is an epidemic disease characterized by its rapid spread within vineyards due to vine-to-vine transmission (EPPO/CABI, 1996). FDp are transmitted by *S. titanus*, a monovoltine leafhopper accidentally introduced to France from North America in the 1950ies. In Europe only species of the *Vitis* genus have been shown to sustain the whole life cycle, from egg to egg, of *S. titanus*; moreover, this leafhopper is considered as oligophagous (Chuche & Thiéry, 2014). Consequently, geographic areas hosting large vector populations can be damaged by strong FD epidemics if FDp are also present. Due to this aspect,

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phytoplasmas associated with FD are quarantine pathogens to be controlled through mandatory measures: (i) promptly eradicating and destroying any plants showing GY type symptoms, even before confirmation of FD infection by laboratory tests where FD is already present, and (ii) compulsory insecticide sprayings to control the *S. titanus* populations. The number of applications varies from one to three per year (targeting nymphs and adults) in commercial vineyards and can be more numerous in nurseries (EFSA, 2014). Even though these measures have reduced the impact of FD, the still broad presence of FDp in different countries indicates that FD epidemic is still important (Botti & Bertaccini, 2007; Jermini *et al*., 2014).

Moreover, recent studies report that the FD epidemiological cycle could be more complex than the exclusive FDp transmission from grapevine to grapevine by *S. titanus*; in fact, (i) FDp related strains are also commonly found in other plant hosts such as *Ailanthus*, *Alnus*, and *Clematis* (EFSA, 2014); (ii) several studies reveal that *S. titanus* can feed on other plant species, on which it can only partially complete its biological cycle (Schvester *et al.*, 1962; Caudwell *et al.*, 1970; Trivellone *et al.*, 2013); these host plants could play a role as source of inoculum for FDp transmission to grapevine (Chuche & Thiery, 2014); (iii) other studies reveal that FDp can be transmitted to grapevine by other insects. In detail, Filippin and colleagues (2009) have demonstrated that FDp can be transmitted from *Clematis vitalba* L. to grapevine by the planthopper *Dictyophara europaea* (L.). The frequency of phytoplasma transmission from *Clematis* to grapevine remains to be determined but phytoplasma transmission cannot provoke an FD outbreak in the absence of the leafhopper *S. titanus* (EFSA, 2014). Recently, the leahoppers *O. alni* (Maixner *et al*., 2000; Arnaud *et al*., 2007; Mehle *et al*., 2011) and *Orientus ishidae* (Matsumura) (Mehle *et al*., 2010, 2011; Gaffuri *et al*., 2011; Koczor *et al*., 2013) have been found infected by FDp related strains in Europe; moreover, latest findings revealed the capability of *O. ishidae* to transmit 16SrV group phytoplasmas to grapevine (Lessio *et al*., 2016).

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Based on such previously described evidences, in the present study carried out in Canton Ticino (Switzerland), a molecular epidemiology approach has been used to test if the FD complex could be considered as an open system by investigating the presence of putative vectors and host plants in the vineyard agro-ecosystem. A special focus has been laid on *O. ishidae*, previously found harboring FDp related strains in the studied area (Trivellone *et al.*, 2016); for the first time, particular attention was paid to investigate the epidemiological role of *O. ishidae* plant hosts in the vineyard and its surroundings.

MATERIALS AND METHODS

Characteristics of the test vineyard

Investigation about the FD epidemiology was carried out in one vineyard, hereby named "the test vineyard", located in Stabio, a town in the Southern wine-growing region of canton Ticino, Switzerland [WGS 84 (lat/lon) 45.85547, 8.92749; Alt 371 m] and in the surrounding vegetation. The test vineyard consists of 21 rows (2177 vines) of Chardonnay vines grafted on 3309 C rootstock. In this vineyard, grapevines are trained using the Guyot system (distance between rows 2 m; plant distance along the row 1 m), occasionally mowing between the rows and chemical weeding along the rows. The vineyard is bordered by woods to the south, meadows to northwest, and vineyards to the east (Figure 1). Based on the directives established by the Cantonal Phytosanitary Service, mandatory destruction of grapevine plants showing GY-typical symptoms are carried out in the vineyard, as well as insecticide treatments against *S. titanus* with the larvicidal insecticide Buprofezin at two time points, at maximum egg hatching and two weeks later, according to monitoring activities and model predictions (Prevostini *et al.*, 2013). Despite these measures, adopted since 2005, and the absence of abandoned vineyards, FD is still present in this area. In

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particular, 3.3% of the grapevine plants in our test vineyard were FDp-positive in 2013 (data from Research Station Agroscope Changins, Switzerland).

Insect monitoring and sampling

The activities were carried out from May to November 2014 with the aim to (i) monitor the presence of *S. titanus* and *O. ishidae* nymphs and adults developing on grapevine and wild plants in the test vineyard and in its surroundings, (ii) survey the leafhopper species known as putative phytoplasma vectors inhabiting the vine plants, (iii) determine the flight dynamics of *S. titanus* and *O. ishidae* by monitoring the presence of their adults spreading/dispersing through/across the test vineyard and in its surroundings.

(i) Three groups each of 30 grapevine plants, randomly selected within the test vineyard, have been sampled weekly from May 13 to September 30 (21 overall samples) with the beating tray method (one hit per plant). Moreover, wild plants hosting known leafhopper phytoplasma vectors, around and within the test vineyard and in neighbouring vineyards, have been occasionally sampled from June 3 to September 2 (38 overall samples) by the beating tray method (up to 4 hits per plant, according to the plant dimension, until reaching 40 hits).

(ii) Fourteen Aeroxon yellows sticky traps (10 x 25 cm) placed horizontally in grapevine canopy (Jermini *et al*., 1992a, 1992b) were used to detect the presence of *S. titanus* and other Auchenorrhyncha on the canopy inside the vineyard from July 01 to November 04. Traps were collected and substituted each week.

These two methods also allowed to simultaneously survey other leafhoppers inhabiting grapevine and wild plants in the test vineyard and in its surroundings.

(iii) Rebell yellow sticky traps (8 x 16 cm), placed vertically outside the plant canopy, were utilized to capture leafhoppers spreading/dispersing through/across the vineyard and the surrounding vegetation, with the specific objective to determine the flight dynamics of *S. titanus*

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and *O. ishidae*. In detail, six Rebell traps (V1 to V6; V, vineyard) were positioned inside the test vineyard (May 13 - November 04), eight (B1 to B8; B, border) on its borders (May 13 - November 04), and nine (W1 to W9; W, woods) outside of it (July 30 - November 04). Particularly, traps W1 to W7 were positioned at the external border of the forest neighbouring to the test vineyard, close to other vineyards of the examined area; traps W8 and W9 were positioned inside the forest neighbouring to the test vineyard (Figure 1). Traps were collected and substituted each week.

Auchenorrhyncha specimens, after being sorted out from the material caught by the traps and the beating tray, were individually identified at the species level with a stereo microscope. All individuals of species belonging to the subfamily Deltocephalinae, considered putative vectors of FDp (Bressan *et al*., 2006), and other leafhopper and planthopper species, reported in the scientific literature as known vectors of phytoplasmas, were preserved in 70 % alcohol for further molecular analyses.

Plant sampling

During a survey on GY carried out from July to September 2014 in the test vineyard, symptomatic leaves were sampled from 74 grapevine plants, showing GY symptoms for the first time. Furthermore, leaf samples were also collected from symptomless woody and shrubby plants of species (*Betula pendula*, *Carpinus betulus*, *Corylus avellana*, *Salix caprea*, *Salix* sp., *Prunus laurocerasus*, *Urtica dioica*) listed in the scientific literature as host plants of *O. ishidae* (https://gd.eppo.int/reporting/article-4763; Günthart *et al*., 2004; Nickel, 2010; Mehle *et al*., 2011). Finally, leaves were also collected from symptomless plants reported as hosts of phytoplasmas closely related to FD (*Clematis vitalba*) (Filippin *et al*., 2009). Leaf samples from Madagascar periwinkle (*Catharanthus roseus* L. (G. Don)) plants, maintained in the greenhouse of the Department of Agriculture and Environmental Sciences (University of Milan, Italy), infected by FDp strains FD92 (subgroup 16SrV-D) and FD70 (subgroup 16SrV-C), '*Ca*. Phytoplasma solani'

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strain STOL (subgroup 16SrXII-A), and '*Ca*. Phytoplasma asteris' strain AY1 (subgroup 16SrI-B), were collected and used as controls in molecular analyses.

Total nucleic acids extraction

Total nucleic acids were extracted from pools of the captured insects (each pool includes one to five specimens captured together on the same trap) following a protocol adapted from Alma *et al.* (1997). Briefly, the ethanol-preserved adults were dried onto filter paper, pooled and homogenised in a pre-warmed (65 °C) CTAB-based buffer (2.5% w/v cetyl-trimethyl-ammonium-bromide (CTAB); 100 mM Tris pH 8.0, 1.4 M NaCl; 50 mM EDTA pH8; 1% PVP-40; 0.5% ascorbic acid). After incubation at 65 °C for 20 min, nucleic acids were extracted with one volume of chloroform:isoamyl alcohol 24:1 v/v solution and then precipitated with the addition of one volume of cold isopropanol at -20 °C for 20 min. The obtained total nucleic acids pellet was washed with 70% ethanol, vacuum dried, redissolved in 50 µL TE pH 8.0 and maintained at -20 °C until further use.

Total nucleic acids were extracted from examined plants using a modified Angelini *et al.* (2001) protocol. Briefly, leaf petioles (1 g) were inserted in extraction bags (12 x 12 cm) (Bioreba, Reinach, Swizterland) and ground in 7 ml of pre-warmed (65 °C) 2.5% CTAB-based buffer (see above) using a Tissue Homogenizer (Sediag, Longvic, France). The solution (1 ml) was held at 65 °C for 30 min. After incubation, nucleic acids were extracted by adding chloroform:isoamyl alcohol 24:1 v/v solution and precipitated by incubation with isopropanol at -20 °C for 10 min. The nucleic acid pellet was washed with 70% ethanol, dried, resuspended in TE buffer, re-precipitated with sodium acetate and absolute ethanol, and re-purified by washing with 70% and 80% ethanol. Obtained nucleic acids pellet was air-dried, suspended in 100 µL of deionised autoclaved water and maintained at -30 °C until further use.

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Detection of Flavescence dorée phytoplasma and related strains

Total nucleic acids extracted from each plant sample and insect pool were used as templates for real-time PCR assays carried out for the FDp and related strains-specific amplification of the gene *rplN*, coding the ribosomal protein L14 (Durante *et al*., 2012), using the commercial kit Real-time PCR (TaqMan probe and primers) (IPADLAB, Lodi, Italy) and TaqMan Universal PCR master mix (Applied Biosystems, Monza, Italy), according to the manufacturer's instructions. Thermocycling was carried out on the StepOnePlus Real-Time PCR System (Applied Biosystems,) and consisted of an initial denaturation at 95 °C for 10 min followed by 40 cycles of 15 sec at 95 °C and 1 min at 62 °C. Total nucleic acids extracted from periwinkle plants infected by phytoplasma strains were used as positive (strains FD92 and FD70) and negative (strains AY1 and STOL) controls; moreover, total nucleic acids from healthy periwinkle plants and reaction mixture without template were used as negative controls. Each sample was analyzed in duplicate.

Flavescence dorée phytoplasma and related strain typing

FDp and related strains, identified in plants and insect pools by real-time PCR assay, were typed by PCR-based amplification and sequence analyses of the gene *map* encoding a methionine aminopeptidase (Arnaud *et al*., 2007). Precisely, total nucleic acids of infected plants and insect pools were employed as templates in nested PCR assays performed using primer pair FD9f5/MAPr1 followed by FD9f6/MAPr2, following the reaction conditions previously described (Arnaud *et al*., 2007). All amplifications were performed using the GeneAmp PCR System 9700 (Applied Biosystem). Positive and negative controls employed in nested PCR amplification of *map* gene were as described above. PCR products were analysed by electrophoresis in 1% agarose gels, stained with ethidium bromide and observed under UV light.

As indicated by Arnaud *et al*. (2007), *map* gene amplicons were typed by restriction fragment length polymorphism (RFLP) analysis performed through double digestion with the

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enzymes *Alu*I and *Eco*72I Fast digest (MBI-Fermentas). Reactions were carried out according to the manufacturer's instructions. RFLP profiles obtained were visualized under UV light in 3% agarose gels stained with ethidium bromide. Attribution of FDp and related strains, identified in the present study, to the *map*-types FD1, 2, and 3 was carried out through the comparison of their *Alu*I-*Eco*72I-RFLP profiles with those described in literature (Arnaud *et al.*, 2007).

Map gene fragments amplified from FDp and related strains, representative of the obtained *Alu*I-*Eco*72I-RFLP profiles and of the phytoplasma hosts, were selected for nucleotide sequence analyses. The *map* gene amplicons were sequenced in both senses (employing primers FD9f6 and MAPr2) by a commercial service (Eurofins, Milan, Italy) to achieve at least 5x coverage per base position. Nucleotide sequence data were assembled by employing the Contig Assembling program of the software BioEdit version 7.0.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). *Map* gene nucleotide sequences of FDp and 16SrV group phytoplasma strains previously published were retrieved from GenBank (Table S1) and utilized for comparison with the sequences obtained in this study. *Map* gene nucleotide sequences were aligned using the "ClustalW Multiple Alignment" application and analyzed for sequence identity determination using the "Sequence Identity Matrix" application of the software BioEdit.

Nucleotide sequences of *map* gene of FDp and related strains and 16SrV group phytoplasma strains from the present study and previously described in GenBank were employed for phylogenetic analyses. Minimum-Evolution method was carried out using the Jukes-Cantor model and bootstrap replicated 1000 times with the software MEGA6 to obtain an unrooted phylogenetic tree (Tamura *et al.*, 2013).

RESULTS

Insect monitoring

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A total of 315 specimens belonging to seven species, previously reported as known or putative phytoplasma vectors, were captured on Rebell and Aeroxon sticky traps positioned inside, on the borders and outside the test vineyard, and by beating tray method. The captured specimens belonged to two Cixiidae species, *Hyalesthes obsoletus* Signoret (15) and *Reptalus cuspidatus* (Fieber) (6), and to five Cicadellidae species, *Orientus ishidae* (245 specimens), *Scaphoideus titanus* (45), *Thamnotettix dilutior* (Kirschbaum) (2), *Fieberiella florii* (Stål) (1), and *Anoplotettix fuscovenosus* (Ferrari) (1), all belonging to the Deltocephalinae subfamily (Table 1).

Captures obtained by beating tray method and Aeroxon traps evidenced that three insect species (*S. titanus*, *O. ishidae*, and *H. obsoletus*) are present on the grapevine canopy in the test vineyard. In detail, it was possible to observe on grapevine canopy (i) three nymphs (two at May 27 and one at July 1st) and 13 adults (two captured by beating tray in August and 11 by Aeroxon trap in August/September) of *S. titanus*, evidencing a sporadic presence of the insect throughout the season (Figure 2A); (ii) 22 adults of *O. ishidae* captured by Aeroxon traps, evidencing a sporadic presence of the insect during the summer and a peak of captures in October (Figure 2A); (iii) three adults of *H. obsoletus* captured in July by Aeroxon traps. Furthermore, from June 21 to July 30, beating tray method allowed the capture of four nymphs of *O. ishidae* from willow (two positive samples out of six), and two nymphs and three adults of *O. ishidae* from hazel tree (three positive samples out of six).

Captures obtained by Rebell traps highlighted that (i) *O. ishidae* adult population was prevalently spread on the borders (111 out of 214) and on the surrounding vegetation (96 out of 214); only seven specimens were captured inside the test vineyard; (ii) the same trend was observed for *S. titanus* (28 specimens out of 29 on the borders and outside the vineyard; only one specimen captured inside the vineyard) (Table 1). Moreover, the obtained results evidenced that specimens of *O. ishidae* (167 out of 214) and *S. titanus* (28 out of 29) were mainly captured on four traps located closely together at the south-western limit of the experimental area. Two traps (B3, B4) were on the

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borders of the test vineyard (103 *O. ishidae* and 7 *S. titanus*), and two (W8, W9) were on the surrounding vegetation, inside the forest where several hazel trees are present (64 *O. ishidae* and 21 *S. titanus*) (Figure 1, Table 1). Concerning other planthoppers and leafhoppers, *R. cuspidatus*, *T. dilutior* and the majority (9 out of 15) of *H. obsoletus* specimens were captured by traps positioned on the vineyard borders. Moreover, unique specimens of *F. florii* and *A. fuscovenosus* were captured inside and outside the vineyard, respectively (Figure 1, Table 1).

Given the numbers of captured specimens per species, it was possible to investigate only the flight dynamic of *O. ishidae* and the pattern of *S. titanus* occurrence in the experimental area. In order to compare the results, data were reported as average weekly captures. Inside and on the borders of the test vineyard, dispersing *O. ishidae* adults were captured continuously by Rebell traps (V1 to V6 and B1 to B8) from the end of June until October, with the presence of two periods of higher catches at (i) end of July / beginning of August and (ii) mid October, respectively. The presence of *S. titanus* was negligible with sporadic catches from August to October (Figure 2B). Outside the vineyard, Rebell traps (W1 to W9) confirmed the pattern of *O. ishidae* captures, as observed inside the vineyard from July to September, but were unable to confirm the presence of dispersing adults during October (Figure 2C). The presence of *S. titanus* outside the vineyard was scarce, with low but regular captures from mid September to mid October (Figure 2C).

FDp and related strain identification

Real-time PCR assays performed to identify FDp and related strains amplified DNA extracted from periwinkle plants infected by FDp strains FD92 and FD70 showed an average Ct (threshold cycle) of 18.05 and 18.88, respectively. No amplification was observed for periwinkle plants infected by phytoplasma strains STOL and AY1, or reaction mixtures without DNA. Only PCR products amplified from insects and plants, with an average Ct < 37, were associated with the presence of FDp and related strains. qPCR assays were carried out on total nucleic acids extracted

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from 119 plant leaf samples and 146 insect pools. FDp and related strain distribution among tested plants is as follows: 70 out of 74 (95%) grapevine samples, 11 out of 31 (35%) hazel tree samples, 3 out of 10 (30%) willow samples, and 3 out of 4 (75%) *Clematis vitalba* samples (Table 2).

Out of 301 leafhoppers captured by sticky traps, 274 specimens were grouped in 146 pools for further molecular analyses. FDp and related strains were detected in 30 out of 146 insect pools (21%). In particular, it was identified in 25 out of 97 (26%) *O. ishidae* pools, 2 out of 29 (7%) *S. titanus* pools, 1 out of 11 (9%) *H. obsoletus* pools and 2 out of 2 (100%) *T. dilutior* pools. All examined pools of *F. florii, R. cuspidatus*, and *A. fuscovenosus* were negative (Table 1).

FDp and related strain typing

Nested PCRs carried out using the primer pair FD9f6/MAPr2 amplified DNA from all the plant and insect samples infected by FDp and related strains, with the exception of 5 hazel leaf samples (out of 11) and 2 willow leaf samples (out of 3) (Table 1 and 2). Enzymatic digestions of FD9f6/MAPr2 amplicons with the enzymes *Alu*I and *Eco*72I produced three restriction profiles among FDp and related strains identified in the present study (Figure 3). The *Alu*I-*Eco*72I-RFLP patterns obtained in this study were compared to those described in the literature (*i.e.* FD1, FD2 and FD3; Arnaud *et al*., 2007). Among the FDp and related strains identified from plants and insects in the test vineyard, the *map*-type FD2 was prevalent (89 out of 108, or 82%), while *map*-types FD1 and FD3 were identified in 13 (12%) and 6 (6%) instances, respectively (Table 1 and 2). FD2 was detected in grapevine (68 plants), hazel trees (1 plant), *O. ishidae* (18 pools) and *T. dilutior* (2 pools). FD1 was identified in grapevine (2 plants), hazel tree (3 plants), willow (1 plant), *O. ishidae* (5 pools), and *S. titanus* (2 pools). FD3 was identified in hazel trees (2 plants), *C. vitalba* (3 plants), *O. ishidae* (2 pools), and *H. obsoletus* (1 pool) (Table 1 and 2).

Nucleotide sequence analyses, performed on 22 FDp and related strains and 16SrV group phytoplasma strains selected on the basis of both their *map*-type and the phytoplasma hosts (Table

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3), confirmed their assignment to *map*-types as determined by the RFLP assays. As a matter of fact, (i) FDp and related strains with an FD1 RFLP profile shared best *map* gene sequence identity (99.8 to 100%) with strain FD70, a FD1 reference strain; (ii) FDp and related strains with an FD2 RFLP profile shared best *map* gene sequence identity (100%) with strain V00-SP5, a FD2 reference strain; (iii) FDp-related and 16SrV group phytoplasma strains with an FD3 RFLP profile shared best *map* gene sequence identity (96.9 to 99.6%) with strain VI04-Toscana1, a FD3 reference strain (Table S2).

Clustering in a phylogenetic tree built using the *map* gene nucleotide sequences isolated from plant and insect hosts in this study clearly confirms the attribution of the FDp and related strains and 16SrV group phytoplasma strains to *map*-types FD1, FD2 and FD3 (Figure 4).

DISCUSSION

Due to the mandatory application of insecticides, the population of the FDp vector *S. titanus* within the test vineyard was very low, as shown by the very small number of specimens (three nymphs and two adults) collected by the beating tray method during the growing season and corroborated by the few captures of adults on the Rebell and Aeroxon yellow sticky trap placed inside the test vineyard. This trend confirmed previous evidences by Trivellone *et al*. (2016), reporting high *S. titanus* populations in Swiss localities where its control was not mandatory, and low abundances in vineyards treated with insecticides twice during the growing season. Previous works have demonstrated that *S. titanus* can survive and feed on several plants (Trivellone *et al*., 2013; Chuche & Thiery, 2014), develop on elm (Gibson, 1973) and lay eggs on elder (Gargani *et al*., 2013). In the present study, the majority of *S. titanus* specimens (28 out of 40) was captured on traps B3, B4, W8 and W9 (Figure 1), placed on the borders and outside the test vineyard, close to a wood of mostly hazel and willow trees, with no wild grapevines to be found. Moreover, the two FDp positive *S. titanus* pools, captured on traps W8 and B4, were infected by the *map*-type FD1

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(Table 1). FD1 was never found before in Switzerland neither in vineyards nor in *S. titanus*, but was the most frequent *map*-type on hazel and willow trees in the experimental area, and affected a low minority (two out of 70) of FDp-infected grapevine plants in the test vineyard (Table 2). On the other hand, *S. titanus* was not captured by beating tray from hazel and willow trees in the surroundings of the test vineyard. Based on such evidences, it is reasonable to raise the question if *S. titanus* can live, but not develop, on wild plants long enough to acquire FDp and related strains.

Despite the compulsory destruction of grapevines with GY typical symptoms and the insecticide treatments, applied at two distinct time points, 74 symptomatic grapevines (3.4%) were identified in the test vineyard in 2014, the same levels as observed in the previous year by Agroscope, suggesting a stable infection rate through the years. This, together with the low populations of *S. titanus* inside and outside the vineyard, as well as the absence of *D. europaea* and *O. alni* [insects known to transmit FDp and related strains from wild plants to grapevine (Chuche & Thiery, 2014)], points at a scenario, in which FDp could be vectored by other phloem-feeding insects. Interestingly, this study revealed an abundant population of the leafhopper *O. ishidae*, recently found able to transmit 16SrV phytoplasmas to vines (Lessio *et al*., 2016). *O. ishidae* was found to harbor FD related phytoplasma in high percentage (26% of the examined pools), prevalently at the borders of the test vineyard and within the wood. Our data confirms the *O. ishidae* contamination rates by FDp related strains recently reported for Canton Ticino, Slovenia and Italy (Mehle *et al*., 2010; Gaffuri *et al*., 2011; Trivellone *et al*., 2016) and its presence in vineyards surrounded by woods (Trivellone *et al*., 2016).

Previous studies report *O. ishidae* as polyphagous, mainly on woody plants (Hamilton, 1985; Nickel, 2010; Mehle *et al*., 2011; https://gd.eppo.int/reporting/article-4763). Here, as previously reported, nymphs and adults of *O. ishidae* were collected from hazel and willow trees by beating tray method; moreover, the highest density of *O. ishidae* (167 specimens) was observed on traps B3, B4, W8 and W9, which were placed in the southwestern side of the experimental area, at

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the border of the test vineyard and within a wood of mostly hazel (*Corylus avellana*), already known as a plant host for '*Candidatus* Phytoplasma asteris' (Cieślińska & Kowalik, 2011), and willow (*Salix* spp.) trees, found infected by FDp related strains (30% of the analyzed samples) for the first time. Moreover, beating tray captures link *O. ishidae* nymphs and adults with hazel and willow, species found infected by FDp related strains in the present study, confirming the role of these plant species as hosts for *O. ishidae* (Lessio *et al*., 2016). On the other hand, *O. ishidae* was not captured by beating tray on *Clematis vitalba*, a plant rarely found in the examined vineyard and previously reported to host FDp (Filippin *et al*., 2009). Even more interestingly, even if no *O. ishidae* specimen was captured by beating tray on grapevine plants, 22 specimens were captured inside the vineyard, mainly on horizontal Aeroxon traps, which are known to catch insects living on the vine canopies, suggesting that *O. ishidae* adults could be present on grapevine for extended periods.

Intriguingly, PCR tests revealed for the first time the presence of 16SrV group phytoplasma in the Cixiidae *Hyalesthes obsoletus*, the vector of '*Candidatus* Phytoplasma solani', associated with Bois noir (Quaglino *et al*., 2013), and FDp related strain in the Cicadellidae *Thamnotettix dilutior*, largely reported as associated with vineyard ecosystems (Bosco *et al*., 1997; Sanna *et al*., 2016). Molecular analyses showed that (i) *H. obsoletus* hosts a 16SrV group phytoplasma strain (Ho1), characterized by the typical RFLP profile of the *map*-type FD3, but very divergent from FDp and related strains of *map*-type FD3 based on sequence identity (from 96.9 to 97.3%); in fact, *map* gene nucleotide sequence of strain Ho1 has 15 SNPs compared to the most closely related strain (FDp strain FG1) in FD3 cluster (data not shown); due to the high polyphagy of *H. obsoletus*, it is reasonable to hypothesize that 16SrV group phytoplasma strain Ho1 was acquired by occasional feeding of the insect on infected source plant(s), which should be identified in further studies; (ii) *T. dilutior* specimens host FDp related strains indistinguishable from *map*-type FD2 phytoplasma strains (sequence identity 100%) identified in grapevine, hazel, and *O. ishidae* in the test vineyard

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(Figure 4, Table S2). These evidences, along with the occurrence of a low population in the test vineyard, suggest that *T. dilutior*, if confirmed as vector, could play a marginal role in FDp transmission to grapevine.

Together with ongoing field surveys and molecular analyses, the data of the present study, based on the detection of FDp and related strains in multiple plants and insects inside and outside the test vineyard, (i) confirm the presence in vineyard agro-ecosystems of different leafhopper and plant species infected with FDp and related strains, (ii) confirm the high FDp related strains-infection rate in *O. ishidae* observed in some European Countries (Mehle *et al*., 2010, 2011; Gaffuri *et al*., 2011; Koczor *et al*., 2013; Trivellone *et al*., 2016), and (iii) show for the first time the high FDp related strain infection rate in hazel, a host plant of *O. ishidae* (Nickel *et al*., 2010; Lessio *et al*., 2016), very common and abundant in Swiss vineyard agro-ecosystems. It is therefore reasonable to hypothesize that the ecological cycle of FDp may be related not exclusively to the grapevine-specific feeding diet of *S. titanus*, but also to that of other insect vector(s) and/or plant host(s). Bressan *et al*. (2006) tested the specificity of vector transmission of FDp on 15 Auchenorrhyncha species, collected in vineyard agro-ecosystem but not developing on grapevine, by abdominal injection of FDp. They demonstrated that three leafhopper species, all belonging to the subfamily Deltocephalinae, transmit FDp. These data suggest that the vector competency of FDp transmission could be widespread and opens the question if in a viticultural area the range/number of effective insect vector species could be determined by the availability of suitable host plant species in a system where FDp strains flow among many different hosts, inside and outside the vineyards.

During the last decades, the Asian Cicadellidae *O. ishidae* has been reported from several European Countries. This species has also been introduced in North America during the last century. *O. ishidae* is a sap-feeder; although some damage (i.e. uniform browning of apple and hazel foliage) has occasionally been reported in the USA, it is not considered to be a major pest of

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cultivated plants. The main concern raised by the introduction of *O. ishidae* in Europe is the possible transmission of phytoplasma diseases (EPPO, 2015). In fact, experiments conducted in the USA have shown that *O. ishidae* can transmit '*Candidatus* Phytoplasma pruni' to celery plants (Rosenberg *et al.*, 1978). Moreover, the present work and recent studies conducted in several European Countries detected the presence of FDp related strains in specimens of *O. ishidae* collected from diseased vineyards (EPPO, 2015). Interestingly, latest findings revealed the capability of *O. ishidae* to transmit 16SrV phytoplasmas to grapevine (Lessio *et al.*, 2016).

In this framework, based on the results obtained in the present study, it is reasonable to assume that the settlement of *O. ishidae* in Canton Ticino at the beginning of the 21st century could have contributed to the persistence of FDp, despite the application of mandatory insecticide treatments, by transmitting FDp from hazel and willow to grapevine, in a way similar to that hypothesized for *O. alni* (Arnaud *et al.*, 2007; Durante *et al.*, 2012).

In the last years, the molecular characterization of phytoplasma species showed molecular diversity, representing ecologically separated populations. The investigation of the genetic diversity among phytoplasmas associated with several diseases allows the identification of strain-specific molecular markers, which are useful to improve the understanding of complex phytoplasma ecologies (Davis *et al.*, 2013; Quaglino *et al.*, 2013). Indeed, among grapevine yellows, the knowledge of the biological complexity of the Bois noir disease was improved through the application of a molecular epidemiology approach (Kostadinovska *et al.*, 2014; Kosovac *et al.*, 2016; Murolo & Romanazzi, 2015; Quaglino *et al.*, 2016). Concerning FD, molecular markers were produced to distinguish FDp strains in different ecological niches (Martini *et al.*, 2002; Botti & Bertaccini, 2007; Filippin *et al.*, 2009; Quaglino *et al.*, 2010; Malembic-Maher *et al.*, 2011; Durante *et al.*, 2012). The most promising candidate for typing closely related strains within the taxonomic group 16SrV is the *map* gene. It distinguishes three *map*-types (FD1, FD2 and FD3), which are associated with distinct geographic areas (Arnaud *et al.*, 2007) and alder yellows strains in Germany

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(Holz *et al*., 2015). In this study, data from the molecular characterization and phylogenetic analyses of FDp and related strains reveal the great prevalence of the *map*-type FD2 in grapevines (97%) and in *O. ishidae* pools (72%); FD2 was found also in hazel and in *T. dilutior*, but not in *S. titanus*. The *map*-type FD1, identified in few grapevines (3%), was found also in *O. ishidae*, *S. titanus*, *C. avellana*, and *Salix* spp.. The *map*-type FD3, not found in grapevine, was identified in *O. ishidae*, *C. avellana*, and *C. vitalba*. Together, these data show that in the test vineyard FD is associated prevalently with phytoplasma strains of *map*-type FD2.

Based on the data obtained in this and in a previous study (Lessio *et al*., 2016), it is reasonable to hypothesize that in the test vineyard the ecology of FDp is related to grapevine-specific cycle of *S. titanus*, the cycle of other polyphagous vectors (e.g. *O. ishidae*), and the presence of other host plants of FDp and related strains (e.g. hazel and willow). Further studies will be needed to prove the role of *O. ishidae* in FD epidemiology. Considering the new scenario describing the FD ecology as an open system, in which the landscape composition can influence the presence of FDp vectors and host plants impacting on FD epidemics, it appears suitable to re-evaluate the disease control strategies.

ACKNOWLEDGEMENTS

We gratefully thank Prof. Alberto Alma (University of Turin) for sharing important information on *O. ishidae* obtained in his own researches, and Dr. Valeria Trivellone (Research Station Agroscope Changins, Cadenazzo, Switzerland) for the technical assistance in insect identification. Moreover, we are grateful to Mister Piercarlo Saglini, the owner of the test vineyard.

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Supporting Information

Casati *et al*. [Insights on Flavescence dorée ecology]

Table S1 *Map* gene nucleotide sequences of 16SrV group strains retrieved from NCBI GenBank for sequence identity and phylogenetic analyses

Strain	*map* type	Host	Location	Accession number
FD V00-SP5 and V00-SP9	FD2	*Vitis vinifera*	Gironde, France	AM384886
FD V02-101 and V01-9	FD1	*Vitis vinifera*	Gironde, France	AM384887
FD V03-9-2, 9-16, and 9-17	FD1	*Vitis vinifera*	Pyrénés Atlantiques, France	AM384888
FD V04-11-19, 11-21, and 11-53	FD1	*Vitis vinifera*	Lot, France	AM384889
FD VI04-C28 and C29	FD3	*Vitis vinifera*	Veneto, Italy	AM384894
FD VI04-Toscana1	FD3	*Vitis vinifera*	Toscana, Italy	AM384895
FD VI04-188-04 and 248-04	FD3	*Vitis vinifera*	Piemonte, Italy	AM384896
FD FD 70	FD3	*Catharanthus roseus*	France	AM238512
PGY PGY-A		*Vitis vinifera*	Rheinland-Pfaltz, Germany	AM384892
PGY PGY-B Yellows (B type)		*Vitis vinifera*	Rheinland-Pfalz, Germany	AM384893
PGY PGY-C EY 38		*Vitis vinifera*	Rheinland-Pfalz, Germany	AM384891
PGY V04-11-01		*Vitis vinifera*	Haut-Rhin, France	AM384890
EY1 E04-D714		*Ulmus glabra*	Haute-Vienne, France	AM384901
AldY AI04-3-13 and AI04-3-7		*Alnus glutinosa*	Basilicata, Italy	AM384884
Ald YWJ1444-32		*Alnus glutinosa*	Pyrénés Orientales, France	AM384897
AldY ALY		*Catharanthus roseus*	Basilicata, Italy	AM384885
AldY A06-30-3	FD1	*Alnus glutinosa*	Gironde, France	FN561864
AldY Ag30 MAC		*Alnus glutinosa*	Macedonia	KJ605451
AldY 74-08-MNE		*Alnus glutinosa*	Montenegro	KC188998
AldY SW7		*Alnus glutinosa*	Germany	KP238304
AldY SW38		*Alnus glutinosa*	Germany	KP238315
AldY SW1		*Alnus glutinosa*	Germany	KP238302
AldY 75-08-MNE		*Alnus glutinosa*	Montenegro	KC188999
AldY Ag26 MAC		*Alnus glutinosa*	Macedonia	KJ605448
AldY Ag27 MAC		*Alnus glutinosa*	Macedonia	KJ605449
AldY Ag29 MAC		*Alnus glutinosa*	Macedonia	KJ605450
AldY A06-30-25		*Alnus glutinosa*	Gironde, France	FN561865
AldY SW13		*Alnus glutinosa*	Germany	KP238307
AldY SW23		*Alnus glutinosa*	Germany	KP238310
AldY A06-30-20		*Alnus glutinosa*	Gironde, France	FN561863
AldY SW4		*Alnus glutinosa*	Germany	KP238303
AldY SW8		*Alnus glutinosa*	Germany	KP238305
AldY SW17		*Alnus glutinosa*	Germany	KP238309
FD FG 1	FD3	*Vitis vinifera*	Serbia	KP238300

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Supporting Information

Casati *et al*. [Insights on Flavescence dorée ecology]

Table S2 Sequence identity of *map* gene of FDp and related strains identified in the present study and 16SrV phytoplasma strains available at NCBI GenBank

#	Type	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	FD1	Vv128	ID																								
2	FD1	Vv129	1	ID																							
3	FD1	Ca249	1	1	ID																						
4	FD1	Ca253	1	1	1	ID																					
5	FD1	Sa252	0,998	0,998	0,998	0,998	ID																				
6	FD1	Oi99	0,998	0,998	0,998	0,998	0,996	ID																			
7	FD1	Oi116	1	1	1	1	0,998	0,998	ID																		
8	FD1	St121	1	1	1	1	0,998	0,998	1	ID																	
9	FD1	St142	1	1	1	1	0,998	0,998	1	1	ID																
10	FD2	Vv178	0,98	0,98	0,98	0,98	0,982	0,978	0,98	0,98	0,98	ID															
11	FD2	Vv220	0,98	0,98	0,98	0,98	0,982	0,978	0,98	0,98	0,98	1	ID														
12	FD2	Ca158	0,98	0,98	0,98	0,98	0,982	0,978	0,98	0,98	0,98	1	1	ID													
13	FD2	Oi12	0,98	0,98	0,98	0,98	0,982	0,978	0,98	0,98	0,98	1	1	1	ID												
14	FD2	Oi32	0,98	0,98	0,98	0,98	0,982	0,978	0,98	0,98	0,98	1	1	1	1	ID											
15	FD2	Td8	0,98	0,98	0,98	0,98	0,982	0,978	0,98	0,98	0,98	1	1	1	1	1	ID										
16	FD2	Td31	0,98	0,98	0,98	0,98	0,982	0,978	0,98	0,98	0,98	1	1	1	1	1	1	ID									
17	FD3	Ca254	0,984	0,984	0,984	0,984	0,985	0,982	0,984	0,984	0,984	0,982	0,982	0,982	0,982	0,982	0,982	0,982	ID								
18	FD3	Ca257	0,984	0,984	0,984	0,984	0,985	0,982	0,984	0,984	0,984	0,982	0,982	0,982	0,982	0,982	0,982	0,982	1	ID							
19	FD3	Ca256	0,984	0,984	0,984	0,984	0,985	0,982	0,984	0,984	0,984	0,982	0,982	0,982	0,982	0,982	0,982	0,982	1	1	ID						
20	FD3	Oi102	0,982	0,982	0,982	0,982	0,984	0,98	0,982	0,982	0,982	0,98	0,98	0,98	0,98	0,98	0,98	0,98	0,998	0,998	0,998	ID					
21	FD3	Oi115	0,984	0,984	0,984	0,984	0,985	0,982	0,984	0,984	0,984	0,982	0,982	0,982	0,982	0,982	0,982	0,982	1	1	1	0,998	ID				
22	FD3	Ho1	0,957	0,957	0,957	0,957	0,959	0,955	0,957	0,957	0,957	0,955	0,955	0,955	0,955	0,955	0,955	0,955	0,973	0,973	0,973	0,971	0,973	ID			
23	FD1	FD70	1	1	1	1	0,998	0,998	1	1	1	0,98	0,98	0,98	0,98	0,98	0,98	0,98	0,984	0,984	0,984	0,982	0,984	0,957	ID		
24	FD2	V00-SP5	0,98	0,98	0,98	0,98	0,982	0,978	0,98	0,98	0,98	1	1	1	1	1	1	1	0,982	0,982	0,982	0,98	0,982	0,955	0,98	ID	
25	FD3	VI04-Toscana1	0,984	0,984	0,984	0,984	0,985	0,982	0,984	0,984	0,984	0,982	0,982	0,982	0,982	0,982	0,982	0,982	0,996	0,996	0,996	0,994	0,996	0,969	0,984	0,982	ID

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Table 1 Insect vector specimens captured by yellow sticky traps in the test vineyard from May to November 2014, and FDp and related strains molecular identification and characterization in pooled insects

Trap		Species [b]																											
		S. titanus					*O. ishidae*					*T. dilutior*				*R. cuspidatus*			*F. florii*			*H. obsoletus*				*A. fuscovenosus*		
		Cpt	And	Pls	Inf	*map*	Cpt	And	Pls	Inf	*map*	Cpt	Pls	Inf	*map*	Cpt	Pls	Inf	Cpt	Pls	Inf	Cpt	Pls	Inf	*map*	Cpt	Pls	Inf
Rebell inside the vineyard	V1																		1	1	0							
	V2	1	1	1	0																								
	V3						5	5	4	0												1	1	1	FD3(1)			
	V4																												
	V5																					2	1	0				
	V6						2	2	2	1	FD2(1)																		
Rebell on the borders	B1															3	3	0											
	B2						1	1	1	0						2	1	0											
	B3	1	1	1	0		88	88	26	10	FD1(2) FD2(8)	1	1	1	FD2(1)	1	1	0											
	B4	6	6	4	1	FD1(1)	15	15	8	2	FD2(2)																		
	B5						2	2	1	0																			
	B6						1	1	1	0		1	1	1	FD2(1)														
	B7						4	4	3	0												5	4	0				
	B8																					4	2	0				
Rebell outside the vineyard [a]	W1						15	15	8	1	FD3(1)																		
	W2						4	4	3	0																			
	W3																												
	W4						4	4	4	2	FD1(1) FD2(1)																		
	W5						4	4	4	2	FD1(1) FD2(1)																		
	W6						2	2	1	0																			
	W7						3	3	3	0																			
	W8	15	15	15	1	FD1(1)	29	29	12	3	FD1(1) FD2(2)																		
	W9	6	6	6	0		35	35	12	4	FD2(3) FD3(1)																		
Aeroxon		11	2	2	0		22	4	4	0												3	3	0				
		40	31	29	2	FD1(2)	236	218	97	25	FD1(5) FD2(18) FD3(2)	2	2	2	FD2(2)	6	5	0	1	1	0	15	11	1	FD3(1)	1	1	0

[a] traps W1 to W7 positioned at the external border of the forest neighboring other vineyards; traps W8 and W9 positioned within the forest

[b] Cpt, captured specimens; And, analyzed specimens; Pls, pools; Inf, infected pools determined by real-time PCR; *map*, *map*-type determined by RFLP analysis of *map* gene amplified by nested PCR

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Table 2 Molecular identification and characterization of FDp and related strains in plants

Host	No. of samples	Real-time PCR	*map* PCR	RFLP *map* types		
				FD1	FD2	FD3
Vitis vinifera	74	70	70	2	68	0
Corylus avellana	31	11	6	3	1	2
Salix sp.	10	3	1	1	0	0
Clematis vitalba	4	3	3	0	0	3

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Table 3 GenBank accession numbers of *map* gene nucleotide sequences amplified from representative FDp, FDp related and 16SrV phytoplasma strains identified in insects and plants

RFLP *map* types	Host	No. of strains	Representative strain	Accession Number
FD1	*Vitis vinifera*	2	Vv128[a]	KX245031
			Vv129[a]	KX245030
	Corylus avellana	3	Ca249[b]	KX245028
			Ca253[b]	KX245033
	Salix sp.	1	Sa252[b]	KX245029
	Orientus ishidae	5	Oi99[b]	KX245034
			Oi116[b]	KX245032
	Scaphoideus titanus	2	St121[a]	KX245035
			St142[a]	KX245036
FD2	*Vitis vinifera*	68	Vv178[a]	KX245026
			Vv220[a]	KX245025
	Corylus avellana	1	Ca158[b]	KX245023
	Orientus ishidae	18	Oi12[b]	KX245021
			Oi32[b]	KX245022
	Thamnotettix dilutior	2	Td8[b]	KX245024
			Td31[b]	KX245027
FD3	*Corylus avellana*	2	Ca254[b]	KX245038
			Ca257[b]	KX245041
	Clematis vitalba	1	Cv256[b]	KX245042
	Orientus ishidae	2	Oi102[b]	KX245040
			Oi115[b]	KX245039
	Hyalesthes obsoletus	1	Ho1[c]	KX245037

[a] FDp strain; [b] FDp related strain; [c] 16SrV phytoplasma strain

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FIGURE LEGENDS

Figure 1. View of the vineyard agro-ecosystem in Stabio, 'Canton Ticino' Region (Switzerland), and position of the yellow sticky traps (Rebell and Aeroxon) used to capture potential phytoplasma insect vectors. Rebell traps inside the vineyards: V1 to V6; Rebell traps on the borders: B1 to P8; Rebell outside the vineyard: W1 to W9. Fourteen aeroxon traps were positioned horizontally on the grapevine canopy inside the test vineyard.

Figure 2. Flight dynamics of *Orientus ishidae* (in blue) and *Scaphoideus titanus* (in red) in the vineyard in Stabio, 'Canton Ticino' Region (Switzerland), determined through the number of specimens captured inside the vineyard by Aeroxon (A) and Rebell (V1 to V6, B1 to B8) (B) traps, and outside the vineyard by Rebell (W1 to W9) traps (C).

Figure 3. Restriction fragment length polymorphism (RFLP) profiles obtained through double digestion with the enzymes *Alu*I and *Eco*72I. RFLP patterns were visualized through electrophoresis in 3% agarose gel. Attribution of RFLP profiles, identified in the present study, was carried out through the comparison with the profiles of the *map*-types FD1 (A), 2 (B), and 3 (C) (Arnaud *et al.*, 2007). Acronyms of the FDp and related strains are reported in Table 3.

Figure 4. Unrooted phylogenetic tree inferred from analyses of nucleotide sequences of *map* gene. Minimum-Evolution method was carried out using the Jukes-Cantor model with the software MEGA6. The reliability of the analyses was subjected to a bootstrap test with 1000 replicates; bootstrap values lower than 50 are not shown. Phytoplasma strains and their nucleotide sequence accession numbers are reported in Table 3 and Table S1. Nucleotide sequences from the present work (Table 3) are written in bold characters.

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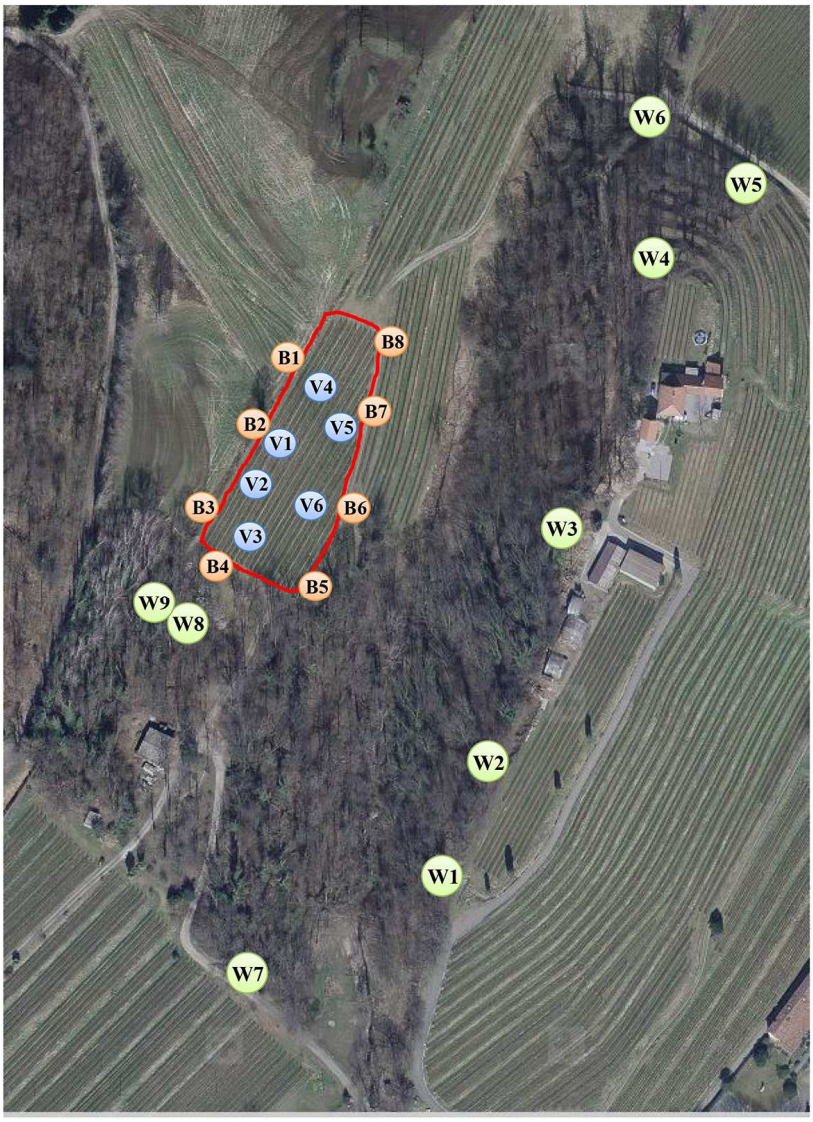

Figure 1

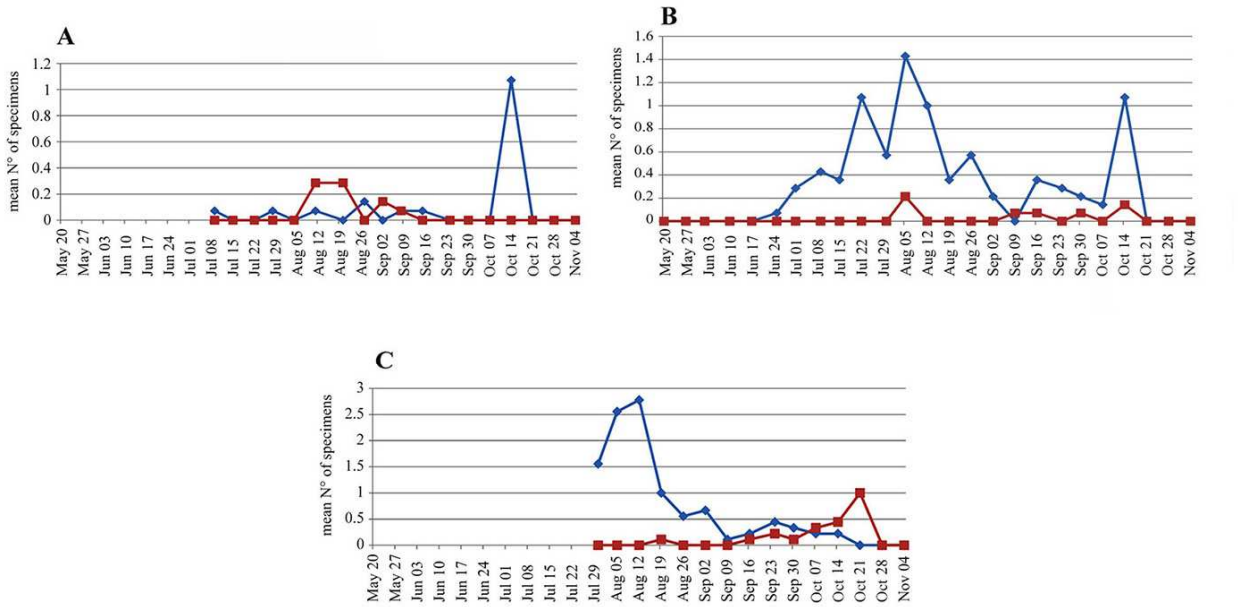

Figure 2

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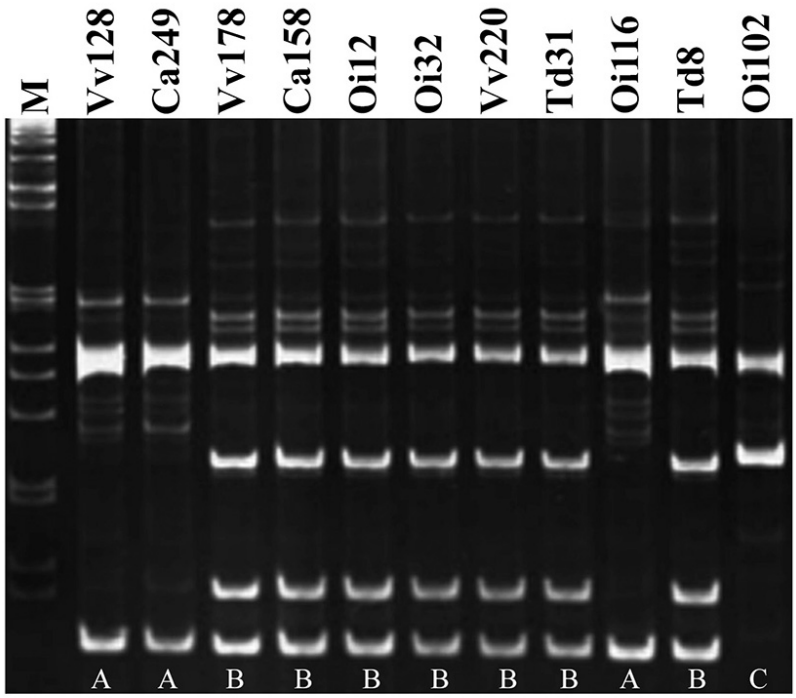

Figure 3

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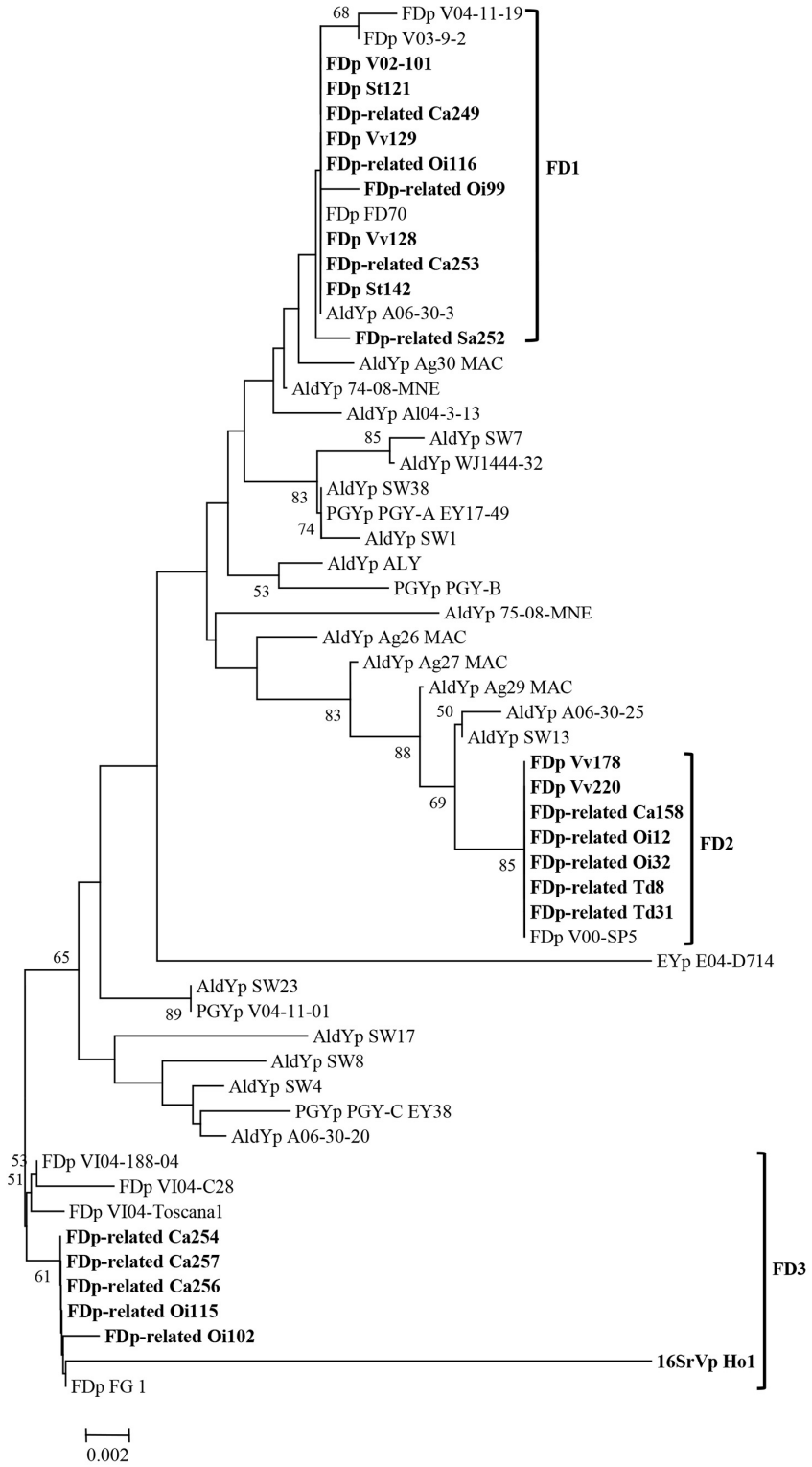
68
FDp V04-11-19
FDp V03-9-2
FDp V02-101
FDp St121
FDp-related Ca249
FDp Vv129
FDp-related Oi116
FDp-related Oi99
FDp FD70
FDp Vv128
FDp-related Ca253
FDp St142
AldYp A06-30-3
FDp-related Sa252
FD1
AldYp Ag30 MAC
AldYp 74-08-MNE
AldYp Al04-3-13
85
AldYp SW7
AldYp WJ1444-32
83
AldYp SW38
PGYp PGY-A EY17-49
74
AldYp SW1
AldYp ALY
53
PGYp PGY-B
AldYp 75-08-MNE
AldYp Ag26 MAC
AldYp Ag27 MAC
AldYp Ag29 MAC
83
50
AldYp A06-30-25
AldYp SW13
88
FDp Vv178
FDp Vv220
FDp-related Ca158
FDp-related Oi12
FDp-related Oi32
FDp-related Td8
FDp-related Td31
FDp V00-SP5
69
85
FD2
65
EYp E04-D714
AldYp SW23
89
PGYp V04-11-01
AldYp SW17
AldYp SW8
AldYp SW4
PGYp PGY-C EY38
AldYp A06-30-20
53
51
FDp VI04-188-04
FDp VI04-C28
FDp VI04-Toscana1
FDp-related Ca254
FDp-related Ca257
61
FDp-related Ca256
FDp-related Oi115
FDp-related Oi102
16SrVp Ho1
FDp FG 1
FD3
0.002

Figure 4