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Molecular characteristics of phytoplasmas associated with *Flavescence dorée* in clematis and grapevine and preliminary results on the role of *Dictyophara europaea* as a vector

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A survey was conducted over several years in Italy and the Balkans in order to gain an understanding of the relationship between the *Flavescence dorée* (FD) phytoplasma isolates found in clematis and grapevine. A total of 399 clematis and 107 grapevine samples were analyzed. The results showed that 36% of the *Clematis vitalba* plant samples were infected by phytoplasmas which, in grapevine, are associated with FD, a quarantine disease in Europe. Infected clematis plants were also found in areas where FD phytoplasma had never previously been reported to infect grapevine, such as Macedonia, Croatia and some areas of Italy and Serbia. Molecular data from three phytoplasma genomic fragments showed the presence of different FD phytoplasma isolates, all belonging to the 16SrV-C subgroup, including the Italian FD-C isolate, the isolate found in Serbia, an isolate similar to the French FD2000 and a new isolate typical of central Italy. A few clematis plants were infected with single nucleotide polymorphism, insertion or deletion mutants of the FD-C isolate. Of all the potential Hemipteran vector species surveyed in Italy and Serbia, only 18 of 527 *Dictyophara europaea* individuals tested proved to be infected with the FD phytoplasma. Preliminary transmission experiments showed that this species is able to transmit the FD phytoplasma from clematis to grapevine. The presence of FD-infected clematis and of *D. europaea* could, therefore, constitute a risk for FD epidemics in the European viticultural regions.

Keywords: Clematis vitalba, *Dictyophara europaea*, grapevine yellows, molecular detection and diagnostics, *Scaphoideus titanus*, *Vitis vinifera*

Introduction

Phytoplasmas are phloem-limited unculturable Mollicutes which cause several hundred diseases in wild and cultivated plants. They are classified on the basis of molecular data obtained from 16S rDNA and other conserved genes into distinct groups, subgroups and species belonging to the newly-established '*Candidatus* Phytoplasma' taxon (IRPCM, 2004).

Clematis vitalba (Ranunculaceae) is a invasive vine-like plant which grows in fields, underbrush and woody areas in many countries in Europe and the rest of the world. In 2004 a phytoplasma belonging to the 16SrV phylogenetic group was found in some clematis plants in north-eastern Italy and southern Serbia. Molecular analyses classified the phytoplasma isolate as FD-C, the same as that present in the adjoining grapevines and in vineyards in the surrounding areas (Angelini *et al*., 2004). FD-C is one of the phytoplasma isolates previously identified in grapevine (Martini *et al*., 1999) and known to cause *Flavescence dorée* (FD), a very serious grapevine yellows disease in vineyards and a quarantine pest in Europe. The most common damage associated with FD is the huge loss of grape and wine production, due to the progressive decline of the plants. In most cases, especially in the more sensitive varieties, the infected grapevines die within a few years.

FD is caused by several isolates which belong to the 16SrV-C and -D phytoplasma phylogenetic subgroups. The FD-D isolate (also called FD92 or FD88), the only FD isolate included in the 16SrV-D subgroup, is the most widespread; it has been detected in grapevine in northern and central Italy, in southern and central France, in the north-eastern areas of Spain and in an area of southern

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Switzerland (Daire *et al*., 1997; Martini *et al*., 1999, 2002; Angelini *et al*., 2001; Arnaud *et al*., 2007). FD70, FD2000 and the related isolates which belong to the 16SrV-C subgroup have only been described in France (Caudwell *et al*., 1970; Boudon-Padieu, 2002; Arnaud *et al*., 2007). Another cluster in the 16SrV-C subgroup included the remaining isolates: FD-C and closely-related isolates, identified only in Italy and the Balkans. The FD-C isolate was involved in an FD epidemic in a small area in north-eastern Italy (Treviso province) (Martini *et al*., 1999); it was also found sporadically in central Italy (Credi *et al*., 2002; Botti & Bertaccini, 2007) and Slovenia (Maixner, 2006). An isolate closely-related to FD-C is present in Serbia (Kuzmanović *et al*., 2008). The FDLomb/Piem isolate, the last one included in this cluster, is found only in the north-western regions of Italy (Lombardy, Piedmont and the Aosta Valley) (Martini *et al*., 2002). FD is transmitted from grapevine to grapevine by the leafhopper vector *Scaphoideus titanus* (Schvester *et al*., 1961; Mori *et al*., 2002). However, no information on the transmission of FD phytoplasmas from clematis to grapevine is available to date.

The aims of this 6-year study were as follows: i) to ascertain the occurrence and geographic distribution of clematis infected with FD phytoplasmas in Italy and the Balkans, especially in areas where FD had never been identified in grapevine; ii) to characterize the phytoplasma isolates in clematis and compare them with those present in grapevine by means of RFLP and nucleotide sequencing; and iii) to gain an understanding of the role of known and potential insect vectors in the transmission of phytoplasma isolates from clematis to grapevine.

Materials and methods

Clematis and grapevine samples

Clematis plants were collected from 2004 to 2007 in northern and central Italy and the Balkans. Plants were collected inside vineyards or at their borders and also in woods, underbrush and meadows situated at a distance of up to 300 m from vineyards. In areas with only a few individual clematis present, every plant was sampled, whereas in areas with many individual clematis present, plants were collected randomly and then grouped to form mixed samples for molecular analyses. Clematis plants showing reddening or yellowing of the leaves were collected separately. A total of 399 *C. vitalba* leaf samples were collected (Table 1). Fourteen *C. flammula*, *C. montana*, *C. recta* and *C. viticella* plants were also collected in northern and central Italy.

Leaves of grapevines showing symptoms were collected from the same areas, in northern and central Italy and the Balkans, for a total of 107 samples. In addition, a few FD-infected grapevine DNA extracts from other regions were kindly supplied by local Institutes, as follows: four samples from Slovenia, obtained from the National Institute of Biology (Ljubljana, Slovenia); four samples from areas in Romagna in central Italy, obtained from the Phytosanitary Service; and two samples from the Marche region in central Italy, obtained from the University of Bologna (Italy).

Clematis and grapevine leaf veins were isolated from all samples, distributed into 1 g aliquots, frozen with liquid nitrogen and stored at –20°C until DNA extraction.

Insect samples

Extensive surveys were performed in Italy (2002–2007) and Serbia (2005–2007) to obtain information about potential FD vectors present in the field. Hemipteran species including leafhoppers, planthoppers and psyllids were collected by sweep net from FD-infected clematis and grapevine plants and from grass in meadows. Insects were collected from eight sites in the Veneto region (Italy) and from 10 sites all over Serbia, and identified as described by Holzinger *et al*. (2003) and Biedermann & Niedringhaus (2004); then samples were frozen with liquid nitrogen and stored at –20°C until DNA extraction.

Acquisition and transmission trials

Transmission trials from FD-infected clematis were performed with three insect species: *S. titanus*, *Euscelidius variegatus* – an experimental vector of FD phytoplasmas to broadbean (*Vicia faba*) plants (Caudwell *et al*., 1972) – and *Dictyophara europaea* (Auchenorrhyncha, Dictyopharidae). Clematis infected with FD were collected in Italy and Serbia and grown in laboratory conditions. The plants were regularly pruned, propagated and PCR-tested for the presence of phytoplasma.

Scaphoideus titanus specimens were hatched from egg-bearing grapevine wood collected in organic vineyards (Bressan *et al*., 2005) and *E. variegatus* colonies were maintained according to Caudwell *et al*. (1972). About 450 *S. titanus* nymphs (L3–L5) were collected from a rearing cage and confined in three cages, each containing one FD-infected clematis plant and about 150 insect specimens. After 3 weeks, the surviving leafhoppers were transferred to caged healthy potted grapevines for 2 weeks and then to caged healthy potted broadbean plants for 1 week. About 200 *E. variegatus* nymphs and young adults were collected from a rearing cage and confined in a cage containing two FD-infected clematis plants. After 3 weeks, the surviving leafhoppers were transferred to caged healthy potted broadbean plants for 11 days. The cages were checked daily and the dead leafhoppers were collected and stored at −80°C until they were tested with PCR.

Dictyophara europaea colonies were established *ex ovo* in a mesh cage outdoors (2·2×2·2×2·5 m). Newly-hatched nymphs were fed on healthy potted seedlings of different plant species sown and growing inside the cage, such as *Linaria vulgaris*, *Crepis foetida*, *Sonchus asper*, *Setaria viridis* and *Elytrigia* (*Agropyron*) *repens*. Adults and 5th instar nymphs of *D. europaea* (a total of 100 individuals) were collected from the rearing cage for acquisition trials. The sources for acquisition were clematis cuttings infected

Table 1 Geographic origin and number of *Clematis vitalba* samples analyzed, together with PCR results on the presence of *Flavescence dorée* (FD) phytoplasma and characterization of the isolate

		PCR results		
Country	Region	No. negative samples	No. FD positive samples	FD isolate type[a]
Italy	Piedmont	3	13	FD-PS, FD-C
Italy	Lombardy	8	3	FD-C
Italy	Trentino Alto Adige	9	0	/
Italy	Veneto	100	53	FD-C, FD-C deletion mutants, FD-C SNP[b] mutant
Italy	Friuli Venezia Giula	15	18	FD-C, FD-C insertion mutant
Italy	Liguria	1	0	/
Italy	Emilia Romagna	3	1	FD-CCI
Italy	Umbria	0	3	FD-CCI
Italy	Latium	8	0	/
Italy	Tuscany	5	8	FD-CCI
Slovenia	Obalno-Kraska	0	2	FD-C
Croatia	Istria	5	5	FD-C
Croatia	Lika/Senj	2	0	/
Serbia	Nišava	65	16	FD-CS
Serbia	Rasina	6	8	FD-CS
Serbia	Belgrade	0	1	FD-CS
Serbia	Šumadija	3	2	FD-CS
Serbia	Braničevo	4	1	FD-CS
Serbia	Bor	5	0	/
Serbia	Jablanica	2	3	FD-CS
Serbia	Pčinja	1	0	/
Serbia	Srem	1	0	/
Serbia	South Banat	7	2	FD-C SNP mutant
Macedonia	Stip	0	3	FD-C
Macedonia	Negotino	1	1	FD-C
Macedonia	Kavadarci	0	2	FD-C
Total		254	145	

[a]For abbreviations and names of the isolates, see the Results section.
[b]SNP: single nucleotide polymorphism.

with FD, which were individually PCR/RFLP-tested and potted in transparent polyvinyl chloride cylinders, ventilated with gauze leads. Two groups of 50 *D. europaea* specimens were allowed to feed on two FD-infected clematis plants for 48 h before being moved to the healthy potted plants in the mesh cage, as described above. After 25 days, in August, the surviving *D. europaea* were moved in groups of six specimens per plant to six healthy grapevine (cv. Plovdina) seedlings, which were individually potted in polyvinyl chloride cylinders of 35 × 10 cm (Plovdina is a red-grape cultivar, very sensitive to FD and used as an FD indicator). The cylinders were checked daily and the dead leafhoppers were immediately collected and stored in 96% ethanol at –20°C until they were tested using PCR.

Phytoplasma reference isolates

The following phytoplasma isolates from the 16SrV group were used as a reference in the PCR/RFLP experiments: FD-C, FD-D and FDLomb/Piem, obtained from leaves of naturally-infected field-grown grapevines in the Veneto and Piedmont regions (Italy); FD70 and ALY (Italian alder yellows), all obtained from experimentally-infected periwinkle (*Catharanthus roseus*) (Angelini *et al*., 2001); and FD2000, obtained from experimentally-infected broadbean plants (Boudon-Padieu, 2002).

DNA extraction and amplification

The DNA was extracted from plants using the CTAB method, according to Angelini *et al*. (2001). Those DNA pellets which were dirty due to the PCR-inhibiting substances contained in most clematis samples were cleaned up with as many as five cycles of washing and isopropanol precipitation, until the pellets were finally clean. The DNA was extracted from insects according to Gatineau *et al*. (2001). Tissue samples from healthy periwinkle and grapevine, both grown from seedlings in insect-proof greenhouse conditions, and egg-hatched healthy specimens of *E. variegatus* and *S. titanus* were used as a negative control in the DNA extraction and amplification.

Diagnosis of both infected and healthy plant and insect samples collected in the field was carried out with a highly sensitive TaqMan real-time PCR system specifically designed for the detection of 16SrV and 16SrXII group phytoplasmas, following the thermal protocol and PCR conditions already described (Angelini *et al*., 2007). The TaqMan assay amplifies short portions in the phytoplasma 16S rRNA gene. Real-time PCR assays were carried out in 96-well plates using a Bio-Rad thermalcycler (iCycler IQ model). Each sample was run at least in duplicate in the same plate.

The negative plant samples were tested again using real-time PCR with primers targeting endogenous grapevine or plant genes, in order to identify possible false negative results. Serial 1:10 dilutions of grapevine DNA samples were tested with a TaqMan assay using primers and a probe which targeted a grapevine gene encoding the chloroplast chaperonin 21, following the thermal protocol and PCR conditions already described (Angelini *et al*., 2007). Serial 1:10 dilutions of clematis DNA samples were tested with a primer pair which targeted the 18S rRNA gene of all plants, using a TaqMan real-time approach; the PCR conditions and thermal protocol were as described in Christensen *et al*. (2004). The DNA samples which gave no positive signal to endogenous genes were further cleaned up as previously described and tested with real-time PCR until they yielded the specific control amplicon.

Amplifications with conventional nested PCR assays were carried out in order to obtain longer amplicons (about 1000 bp), useful for the subsequent characterization of the phytoplasma isolates by means of RFLP and sequencing. Three genomic regions were amplified from field-collected plants and insects: i) the 16S–23S ribosomal RNA genes; ii) a fragment of the *rpl22* and *rps3* genes, encoding the L22 and S3 ribosomal proteins; and iii) the FD9 marker, which contains the 3′ end of the *rplO* gene, encoding the L15 50S ribosomal protein, and a long fragment of the *secY* gene, encoding a translocase protein. The following primer pairs were used: i) P1/P7 (Deng & Hiruki, 1991; Smart *et al*., 1996), followed by 16r758f/M23Sr (Gibb *et al*., 1995; Padovan *et al*., 1995), which are universal primer pairs for phytoplasmas; ii) rp(V)F1/R1 (Lee *et al*., 1998), followed by rp(V)F1A/R1A (Lee *et al*., 2004) and iii) FD9f/r (Daire *et al*., 1997), followed by FD9f/r2, FD9f2/r or FD9f3/r2 (Angelini *et al*., 2001). All the latter primers are specifically designed for 16SrV group phytoplasmas. The concentration of reagents and PCR conditions were according to Angelini *et al*. (2001) and Martini *et al*. (2002). The PCR products were analysed by 1% agarose gel electrophoresis in TBE buffer (Tris-Borate 90 mM, EDTA 1 mM), stained with ethidium bromide and visualized under a UV transilluminator.

In the transmission trials, the DNA was extracted from individual *E. variegatus* and *D. europaea* specimens and from 4-specimen batches of *S. titanus* leafhoppers; the amplification of the FD9 fragment was carried out in order to detect the infected specimens.

RFLP analyses and DNA sequencing

Aliquots of the PCR products obtained from all the positive samples were digested with restriction enzymes, according to the manufacturer's instructions. The 16r758f/M23Sr fragments were processed using *Taq*I, the rpVF1A/R1A fragments using *Hpa*II and the FD9f3/r2 fragments using *Taq*I, *Mse*I, *Alu*I and *Hpy*188I endonucleases (New England Biolabs and MBI Fermentas). The ALY, FD70, FD2000, FDLomb/Piem, FD-D and FD-C phytoplasma isolates were used as a reference to compare the restriction patterns in the three fragments. Restriction products were separated by 10% polyacrylamide gel electrophoresis in TBE buffer, stained with ethidium bromide and visualized under a UV transilluminator.

Only the FD9 amplicon, which is known to be more variable than the other DNA regions examined, was sequenced. Nucleotide sequences were obtained for 22 clematis plants, 11 grapevines and three *D. europaea* specimens which were PCR-positive. FD9f/r2 and FD9f2/r amplicons (about 1200 bp) were sequenced using automated equipment (BMR Service, Padova, Italy). Intermediate primers were used to allow the sequences to overlap. Sequences from each DNA region were assembled after each nucleotide position had been covered at least 2–3 times by sequencing. The EMBOSS program (http://www.ebi.ac.uk/) was used for comparison. Alignment and comparison with phytoplasma sequences from GenBank was carried out using CLUSTALW2 (http://www.ebi.ac.uk/). Phylogenetic analyses were conducted in MEGA4 using the neighbour-joining method. The bootstrap consensus tree was inferred from 1000 replicates.

Statistical analyses

Statistical analyses were performed with PracticStat software.

Results

Occurrence of phytoplasmas in clematis and grapevine plants

The cleanup procedure with multiple isopropanol precipitation steps allowed the PCR-inhibiting substances contained in several clematis samples to be eliminated. Although the DNA yield was lower at the end of the procedure, the purity of the cleaned pellets was greatly increased and the PCR amplification of endogenous targets was achieved in all cleaned samples.

PCR analyses using primers specifically designed for phytoplasmas showed that only *C. vitalba* was infected, and not samples of the other clematis species collected. Clematis plants infected by FD phytoplasma were present to varying degrees in almost all the areas investigated (Table 1). As far as Italy was concerned, infected clematis plants were identified in all but two regions out of seven in the north (Liguria and Trentino Alto Adige) and all but one out of three in the centre (Latium). In Croatia,

negative results were obtained only from samples collected in the south. In Serbia, infected clematis plants were found in all but three out of ten regions investigated (Bor, Pčinja and Srem), while all the regions investigated in Macedonia and Slovenia contained infected clematis. In total, more than 36% of the samples were infected by the FD phytoplasma. In the extensively surveyed countries of Italy and Serbia, the percentage of positive samples was 39% and 26%, respectively. FD-infected clematis plants were also detected in geographic regions where FD had never previously been reported, such as Macedonia, Croatia, some areas of central and north-eastern Italy and north-eastern Serbia.

The PCR data obtained from 242 clematis samples collected in regions with known presence of FD in grapevine were analysed in order to find out whether FD-infected clematis plants were more widespread in vineyards or in areas at least 300 m away from them. The statistical analyses showed that the differences were not significant ($P > 0{\cdot}05$): the level of infection was similar (41·3 versus 35·9%).

Clematis plants in the field often showed symptoms of reddening, yellowing and rolling of the leaves at the end of the summer. A statistical analysis was carried out on 238 samples, previously classified on the basis of symptoms: about half of them showed these symptoms, while the others did not display any anomaly in their general appearance or in leaf colouration. An association between the presence/absence of symptoms and the presence/absence of FD-phytoplasma in clematis was ascertained in 77% of the cases. In particular, 79 out of 116 clematis plants with symptoms were found to host the phytoplasma, while 103 out of 122 clematis samples without symptoms were negative in the molecular assays.

PCR analyses were carried out on grapevines collected from all regions where clematis plants had been taken. Wherever possible, the grapevine samples were collected from vineyards located near the sites where the clematis plants had also been collected. In Italy, FD phytoplasma was detected in grapevines from all the investigated regions except Latium. In Serbia, FD-infected grapevines were identified in all the regions surveyed, except for sites in Braničevo and South Banat. The FD phytoplasma was not detected in any grapevine samples from Croatia and Macedonia. Grapevine samples from Slovenia, provided by the National Institute of Biology in Ljubljana, proved to be infected with the FD phytoplasma.

RFLP characterization of phytoplasmas present in clematis and grapevine

The 16r758f/M23Sr, rp(V)F1A/R1A and FD9f3/r2 products from all the infected clematis and grapevines and from the phytoplasma reference isolates were digested separately with restriction enzymes.

The RFLP analyses of the 16S rRNA gene showed that all the clematis phytoplasmas belonged to the 16SrV-C subgroup. *Hpa*II digestion of amplicons which encompassed genes for the L22 and S3 ribosomal proteins showed that all the phytoplasmas present in clematis displayed RFLP patterns which were identical to those of the FD-C isolate from grapevine, except for five samples from Piedmont, which exhibited RFLP profiles which were similar to the FD70, FD-D, FD2000, FDLomb/Piem and ALY isolates (Fig. 1).

The RFLP patterns in the *rplO-secY* gene (FD9 fragment) of phytoplasmal DNA from clematis samples were most complex. Using *Taq*I, *Alu*I and *Mse*I endonucleases, another seven samples showed unusual profiles: two clematis samples collected in Friuli Venezia Giulia and five samples collected from the same site in the Veneto region. The digestion profile from the latter samples suggested the presence in these amplicons of an insertion (Fig. 2, lane CL-UD47) or deletion (Fig. 2, lanes CL-TV33 and

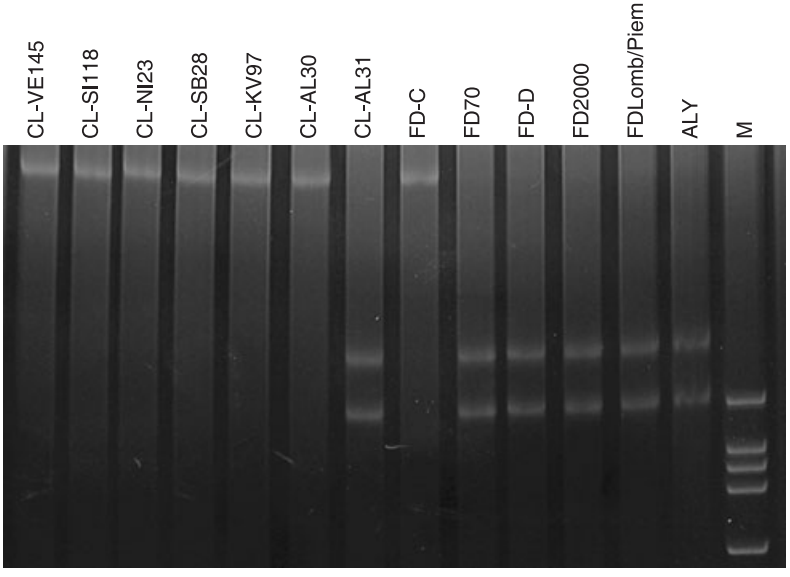

Figure 1 Polyacrylamide gel (10%) showing RFLP patterns in nested-PCR products for clematis (*Clematis vitalba*) samples and reference 16SrV-group phytoplasma isolates obtained from grapevine (*Vitis vinifera*) or periwinkle (*Catharanthus roseus*). The rp(V)F1A/R1A fragments, encompassing the genes that encode the L22 and S3 ribosomal proteins, were digested using *Hpa*II restriction endonuclease. Phytoplasmas: lanes 1–5, clematis samples from Italy (Veneto and Tuscany), Serbia (Nišava and South Banat) and Macedonia (Kavadarci); lanes 6–7, clematis samples from Piedmont, Italy; FD-C, FD70, FD-D, FD2000, FDLomb/Piem, reference *Flavescence dorée* isolates; ALY, alder yellow phytoplasma; M: molecular weight marker, pBR322/*Hae*III digested (Sigma Aldrich) giving fragment sizes from top to bottom of: 587, 540, 504, 458, 434 bp.

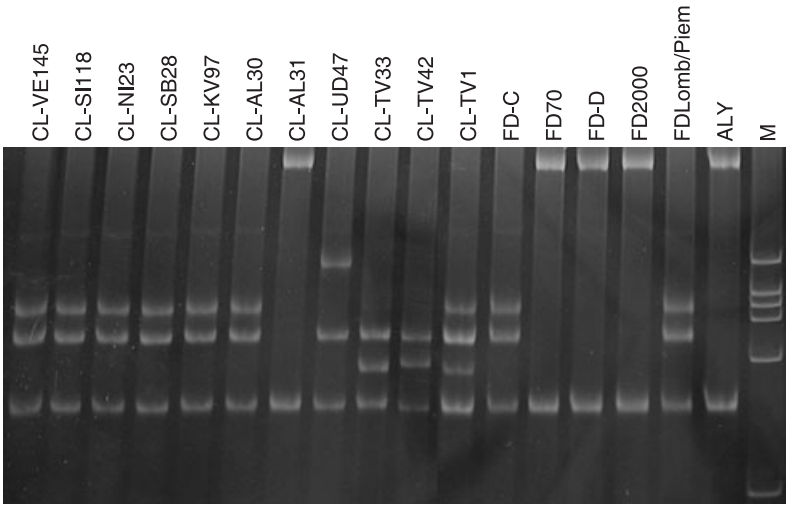

Figure 2 Polyacrylamide gel (10%) showing RFLP patterns in non ribosomal nested-PCR products for clematis (*Clematis vitalba*) samples and phytoplasma isolates obtained from grapevine (*Vitis vinifera*) or periwinkle (*Catharanthus roseus*). The FD9f3/r2 fragments, encompassing the genes that encode the L15 and SecY proteins, were digested using restriction *Taq*I endonuclease. Phytoplasmas: lanes 1–5, clematis samples from Italy (Veneto and Tuscany), Serbia (Nišava and South Banat) and Macedonia (Kavadarci); lanes 6–7, clematis samples from Piedmont, Italy; lanes 8–10, clematis with FD-C insertion and deletion mutants, northern Italy; lane 11, clematis with mixed infection (FD-C + FD-C deletion mutant); FD-C, FD70, FD-D, FD2000, FDLomb/Piem, reference *Flavescence dorée* isolates; ALY, alder yellow phytoplasma; M: molecular weight marker, pBR322/*Hae*III digested (Sigma Aldrich) giving fragment sizes from top to bottom of: 587, 540, 504, 458, 434, 267 bp.

CL-TV42). Moreover, two samples showed the presence of mixed infection with two different phytoplasmas: the FD-C isolate and one of the deletion mutants (Fig. 2, lane CL-TV1). The five samples from Piedmont displayed RFLP patterns which, in this genomic fragment digested with the three enzymes, were always similar to the FD-D, FD2000 and ALY isolates (Fig. 2, lane CL-AL31). The amplicons from the latter samples were digested using *Hpy*188I to ascertain their similarity to the grapevine FD2000 or alder ALY isolates. The results showed that all five samples from Piedmont and the FD2000 isolate had the same *Hpy*188I RFLP profile (data not shown).

The RFLP analyses of the phytoplasmal DNA from the grapevine samples showed the presence of FD and *Bois noir* (BN), a different grapevine yellows disease. BN was found in all the areas investigated in Italy and the Balkans. The grapevines infected with the FD phytoplasma showed different RFLP patterns in each of the three genomic regions, allowing typical profiles corresponding to the FD-D, FD-C and FDLomb/Piem isolates to be identified (data not shown). The FD-D isolate was found in all the northern Italian regions surveyed (the Aosta Valley, Piedmont, Lombardy, the Veneto, Trentino Alto Adige, Friuli Venezia Giulia), as well as in Emilia Romagna (central Italy) and in Slovenia. Phytoplasmas showing RFLP patterns similar to FD-C were found in grapevine in Serbia, Slovenia and in the following Italian regions: the Veneto, Emilia Romagna, Tuscany, Umbria and the Marche. The FDLomb/Piem isolate was found only in north-western Italy, i.e. in the Lombardy, Piedmont and Aosta Valley regions.

Nucleotide sequencing of clematis and grapevine FD phytoplasmas

RplO-secY (FD9) amplicons, obtained from a selection of clematis and grapevine samples infected with phytoplasmas belonging to the 16SrV-C subgroup, were sequenced and the results were deposited in GenBank (Table 2). Many different FD variants were found.

Clematis phytoplasmas from almost all of northern Italy and from Slovenia, Croatia and Macedonia proved to have the same nucleotide (nt) sequence as the FD-C reference phytoplasma. All the phytoplasma-infected clematis from central Italy (Emilia Romagna, Umbria and Tuscany) contained an FD-C SNP (single nt polymorphism) mutant, named FD-CCI isolate (FD-C Central Italy). Two infected clematis from Serbia contained the typical Serbian isolate (Kuzmanović *et al*., 2008), called FD-CS in the present paper for clarity. The phytoplasma infecting the few clematis plants from Piedmont, which had previously shown an RFLP pattern similar to FD2000 in the *rplO-secY* DNA region, showed two nt differences compared to FD2000 and 100% nt identity with a phytoplasma isolate (GenBank Acc. no. AM397286) which had recently been found in four grapevines in the Savoie region of western France (Arnaud *et al*., 2007); the isolate is denoted FD-PS (Piedmont Savoie) in the present paper. The clematis phytoplasma amplicons which showed different lengths in the RFLP experiments confirmed that they had a deletion of 51 and 57 nt and an insertion of 47 nt, respectively. The deletions were located in the same position in the translocase gene and entailed the

Table 2 List of the *Flavescence dorée* (FD) phytoplasma isolates sequenced in the *rplO-secY* gene region and characteristics of the samples

Isolate	Host	Geographic origin	Nt differences with FD-C isolate[a]	GenBank Accession no.	FD isolate type
CL-TV37	Clematis	Veneto – Italy	0	FJ648472	FD-C
CL-TV33	Clematis	Veneto – Italy	51 (del)	FJ648473	FD-C deletion mutant
CL-TV42	Clematis	Veneto – Italy	57 (del)	FJ648474	FD-C deletion mutant
CL-TV111	Clematis	Veneto – Italy	1	FJ648475	FD-C SNP mutant
CL-VE145	Clematis	Veneto – Italy	0	FJ648476	FD-C
Vv-TV28	Grapevine	Veneto – Italy	0	FJ648458	FD-C
Vv-TV229	Grapevine (rootstock)	Veneto – Italy	0	FJ648459	FD-C
De-TV222	*D. europaea*	Veneto – Italy	0	FJ648469	FD-C
CL-UD147	Clematis	FriuliVG – Italy	47 (ins)	FJ648477	FD-C insertion mutant
CL-GO199	Clematis	FriuliVG – Italy	0	FJ648478	FD-C
CL-AL30	Clematis	Piedmont – Italy	0	FJ648479	FD-C
CL-AL31	Clematis	Piedmont – Italy	19	FJ648480	FD-PS
Vv-AL23	Grapevine	Piedmont – Italy	0	FJ648460	FD-C
CL-PV91	Clematis	Lombardy – Italy	0	FJ648481	FD-C
CL-PV109	Clematis	Lombardy – Italy	0	FJ648482	FD-C
CL-SI118	Clematis	Tuscany – Italy	1	FJ648483	FD-CCI
Vv-SI257	Grapevine	Tuscany – Italy	1	FJ648461	FD-CCI
CL-RA78	Clematis	Emilia Romagna – Italy	1	FJ648484	FD-CCI
Vv-RA1	Grapevine	Emilia Romagna – Italy	1	FJ648462	FD-CCI
Vv-FC1	Grapevine	Emilia Romagna – Italy	1	FJ648463	FD-CCI
Vv-AP1	Grapevine	Marche – Italy	2	FJ648464	FD-CCI SNP mutant
Vv-AP2	Grapevine	Marche – Italy	2	FJ648465	FD-CCI SNP mutant
CL-PG40	Clematis	Umbria – Italy	1	FJ648485	FD-CCI
CL-PG49	Clematis	Umbria – Italy	1	FJ648486	FD-CCI
Vv-PG204	Grapevine	Umbria – Italy	1	FJ648466	FD-CCI
CL-SLO169	Clematis	Slovenia	0	FJ648487	FD-C
Vv-SLO1	Grapevine	Slovenia	2	FJ648467	FD-C SNP mutant
CL-HR179	Clematis	Istria – Croatia	0	FJ648488	FD-C
CL-NI23	Clematis	Nišava – Serbia	1	FJ648489	FD-CS
Vv-NI10	Grapevine	Nišava – Serbia	1	FJ648468	FD-CS
De-NI10	*D. europaea*	Nišava – Serbia	1	FJ648470	FD-CS
CL-BR30	Clematis	Braničevo – Serbia	1	FJ648490	FD-CS
CL-SB28	Clematis	South Banat – Serbia	1	FJ648491	FD-C SNP mutant
De-SB18	*D. europaea*	South Banat – Serbia	1	FJ648471	FD-C SNP mutant
CL-KV97	Clematis	Kavadarci – Macedonia	0	FJ648492	FD-C
CL-NG98	Clematis	Negotino – Macedonia	0	FJ648493	FD-C

[a]GenBank Accession no. AF458378 and AY197665.

lack of 27 and 29 amino acids, respectively, in the encoded protein. The insertion, on the other hand, was located in the intergenic sequence between the L15 ribosomal protein and the translocase genes. Other FD clematis phytoplasma revealed SNP mutations compared to the FD-C phytoplasma (Table 2).

The sequencing of the *rplO-secY* amplicons from grapevines infected with 16SrV-C subgroup phytoplasmas showed that most of the samples hosted the isolate typical of the investigated areas. In particular, grapevines from the Veneto were infected with the FD-C isolate, as expected; grapevines from central Italy proved to host a phytoplasma slightly different from FD-C because of an SNP mutation, the same one that was present in clematis collected from these regions (i.e. FD-CCI); grapevines from Serbia showed the presence of the typical Serbian isolate, the same one that was present in clematis from those regions. Other mutants were sporadically identified: one sample from Slovenia revealed two SNP mutations compared to FD-C; another two samples from the Marche region in central Italy showed an SNP mutation compared to FD-CCI and two SNP mutations compared to FD-C. Unlike for clematis, no insertion or deletion mutants were identified.

All the phytoplasma sequences obtained from clematis and grapevine in the *rplO-secY* gene, together with the FD sequences obtained from GenBank, were used to construct a phylogenetic tree (Fig. 3). All but one of the phytoplasmas found in clematis were grouped into a separate cluster, together with the all phytoplasmas belonging to the 16SrV-C subgroup found in grapevine in Italy and the Balkans so far; all the mutants, from grapevine and clematis, were also included in this cluster. The FD-PS phytoplasma isolate, found in five clematis plants from Piedmont and identical to the isolate detected in grapevine in western France, was the only one to be more divergent, as it was grouped into a different cluster,

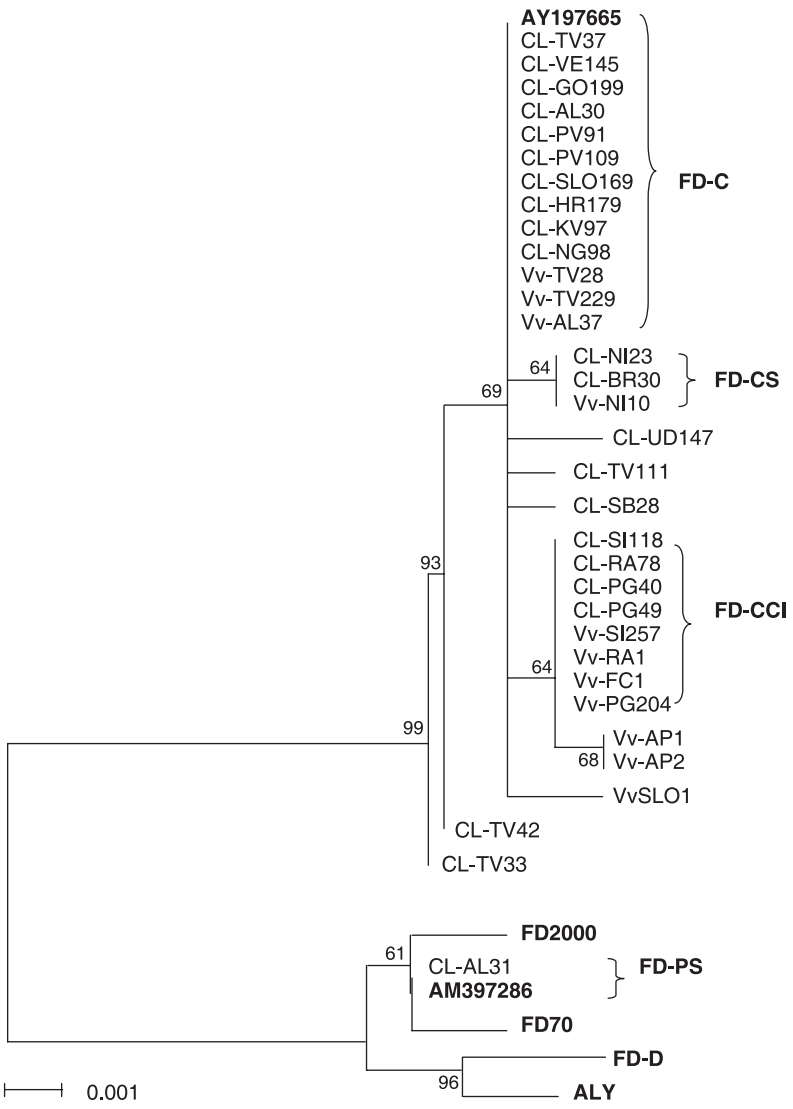

Figure 3 Phylogenetic tree constructed with sequencing data obtained from clematis (*Clematis vitalba*) and grapevine (*Vitis vinifera*) phytoplasmas in the *rplO-secY* gene amplicon. Bar lengths are proportional to the number of base substitutions per site. For abbreviations, see Table 2.

together with the FD2000 and FD70 phytoplasma isolates from French grapevines.

Insect surveys

Of all the potential Hemipteran vectors surveyed – a total of 961 insect specimens belonging to 27 species – only 20 out of 180 (11%) of *S. titanus* individuals and 18 out of 527 (3·4%) of *D. europaea* individuals proved to be infected with the FD phytoplasma (Table 3). The FD phytoplasma was always identified in adults, while all the nymphs collected were negative. *Scaphoideus titanus* individuals were consistently found in grapevine, but some specimens were also collected from clematis, while *D. europaea* individuals were consistently collected from both plants. All seven FD-positive *D. europaea* specimens from Italy were collected from clematis, while, in Serbia, four FD-infected specimens were collected from grapevine and seven from clematis.

All the PCR amplicons from the *D. europaea* phytoplasmas showed an RFLP profile which was identical to the FD-C isolate in the FD9 marker. The sequencing of *rplO-secY* gene amplicons from three *D. europaea* specimens showed that the insects were always infected with the same isolate which was present in the clematis and/or grapevine plants of the region where they were collected. One *D. europaea* specimen from the Veneto contained the FD-C isolate, one *D. europaea* specimen from Serbia contained the Serbian FD-CS isolate, while the other specimen, collected from the South Banat region in Serbia, was infected with the FD-C SNP mutant present in the clematis plants of that area (Table 2).

Acquisition and transmission trials

Many *S. titanus* and *E. variegatus* specimens, placed to feed on FD-infected clematis plants, were not able to survive for a long time. A high rate of mortality was observed

Table 3 Complete list of insect species and individuals collected by sweep net surveys in Italy (2002–2007) and Serbia (2005–2007) and their sanitary status

Country/Year	Insect species	No. individuals	No. FD-infected
Italy	*Hyalesthes obsoletus*	10	0
2002–2007	*Dictyophara europaea*	184	7
	Philaenus spumarius	1	0
	Empoasca vitis	7	0
	Metcalfa pruinosa	4	0
	Stictocephala bisonia	1	0
	Zygina rhamni	1	0
	Macrosteles sp.	3	0
	Euscelis incisus	8	0
	Euscelidius variegatus	4	0
	Psammotettix sp.	17	0
	Scaphoideus titanus	78	6
	species 1 (Psylloideae)	2	0
Serbia	*Cixius similis*	3	0
2005–2007	*Hyalesthes obsoletus*	16	0
	Asirica clavicornis	3	0
	Stenocranus major	2	0
	Laodelphax striatella	5	0
	Dictyophara europaea	343	11
	Philaenus spumarius	27	0
	Issus coleoptratus	3	0
	Agalmatium flavescens	1	0
	Aphrophora alni	37	0
	Fieberiella septentrionalis	1	0
	Neoaliturus fenestratus	3	0
	Mocydia crocea	8	0
	Mocydiopsis intermedia	1	0
	Euscelis incisus	25	0
	Psamottetix alienus	38	0
	Scaphoideus titanus	102	14
	Jassargus obtusivalvis	21	0
	Cosmottetix sp.	1	0
	Enantiocephalus cornutus	1	0
Total		961	38

in both cases from the first days of the experiments. Most of the *S. titanus* samples did not show the positive band after the specific PCR for FD phytoplasma. Only two batches of four individuals which had fed for 10 days on clematis gave a faint signal on agarose gels. The *E. variegatus* specimens were all negative using the FD-specific PCR amplification.

The transmission experiments with the *D. europaea* individuals gave completely different results. A total of 36 individuals out of 100 used for the acquisition trial from FD-CS-infected clematis were still alive after 28 days and were moved to grapevine seedlings, where the species survived for 1–7 days (average 3·8 ± 1·7). The PCR/RFLP tests carried out after they died showed that five specimens out of 36 (14%) were infected with the same FD phytoplasma that was present in the clematis plants. Two of them survived for 3 days, one individual for 5 days and two individuals for 6 days on grapevine. Three out of six grapevine seedlings on which these individuals were fed developed symptoms of grapevine yellows 12 months later. The PCR/RFLP assay conducted on these plants confirmed the presence of the FD phytoplasma. The five FD-positive *D. europaea* individuals were fed precisely on these three grapevine seedlings. The three remaining grapevine seedlings were symptomless and PCR-negative, as well as the *D. europaea* specimens that had been fed on the latter plants.

Discussion

The wide geographical survey conducted in this work revealed a significant occurrence of FD phytoplasma in *C. vitalba*, as approximately 36% of the collected clematis samples were infected. This phenomenon was not restricted to a few geographical areas, but involved almost all the regions investigated, spanning from central Italy to Macedonia, via northern Italy, Slovenia, Croatia and Serbia.

FD-infected clematis plants were also present in areas where FD-C and related phytoplasma isolates had never previously been recorded in grapevine and only BN or FD-D had been detected up to that time (Šeruga *et al.*, 2000, 2003). Moreover, FD-infected clematis plants were also found far away from FD-infected vineyards. These findings suggest that *C. vitalba* could be an original host of those FD isolates closely related to FD-C.

The population of FD phytoplasma isolates in the clematis was quite heterogeneous, though all of them belonged to the 16SrV-C subgroup and almost all of them were closely-related to FD-C. The spatial distribution of the isolates depended mainly on the territory. A close genetic similarity was found between FD isolates belonging to the 16SrV-C group present in clematis and grapevine plants coming from the same location. Indeed, the FD-C, FD-CS and FD-CCI isolates typical of north-eastern Italy, Serbia and central Italy, respectively, were found in the same areas both in clematis and grapevine. An apparent exception was the FD-PS phytoplasma isolate, found in clematis plants from Piedmont, which has not been found in grapevine in this work; however, a few FD-infected grapevines collected in the same areas in 2004–2005 showed RFLP patterns which were identical to those obtained from the FD-PS isolate (C. Marzachì, CNR Torino, Italy, personal communication). Moreover, the FD-PS isolate showed 100% nt identity with a grapevine FD isolate from Savoie (Arnaud *et al.*, 2007), the French region closest to Piedmont.

This spatial association suggests that it is possible for FD phytoplasmas to be transmitted from clematis to grapevine and that this has indeed happened. Research into the vectors involved could, therefore, be a critical issue in the epidemiology and control of FD disease (Boudon-Padieu, 2002). *Scaphoideus titanus* and *E. variegatus* were both good potential candidates for the transmission of the FD phytoplasma from clematis to grapevine. *Scaphoideus titanus* is the only known and highly efficient vector of FD phytoplasmas from one grapevine to another, it is able to survive and feed on many plant

species in experimental conditions (Caudwell *et al*., 1970; Alma *et al*., 2001; Bressan *et al*., 2005) and it was sporadically observed on *C. vitalba* by the authors and by other researchers (F. Pavan, Univ. Udine, Italy, personal communication); *Euscelidius variegatus*, on the other hand, is able to acquire and transmit FD phytoplasma from and to broadbean and some other plants in experimental conditions (Caudwell *et al*., 1972; Bressan *et al*., 2006). However, the results of the present work seem to exclude *S. titanus* and *E. variegatus* as possible vectors of FD phytoplasma from clematis to grapevine because both species were only able to survive on clematis in experimental conditions for a very short time and could not acquire FD phytoplasma from this plant.

The results of this work showed that a significant rate of FD-positive *D. europaea* specimens (approximately 3·5%) was found in field conditions and that in experimental conditions this species was able to acquire FD-CS phytoplasma from clematis and to transmit it to grapevine. The number of *D. europaea* specimens which acquired the pathogen from clematis was not very high (14%); however, only one infected *D. europaea* specimen would have been sufficient to infect grapevine, when forced to feed on grapevine alone. *Dictyophara europaea* can, therefore, play an important role in the epidemiology of FD phytoplasma in all the viticultural areas where FD-infected clematis plants are found. The importance of these findings has to be evaluated in the frame of the ecological behaviour of the species in different environments and climates. *Dictyophara europaea* is a polyphagous planthopper that occurs commonly on clematis, where it can live and feed, as reported in this work. It was consistently observed on grapevine in the Italian and Serbian areas investigated. In other regions, with different ecological conditions, *D. europaea* has been found on many plant species, including *Vitis* spp., but not on clematis (Lessio & Alma, 2008).

All these findings, together with the high prevalence of naturally FD-infected clematis plants, confirm that *D. europaea* is an occasional vector of FD phytoplasma from clematis to grapevine. In the past, the grapevine-*S. titanus* cycle was believed to be the only mechanism involved in the FD epidemiology in Europe. However, this work demonstrates that this cycle is not closed, as FD phytoplasma can be transmitted to grapevine from clematis, a non-grapevine host plant, by an occasional vine-feeder, *D. europaea* (Fig. 4). In particular, the capacity of the planthopper to transmit FD from clematis to grapevine could easily explain the sporadic presence of FD phytoplasmas in grapevine plants in some regions of central Italy (Credi *et al*., 2002; this work; V. Vicchi, Phytosanitary Service Emilia Romagna, Italy, personal communication), where, at the time of these findings, *S. titanus* was not present.

The results of this work can provide a very likely explanation for the outbreak of FD in the Veneto (north-eastern Italy), central Italy and Serbia, caused by the FD-C, FD-CCI and FD-CS phytoplasma isolates, respectively. It might be assumed that in these regions, those FD isolates were originally present in clematis, as natural

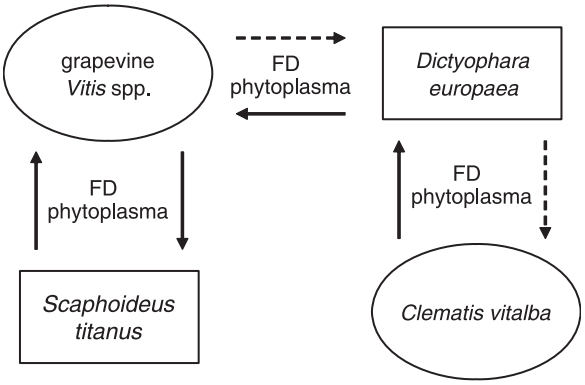

Figure 4 The cycle of *Flavescence dorée* phytoplasma as determined in this work. Dotted lines indicate possible transmission; no data available yet.

pathogens; *D. europaea* individuals fed on clematis and occasionally transmitted the pathogen to grapevine, but no epidemic took place in the vineyard. *Scaphoideus titanus*, a nearctic vine-feeding species introduced into Europe at the beginning of the 20th Century, proved to be a competent and efficient vector of the FD phytoplasma on grapevine and caused the FD epidemics, as suggested previously (Boudon-Padieu, 2002). In short, the FD pathogen was allowed to gain a new ecological niche, in which it was aggressive and dangerous. Thus, clematis and *Dictyophara europaea* have a primary role in the introduction of FD phytoplasma into the grapevine-*S. titanus* niche and in generating primary infection sites.

The high prevalence of FD-infected clematis plants, the capacity of *D. europaea* to acquire and transmit the FD phytoplasma and its ecological behaviour suggest that this planthopper could also be the natural vector of the phytoplasma from clematis to clematis. However, further transmission trials should be performed to confirm this hypothesis. Only species belonging to the Cicadellidae family (Cicadomorpha infraorder) have to date been found to have the vector competency for FD phytoplasma (Bressan *et al*., 2006). *Dictyophara europaea* belongs to the Dictyopharidae family (Fulgoromorpha infraorder), thus it is phylogenetically quite distant. Divergent mechanisms that allow the insect to be a competent vector could be present, and further studies are therefore required on the interactions between the FD phytoplasma and *D. europaea*.

In this work, clematis and *D. europaea* were never found to be infected with FD-D, the most common grapevine FD isolate in Europe. The most reasonable hypothesis could be that other host plants and vectors do exist and are linked to FD isolates which are phylogenetically different from the FD-C cluster (Fig. 3). Recently, alder was also supposed to be an original European host to FD phytoplasma (Arnaud *et al*., 2007). Alder has long been found infected by 16SrV group phytoplasmas (Maixner & Reinert, 1999). However, recent investigations

showed that it can host some phytoplasma isolates which are very similar to FD70, FD2000 and FD-D, but not FD-C or closely-related isolates (Angelini *et al*., 2003; Arnaud *et al*., 2007; Malembic-Maher *et al*., 2007). Further studies and research regarding alder, other spontaneous FD-host plants and potential vectors need to be performed to add new information to the ecological cycles of the FD phytoplasmas in Europe.

The presence of FD phytoplasma reservoirs other than grapevine and the occasional transmission of FD phytoplasmas by means of *D. europaea* or other vectors should be taken into account in order to assess the true risk of FD epidemics. Indeed, the control strategies currently used in Europe for grapevine yellows epidemics are not effective against pathogen sources and vectors originating outside the vineyard. Moreover, no insecticide treatments against *S. titanus* are carried out where FD disease does not exist. Management of FD disease should therefore consider, as a preventive measure, reducing *S. titanus* populations in vineyards even in regions where FD epidemics do not occur (Boudon-Padieu, 2002).

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