Journal of Plant Pathology (2023) 105:1511–1519
https://doi.org/10.1007/s42161-023-01474-2

ORIGINAL ARTICLE

Identification of phytoplasmas associated with grapevine 'bois noir' and flavescence dorée in inter-row groundcover vegetation used for green manure in Franciacorta vineyards

Abdelhameed Moussa[1,2] · Enea Guerrieri[3] · Sara Torcoli[4] · Flavio Serina[4] · Fabio Quaglino[2] · Nicola Mori[3]

Received: 20 December 2022 / Accepted: 19 July 2023 / Published online: 8 August 2023

Abstract
'Bois noir' (BN) and flavescence dorèe (FD), the two main diseases of the grapevine yellows complex associated with genetically distinct phytoplasmas, have a complex epidemiology including multiple insect vectors and reservoir plants. This study investigated the presence of BN and FD phytoplasmas in nine groundcover plant species commonly utilized for inter-row vineyard green manure in Franciacorta (North Italy). The activities conducted in 2020 included monitoring and sampling groundcover plant species and symptomatic grapevines in September, and phytoplasma identification and typing by amplification and sequence analyses of *stamp* and *map* genes. Molecular analyses identified BN phytoplasma (strains carrying the *stamp* gene sequence variants St5, St19, St30) and FD phytoplasma (strains carrying the *map* gene sequence variant M54) in 72% and 28% of symptomatic grapevines, respectively. BN phytoplasma strains St5 and St30 were found also in *Eruca sativa*, *Vicia sativa*, and *Polygonum fagopyrum*. FD phytoplasma strain M54 was found also in *Vicia faba*, *Trifolium incarnatum*, and *Polygonum fagopyrum*. These results reinforced the evidence of the increasing range of BN and FD phytoplasma alternative plant hosts and suggested a criterium for the selection of the groundcover plant species utilized for green manure, excluding the ones putatively involved in BN and FD diffusion.

Keywords '*Candidatus* Phytoplasma solani' · 16SrV phytoplasmas · *Stamp* · *Map* · Viticulture · Weeds

Introduction

Phytoplasmas are cell wall-less prokaryotic unicellular microorganisms belonging to the Mollicutes class. They are phloem-limited obligate intracellular parasites of a broad range of plants in which they are associated with several diseases (Bertaccini 2007). They are difficult to be cultivated in axenic conditions and are plant-to-plant transmitted by insect vectors (phloem-feeders) (Weintraub and Beanland 2006). Based on molecular and other biological features phytoplasma strains have been classified into 49 species within the provisional genus '*Candidatus* Phytoplasma' and taxonomic groupings have also been delimited according to the DNA sequence coding for their 16S ribosomal RNA (Bertaccini et al. 2022).

The two main phytoplasma-associated diseases belonging to the grapevine yellows (GY) complex are 'bois noir' (BN) and flavescence doreé (FD), widespread and destructive throughout the Mediterranean area, central and eastern Europe (Dermastia et al. 2017). The symptoms that BN and FD cause on grapevine are undistinguishable and include color alteration and downward curling of the leaves, total or partial lack of lignification of the new shoots, and berry shrivel. Although the identical symptoms, BN and FD are

Abdelhameed Moussa and Enea Guerrieri equally contributed to the work.

✉ Fabio Quaglino
fabio.quaglino@unimi.it

1 Competence Centre for Plant Health, Free University of Bozen-Bolzano, Piazza Università 5, Bolzano 39100, Italy

2 Department of Agricultural and Environmental Sciences - Production, Landscape, Agroenergy, University of Milan, Via Celoria 2, Milan 20133, Italy

3 Department of Biotechnology, University of Verona, Via della Pieve 70, San Floriano, Verona 37020, Italy

4 Franciacorta Consortium, Via Verdi 53, Erbusco 25030, BS, Italy

associated with genetically distinct phytoplasmas (Belli et al. 2010).

'*Candidatus* Phytoplasma solani' (CaPsol) strains, taxonomic subgroup 16SrXII-A, are associated with BN (Quaglino et al. 2013). In European and Mediterranean countries, CaPsol strains are transmitted to grapevine by the insect vector *Hyalesthes obsoletus* Signoret, a polyphagous cixiid completing its biological cycle preferentially on *Convolvulus arvensis* L. and *Urtica dioica* L.. During its feeding activity on such plants, *H. obsoletus* can acquire CaPsol and occasionally transmit it to grapevine, a dead-end host for the pathogen (Langer and Maixner 2004). Other studies reported the presence of additional insect vectors able to transmit CaPsol to grapevine: *Reptalus panz*eri (Löw) in Serbia (Cvrkovic et al 2014), *R. artemisiae* (Becker) in France and Italy (Chuche et al. 2016; Pierro et al. 2020), *Aphrodes makarovi* Zachvatkin, *Dicranotropis hamata* Boheman, *Dictyophara europaea* Spinola, *Euscelis incisus* (Kirschbaum), *Euscelidius variegates* Kirschbaum, *Laodelphax striatellus* (Fallén), *Philaenus spumarius* (Linnaeus), and *Psammotettix alienus/confinis* (Dahlbom) in northern Italy (Quaglino et al. 2019). Furthermore, spontaneous weeds commonly present within the vineyard inter-row groundcover vegetation (*Chenopodium album* L., *Malva sylvestris* L., *Polygonum aviculare* L., *Trifolium repens* L.) were found hosting CaPsol and supporting the *H. obsoletus* survival for around two weeks, confirming that BN epidemiology is influenced by several weeds and their distribution patterns inside and outside vineyards (Mori et al. 2015a, b; Quaglino et al. 2021).

Phytoplasma strains belonging to the taxonomic group 16SrV, subgroups -C and -D, are associated with FD. FD phytoplasmas (FDp) are efficiently vine-to-vine transmitted by the ampelophagous insect vector *Scaphoideus titanus* Ball, determining highly epidemic diffusion of the disease (Arnaud et al. 2007; Chuche and Thiery 2014). For that reason, FDp is a quarantine pathogen, and its control is regulated by mandatory measures including the eradication of infected grapevines and the insecticide treatments against *S. titanus* (Belli et al. 2010). Further evidence highlighted that FD epidemiology is not limited to the close pathosystem "grapevine - *S. titanus*". It was demonstrated that (i) *D. europaea* is a vector of FDp from *Clematis vitalba* L. to grapevine (Filippin et al. 2009), (ii) *Orientus ishidae* (Matsumura) can acquire FDp from multiple plant sources and transmit it to grapevine and *Alnus glutinosa* L. (Mehle et al. 2010; Gaffuri et al. 2011; Parise 2017), (iii) *Allygus* spp. are vectors of FDp from alder to alder and faba bean (Malembic-Maher et al. 2020).

Furthermore, studies conducted in the last years have led to the identification of insects able to acquire FDp, such as *Phlogotettix cyclops* (Mulsant & Rey) (Strauss et al. 2018) and *Hishimonus hamatus* Kuoh (Belgeri et al. 2022). Interestingly, other studies demonstrated that *S. titanus* is able to survive on herbaceous (*Amaranthus* sp., *Chenopodium* sp., *Convolvulus* sp., *Daucus carota* L., *Onoclea sensibilis* L., *Polygonum* sp., *Solidago* sp.) and woody (*Crataegus* sp., *Juniperus virginiana* L., *Malus* sp., *Prunus persica* (L.) Batsch, *Salix* sp., *Ulmus americana* L.) plants (Chuche and Thiery 2014). Furthermore, based on observations conducted in vineyards, it was noticed that *S. titanus*, knocked down to the ground following intense rains, can survive up to 3–4 days on several weeds such as *Cyperus rotundus* L., *Dandelion officinale* Weber, *Galinsoga parviflora* Cav., and *Trifolium repens* L. (Mori, personal communication). Based on this evidence, it is reasonable to hypothesize that also weeds could be involved in FDp spreading.

Green manure is a common agronomic practice in vineyards. It is conducted to improve soil quality and consists in growing in the vineyard inter-rows and ploughing into the terrain mixtures of selected grasses and legume plants (groundcover crops). Several benefits are generated by this practice: erosion and leaching are reduced by the improvement of the soil physical structure (Ingels et al. 2005); activity of N cycle enzymes is enhanced by the higher carbon availability for the soil microorganisms (Okur et al. 2016); pest control is improved by the attractiveness of the selected groundcover plants for natural predators/parasitoids (Irvin et al. 2014); the grape organoleptic features are improved (Rotaru et al. 2011). Although the agronomic advantages of green manure have been recognized, the phytosanitary consequences on grapevine diseases should be considered.

In this study, we investigated the role of nine ground cover plant species (*Eruca sativa* Miller, *Sinapis arvensis* L., *Lobularia maritima* (L.) Desv., *Phacelia tanacetifolia* Benth., *Vicia sativa* L., *Vicia faba* var. minor Beck, *Trifolium incarnatum* L., *Trifolium alexandrinum* L., *Polygonum fagopyrum* L.), commonly used for inter-row green manure of vineyards in Franciacorta (northern Italy), as alternative plant hosts of phytoplasmas associated with BN and FD, putatively involved in the spread of such diseases.

Materials and methods

Field surveys

In September 2020, typical GY symptoms (color alteration and downward curling of the leaves, total or partial lack of lignification of the new shoots, and berry shrivel) were monitored in two Chardonnay organic vineyards localized in Gussago (45°35′38.12″N, 10°09′34.32″E) and Provaglio d'Iseo (45°63′59.95″N, 10°06′24.26″E) in the Franciacorta (BS, Italy) grape growing area. Both vineyards were

Table 1 Molecular detection and typing of GY associated phytoplasmas in Gussago vineyard

Species	No. of plants[1]		CaPsol strain[2] (No. of plants)
	Collected	Infected by CaPsol (%)	
Vitis vinifera L.	60	60 (100%)	**St5** (23), St19 (23), **St30** (14)
Convolvulus arvensis L.	35	7 (20%)	St1 (1), **St5** (4), St10 (2)
Eruca sativa Miller	67	6 (9%)	St1 (2), **St5** (4)
Phacelia tanacetifolia Benth.	20	0	
Polygonum fagopyrum L.	47	4 (9%)	St10 (4)
Sinapis arvensis L.	20	0	
Trifolium alexandrinum L.	18	0	
Trifolium incarnatum L.	47	0	
Vicia faba var. minor Beck	20	0	
Vicia sativa L.	46	13 (28%)	St1 (2), **St5** (7), **St30** (4)

[1] No plants were found infected by 16SrV phytoplasmas

[2] Strains identified in grapevine and groundcover vegetation are written in bold

Table 2 Molecular detection and typing of GY associated phytoplasmas in Provaglio d'Iseo vineyard

Species	No. of plants			CaPsol strain[1] (No. of plants)	FDp strain[1] (No. of plants)
	Collected	Infected by CaPsol (%)	Infected by 16SrV phytoplasmas (%)		
Vitis vinifera L.	55	23 (42%)	32 (58%)	**St5** (15), St19 (7)	**M54** (32)
Convolvulus arvensis L.	20	7 (35%)	0	**St5** (7)	
Lobularia maritima (L.) Desv.	20	0	0		
Phacelia tanacetifolia Benth.	8	0	0		
Polygonum fagopyrum L.	16	3 (18%)	5 (31%)	**St5** (3)	**M54** (5)
Trifolium incarnatum L.	16	0	3 (19%)	**M54** (3)	
Vicia faba var. minor Beck	16	0	1 (6%)	**M54** (1)	

[1] Strains identified in grapevine and groundcover vegetation are written in bold

managed by inter-row green manure with cover vegetation constituted by eight species (*Eruca sativa* Miller, *Phacelia tanacetifolia* Benth., *Polygonum fagopyrum* L., *Sinapis arvensis* L., *Trifolium alexandrinum* L., *Trifolium incarnatum* L., *Vicia sativa* L., *Vicia faba* var. minor Beck) in Gussago vineyard, and five species (*Lobularia maritima* (L.) Desv., *P. tanacetifolia*, *P. fagopyrum*, *T. incarnatum*, *V. faba*) in Provaglio d'Iseo. Leaves and petioles were collected from 531 plants: 115 symptomatic grapevines (60 in Gussago, 55 in Provaglio d'Iseo), 55 symptomless plants of *Convolvulus arvensis* (the main BNp reservoir plant) (35 in Gussago, 20 in Provaglio d'Iseo), and 361 symptomless plants of ground cover vegetation (285 in Gussago, 76 in Provaglio d'Iseo) (Tables 1 and 2). Collected leaves and petioles were stored at -20 °C until molecular analyses.

Phytoplasma detection

Total nucleic acids (TNAs) were extracted from 1 g of petiole and/or leaf tissue of all the collected plant samples using a CTAB-based extraction protocol previously described (Angelini et al. 2001). Obtained TNAs were solved in 150 µl distilled sterile water, their quality and concentration were measured by Nanodrop system, and they were stored at -20 °C until further use. Extracted TNAs were utilized as templates in nested PCR reactions conducted for CaPsol and 16SrV phytoplasma specific identification through the amplification of the *stamp* and *map* gene, respectively. For *stamp* gene, direct PCRs were performed using the primer pair StampF/StampR0, followed by nested PCRs with the primer pair StampF1/StampR1; primer sequences and reaction conditions were as previously described (Fabre et al. 2011). For *map* gene, direct PCRs were performed using the primer pair FD9F5/MapR1, followed by nested PCRs with the primer pair FD9F6/MapR2; primer sequences and reaction conditions were as previously described (Arnaud et al. 2007). Total nucleic acids from periwinkle plants infected by phytoplasma strains STOL ('*Ca*. P. solani', subgroup 16SrXII-A, Acc. No. AF248959; Quaglino et al. 2013), AY1 ('*Ca*. P. asteris', subgroup 16SrI-B, M30790; Lee et al. 2004a, b) and EY1 ('*Ca*. P. ulmi', subgroup 16SrV-A, AY197655; Lee et al. 2004a, b) were used as reference controls. The reaction mixture devoid of nucleic acids was used as negative control. PCR products were verified by electrophoresis on 1% agarose gel in TBE buffer and visualized under a UV transilluminator.

Phytoplasma typing and phylogenetic analysis

Two StampF1/StampR1 and FD9F6/MapR2 amplicons, obtained in separate reactions from each analyzed plant, were sequenced in both strands (4X coverage per base position)

by a commercial sequencing service (Eurofins Genomics, Germany). Nucleotide sequences were assembled by the Contig Assembling Program and trimmed to the *stamp* gene start and stop codons and to the *map* gene annealing sites of the FD9F6 and MapR2 primers in the software BioEdit version 7.2.6 (Hall 1999). *Stamp* gene nucleotide sequences, obtained in this study from CaPsol strains identified in grapevines and groundcover plants, were aligned using the ClustalW Multiple Alignment program in the software BioEdit and analyzed by Sequence Identity Matrix to calculate their genetic diversity. The same procedure was utilized for *map* gene nucleotide sequences of 16SrV phytoplasma strains identified in this study. Finally, the obtained *stamp* and *map* sequences were aligned respectively with representative sequences of previously defined *stamp* (Pierro et al. 2018a, b) and *map* (Malembic et al. 2020) sequence variants. A nucleotide sequence identity of 100% was necessary for the sequence variant attribution.

Nucleotide sequences of CaPsol representative strains of *stamp* sequence variants and 16SrV phytoplasma strains representative of *map* sequence variants, identified in this and in previous studies (Pierro et al. 2018a, b; Malembic et al. 2020), were aligned and used for generating unrooted phylogenetic trees by Neighbor-Joining method performed using the Jukes-Cantor model and bootstrap replicated 1000 times in the MEGAX software (Kumar et al. 2018).

Results and discussion

Grapevine yellows phytoplasma detection in grapevines and groundcover plants

Nested PCRs allowed identifying CaPsol and 16SrV phytoplasmas respectively in 23% (123 out of 531) and 8% (41 out of 531) of analyzed plants. CaPsol was detected in 72% (83 out of 115) of symptomatic grapevines, 25% (14 out of 55) of bindweed plants, and in 11% (40 out of 361) of groundcover plants. Phytoplasmas of 16SrV group (including FDp) were detected in 28% (32 out of 115) of symptomatic grapevines and 2% (9 out of 361) of groundcover plants (Tables 1 and 2). These data confirmed the prevalence of BN disease in Franciacorta vineyards (Quaglino et al. 2019, 2021).

In Gussago vineyard, 16SrV phytoplasmas were not detected in both symptomatic grapevines and groundcover vegetation; CaPsol was identified in all symptomatic grapevines, in 20% of bindweed plants, and in the groundcover plant species *E. sativa* (9% of the examined plants), *P. fagopyrum* (9%), and *V. sativa* (28%) (Table 1). To the best of our knowledge, such plant species were never reported before as CaPsol host plants. The remnant five species were found not infected by CaPsol (Table 1).

In Provaglio d'Iseo vineyard, 16SrV phytoplasmas and CaPsol were detected respectively in 58% and 42% of examined symptomatic grapevines. Moreover, CaPsol was identified in 35% of bindweed plants and 18% of *P. fagopyrum* plants; 16SrV phytoplasmas were detected also in *P. fagopyrum* (31% of the examined plants), *T. incarnatum* (19%), and *V. faba* (6%) (Table 2). To the best of our knowledge, *P. fagopyrum* and *T. incarnatum* were never reported before as 16SrV phytoplasma host plants, while *V. faba* is known as the main experimental host of FDp (Caudwell et al. 1970).

Molecular typing of CaPsol strains

Nucleotide sequence analyses, carried out on *stamp* gene nested PCR products (from 83 symptomatic grapevines, 14 bindweed plants, and 40 groundcover plants) revealed the presence of four distinct *stamp* gene sequence variants within CaPsol strain populations identified in Gussago and Provaglio d'Iseo vineyards. Such *stamp* sequence variants were undistinguishable from previously reported sequence variants St1 (representative strain Rqg50, Acc. No. KC703019), St5 (representative strain GGY, FN813256), St10 (representative strain PO, FN813270), St19 (representative strain CrHo13_1183, KJ469719), and St30 (representative strain Vv24, KC703022) (Pierro et al. 2018a, b). Previous studies evidenced the prevalence of these CaPsol strains in symptomatic grapevines, insect vectors, and reservoir plants in Franciacorta (Quaglino et al. 2019, 2021). The prevalent CaPsol strain, identified in 51% of CaPsol-infected plants in both Gussago and Provaglio d'Iseo vineyards, was typed by the sequence variant St5 followed by CaPsol strains carrying the sequence variants St19 (25%), St30 (15%), St10 (5%), and St1 (4%) (Tables 1 and 2). As *stamp* gene nucleotide sequence variants carried by CaPsol strains identified in the present study are identical (100% sequence identity) with variants already published and available in NCBI GenBank, they were not deposited to GenBank to avoid unnecessary repetitions.

To hypothesize its putative role in GY epidemiology, an alternative plant host should carry the same phytoplasma strain identified in grapevines exhibiting typical GY symptoms (Mori et al. 2015b; Quaglino et al. 2021). Here, CaPsol strains carrying the sequence variants St5 and St30 were identified in grapevines, *C. arvensis* (St5 in both Gussago and Provaglio d'Iseo), *E. sativa* (St5 in Gussago), *P. fagopyrum* (St5 in Provaglio d'Iseo), and *V. sativa* (St5 and St30 in Gussago). This evidence reinforced the role of bindweed as CaPsol reservoir plant (Langer et al. 2004). Interestingly, as reported in previous studies conducted in northern Italy

(Mori et al. 2015; Quaglino et al. 2021), CaPsol-infected bindweed plants were symptomless, while in other geographic regions (i.e., Serbia and Georgia) most bindweeds carrying CaPsol showed typical symptoms such as yellowing, reddening, dwarfism, and leaf malformation (Quaglino et al. 2016; Jović et al. 2021). This difference could be related to the genetic diversity among CaPsol strains prevalently identified in bindweeds in these regions: CaPsol strains carrying the *stamp* gene variant St5, St8 and St10 in northern Italy (Quaglino et al. 2021; this study), St1 and St2 in Serbia (Jović et al. 2021), St15 and St37 in Georgia (Quaglino et al. 2016). Moreover, obtained data highlighted the presence of three CaPsol alternative plant hosts, putatively related to feeding activities of additional CaPsol insect vectors recently reported (Quaglino et al. 2019). To study the role of such CaPsol plant hosts as reservoir plants, acquisition and transmission trials with insect vectors are required. Interestingly, in both examined vineyards, CaPsol strains carrying *stamp* variant St19 were identified exclusively in symptomatic grapevines, reinforcing the hypothesis of the existence of a BN epidemiological pattern including insect vectors able to transmit CaPsol vine-to-vine (Quaglino et al. 2019). On the other hand, CaPsol strains carrying *stamp* variants St1 and St10 were identified exclusively in groundcover vegetation: St1 in *C. arvensis*, *E. sativa*, and *V. sativa*; St10 in *C. arvensis* and *P. fagopyrum*. Interestingly, CaPsol strain St10, never reported before in grapevine, was recently found as the prevalent strain associated with BN in Tuscany vineyards and related to a newly proposed epidemiological pattern involving *R. artemisiae* as main vector (Pierro et al. 2020).

Phylogenetic analyses evidenced that CaPsol strains St5 and St30, identified in grapevine, *E. sativa*, *P. fagopyrum*, and *V. sativa*, and strains St1 and St10, identified exclusively in groundcover vegetation, belong to bindweed-related subclusters b-I and b-II, while CaPsol strains St19, identified exclusively in grapevines, belong to nettle-related subcluster a2 (Fig. 1) (Atanasova et al. 2015). This evidence suggests that groundcover vegetation used for green manure could play a role in the diffusion of bindweed-related CaPsol strains. On the other hand, considering the absence of nettle within and around the examined vineyards, it is reasonable to hypothesize that CaPsol nettle-related strains could be closely associated with a vine-to-vine transmission pattern.

As perennial plants constitute the main phytoplasma source involved in the epidemiology of phytoplasma-associated diseases, it is interesting that groundcover plants used for green manure, identified in the present study as CaPsol alternative plant hosts putatively involved in pathogen spread, are annual species. Two hypotheses have been articulated for explaining the possible role of annual plants in BN epidemiology: (i) CaPsol strains, infecting annual plants, can be vertically transmitted throughout seasons by seeds; (ii) CaPsol strains can be acquired from annual plants and transmitted to grapevine within the same season by alternative vectors living as adults in the vineyards longer than *H. obsoletus* (Mori et al. 2015b). This last hypothesis is supported by the recent finding of alternative CaPsol vectors (*Dictyophara europaea*, *Dicranotropis hamata*, *Philaenus spumarius*, *Euscelis incisus*, *Euscelidius variegatus*) in Franciacorta, characterized by a longer adult stage or able to overwinter as nymphs or adults, carrying CaPsol to the next season (Quaglino et al. 2019). On the other hand, it should be considered that annual plants, identified in this study as CaPsol alterative plant hosts, can represent dead-end hosts of the phytoplasma.

Molecular typing of 16SrV phytoplasma strains

Nucleotide sequence analyses, carried out on *map* gene nested PCR products (from 32 symptomatic grapevines and 9 groundcover plants of the species *P. fagopyrum*, *T. incarnatum*, *V. faba*) revealed the presence of a unique *map* sequence variant within 16SrV phytoplasma strain population in Provaglio d'Iseo vineyard (Table 2). Such *map* variant was undistinguishable from the sequence variant M54 (representative strain VF-00-SP5, Acc. No. AM384886), recently reported in FDp strains associated with FD outbreaks (epidemic strains) (Malembic-Maher et al. 2020). As *map* gene nucleotide sequence variants carried by FDp strains identified in the present study are identical (100% sequence identity) with variants already published and available in NCBI GenBank, they were not deposited to GenBank to avoid unnecessary repetitions. Phylogenetic analyses confirmed that FDp strain M54 (subgroup 16SrV-D) belongs to Map-FD2 cluster, including other epidemic FDp strains (M38, M121) classified in taxonomic subgroup 16SrV-C (Fig. 2) (Malembic-Maher et al. 2020). Up to now, M54 was reported as the prevalent FDp strain in vineyards from different French viticultural areas, Switzerland, and northwestern Italy (Casati et al. 2017; Rossi et al. 2019; Malembic-Maher et al. 2020). Even if strain M54 was identified mainly in symptomatic grapevines and *S. titanus*, it was found also in *Corylus avellana* L. and gone-wild *Vitis vinifera* L. and the insect vector *O. ishidae*, suggesting that its epidemics can be related not exclusively to the close pathosystem grapevine-*S. titanus* (Casati et al. 2017; Rossi et al. 2019). Interestingly, in the present study FDp strain M54 was reported as the unique FDp strain infecting grapevine in the examined vineyard in Franciacorta. Moreover, it was firstly identified in alternative plant hosts (*P. fagopyrum*, *T. incarnatum*, *V. faba*) whose role as reservoirs in FDp diffusion could be related to the activity of *S. titanus*, recently reported as able to adapt to other plants (Chuche

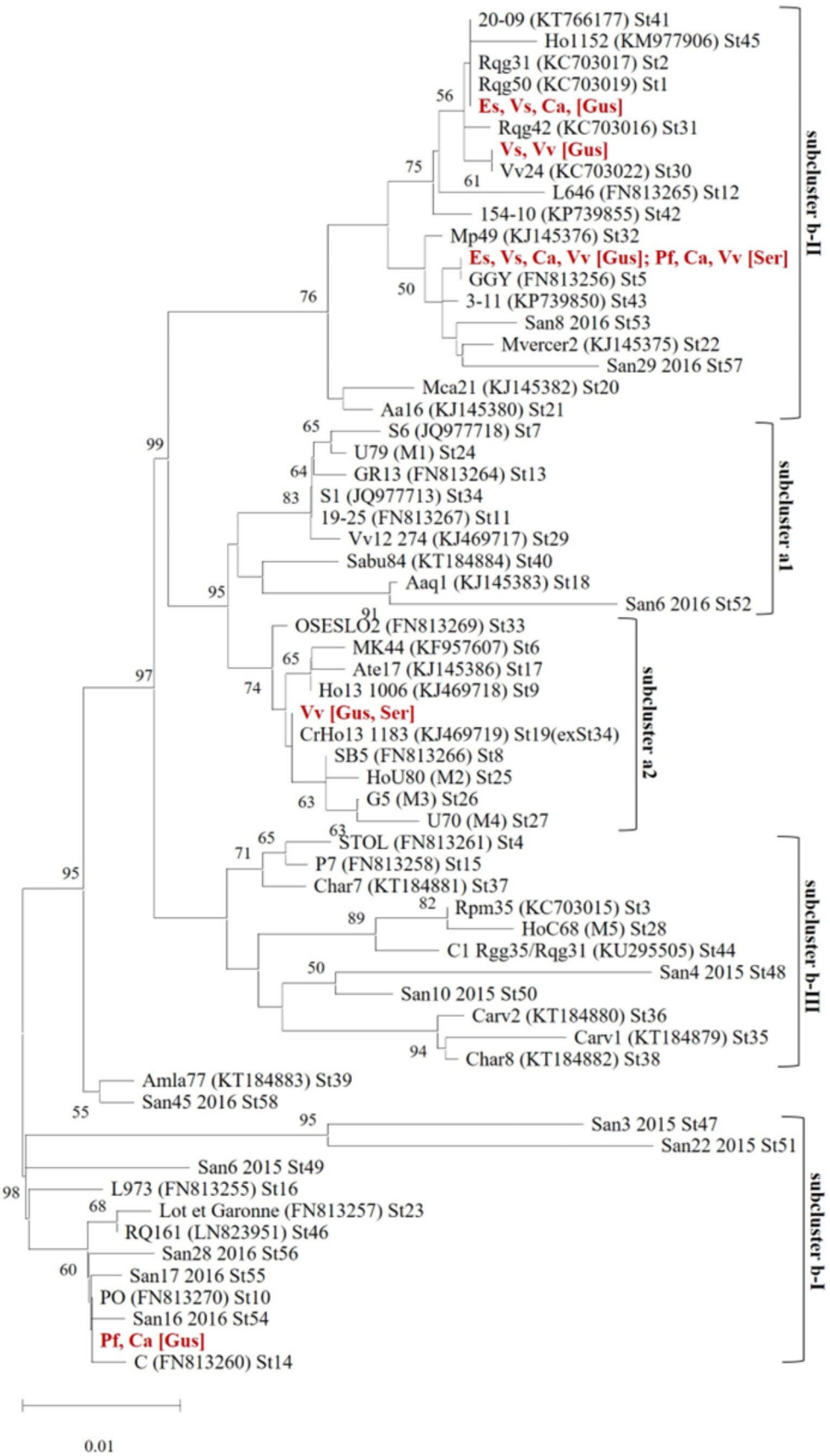

Fig. 1 Unrooted phylogenetic tree inferred from *stamp* gene nucleotide sequences of CaPsol strains representative of *stamp* sequence variants previously described and identified in this study (Tables 1 and 2); minimum evolution analysis was performed using the neighbour-joining method and bootstrap replicated 1,000 times. Names of strains are reported on the image. Strains from the present study, written in red-bold, are indicated using acronyms of their host plants and the related vineyard as follows: Ca (*Convolvulus arvensis*), Es (*Eruca sativa*), Pf (*Polygonum fagopyrum*), Vs (*Vicia sativa*), Vv (*Vitis vinifera*); Gus (Gussago), Ser (Provaglio d'Iseo). GenBank accession number of each sequence is given in parenthesis. Clusters are shown as delimitated by parentheses

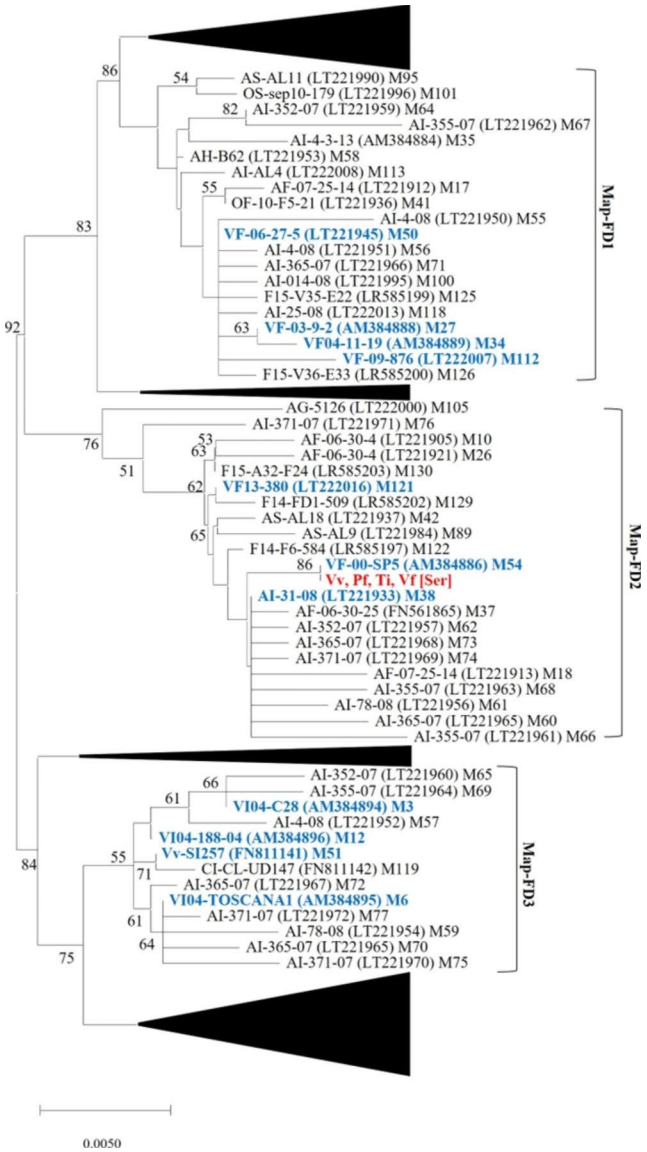

Fig. 2 Unrooted phylogenetic tree inferred from *map* gene nucleotide sequences of 16SrV strains representative of *map* sequence variants previously described (Malembic-Maher et al. 2020) and identified in this study (Table 2); minimum evolution analysis was performed using the neighbour-joining method and bootstrap replicated 1,000 times. Names of strains are reported on the image. Strains from the present study, written in red bold, are indicated using acronyms of their host plants and the related vineyard as follows: Pf (*Polygonum fagopyrum*), Ti (*Trifolium incarnatum*), Vf (*Vicia faba*), Vv (*Vitis vinifera*); Ser (Provaglio d'Iseo). Epidemic FDp strains are written in blue bold. GenBank accession number of each sequence is given in parenthesis. Clusters are shown as delimitated by parentheses

et al. 2014) and to become infective as adult in a short time frame (1–2 weeks) (Alma et al. 2018). Additionally, polyphagous insect vectors with long flight periods (i.e., *D. europaea*) could be able to acquire FDp from annual plant hosts and transmit it to grapevine.

Conclusions

Green manure of inter-row groundcover vegetation is an agronomic practice which utilization is increasing particularly in organic vineyard management in Europe. This study demonstrated that some plant species frequently employed for green manure can harbor phytoplasmas associated with both BN and FD. The evidence that CaPsol and FDp strains, identified in groundcover plant species, are undistinguishable from strains infecting grapevines, suggests that such plants could represent inoculum sources involved in the spread to grapevines of GY-associated phytoplasmas. These results reinforced the evidence of the increasing range of CaPsol and FDp alternative plant hosts and suggested a criterium for the selection of the groundcover plant species utilized for green manure, excluding the ones putatively involved in BN and FD diffusion.

Funding This study was funded by Franciacorta Consortium.
Open access funding provided by Università degli Studi di Milano within the CRUI-CARE Agreement.

Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

References

Alma A, Lessio F, Gonella E, Picciau L, Mandrioli M, Tota F (2018) New insights in phytoplasma-vector interaction: acquisition and inoculation of flavescence dorée phytoplasma by *Scaphoideus titanus* adults in a short window of time. Ann Appl Biol 173:55–62. https://doi.org/10.1111/aab.12433

Angelini E, Clair D, Borgo M, Bertaccini A, Boudon-Padieu E (2001) Flavescence dorée in France and Italy: occurrence of closely related phytoplasma isolates and their near relationships to palatinate grapevine yellows and an alder yellows phytoplasma. Vitis 40:79–86. https://doi.org/10.5073/vitis.2001.40.79-86

Arnaud G, Malembic-Maher S, Salar P, Bonnet P, Maixner M, Marcone C, Boudon-Padieu E, Foissac X (2007) Multilocus sequence

typing confirms the close genetic interrelatedness of three distinct flavescence dorée phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. App Environ Microbiol 73:4001–4010. https://doi.org/10.1128/AEM.02323-06

Atanasova B, Jakovljević M, Spasov D, Jović J, Mitrović M, Toševski I, Cvrković T (2015) The molecular epidemiology of bois noir grapevine yellows caused by '*Candidatus* Phytoplasma solani' in the Republic of Macedonia. Eur J Plant Pathol 142:759–770. https://doi.org/10.1007/s10658-015-0649-0

Belgeri E, Rizzoli A, Jermini M, Angelini E, Filippin L, Rigamonti E (2022) First report of flavescence dorée phytoplasma identification and characterization in three species of leafhoppers. J Plant Pathol 104:375–379. https://doi.org/10.1007/s42161-021-01012-y

Belli G, Bianco PA, Conti M (2010) Grapevine yellows: past, present and future. J Plant Pathol 92:303–326. https://doi.org/10.4454/jpp.v92i2.172

Bertaccini A (2007) Phytoplasmas: diversity, taxonomy, and epidemiology. Front Biosci 12:673–689. https://doi.org/10.2741/2092

Bertaccini A, Arocha-Rosete Y, Contaldo N, Duduk B, Fiore N, Montano HG, Kube M, Kuo CH, Martini M, Oshima K, Quaglino F, Schneider B, Wei W, Zamorano A (2022) Revision of the '*Candidatus* Phytoplasma' species description guidelines. Int J Syst Evol Microbiol 72:005353. https://doi.org/10.1099/ijsem.0.005353

Casati P, Jermini M, Quaglino F, Corbani G, Schaerer S, Passera A, Bianco PA, Rigamonti IE (2017) New insights on Flavescence dorée phytoplasma ecology in the vineyard agro-ecosystem in southern Switzerland. Ann Appl Biol 171:37–51. https://doi.org/10.1111/aab.12359

Caudwell A, Kuszala C, Bachelier JC, Larrue J (1970) Transmission of the golden flavescence of vines to herbaceous plants by the prolongation of the time during which *S. littoralis* can be used and the study of its survival on a large number of plant species. Ann Phytopathol 2:415–428

Chuche J, Thiery D (2014) Biology and ecology of the flavescence dorée vector *Scaphoideus titanus*: a review. Agron Sustain Dev 34:381–403. https://doi.org/10.1007/s13593-014-0208-7

Chuche J, Danet J-L, Salar P, Thiery D (2016) Transmission of '*Candidatus* Phytoplasma solani' by *Reptalus quinquecostatus* (Hemiptera: Cixiidae). Ann Appl Biol 169:214–223. https://doi.org/10.1111/aab.12291

Cvrković T, Jović J, Mitrović M, Krstić O, Toševski I (2014) Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of "bois noir. Plant Pathol 63:42–53. https://doi.org/10.1111/ppa.12080

Dermastia M, Bertaccini A, Constable F, Mehle N (2017) Grapevine yellows diseases and their phytoplasma agents. Springer, Cham, Switzerland. https://doi.org/10.1007/978-3-319-50648-7

Fabre A, Danet JL, Foissac X (2011) The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection. Gene 472:37–41. https://doi.org/10.1016/j.gene.2010.10.012

Filippin L, Jović J, Cvrković T, Forte V, Clair D, Toševski I, Boudon Padieu E, Borgo M, Angelini E (2009) Molecular characteristics of phytoplasmas associated with flavescence dorée in clematis and grapevine and preliminary results on the role of *Dictyophara europaea* as a vector. Plant Pathol 58:826–837. https://doi.org/10.1111/j.1365-3059.2009.02092.x

Gaffuri F, Sacchi S, Cavagna B (2011) First detection of the mosaic leafhopper, *Orientus ishidae*, in northern italian vineyards infected by the flavescence dorée phytoplasma. New Dis Rep 24:22. https://doi.org/10.5197/j.2044-0588.2011.024.022

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95–98

Ingels CA, Scow KM, Whisson DA, Drenovsky RE (2005) Effects of cover crops on grapevines, yield, juice composition, soil microbial ecology, and gopher activity. Am J Enol Vitic 56:19–29

Irvin NA, Pinckard TR, Perring TM, Hoddle MS (2014) Evaluating the potential of Buck wheat and Cahaba vetch as nectar producing cover crops for enhancing biological control of *Homalodisca vitripennis* in California vineyards. Biol Control 76:10–18. https://doi.org/10.1016/j.biocontrol.2014.04.006

Jović J, Marinković S, Jakovljević M, Krstić O, Cvrković T, Mitrović M, Toševski I (2021) Symptomatology, (co)occurrence and differential diagnostic PCR identification of '*Ca*. Phytoplasma solani' and '*Ca*. Phytoplasma convolvuli' in field bindweed. Pathogens 10:160. https://doi.org/10.3390/pathogens10020160

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol 35:1547–1549. https://doi.org/10.1093/molbev/msy096

Langer M, Maixner M (2004) Molecular characterization of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA. Vitis 43:191–199. https://doi.org/10.5073/vitis.2004.43.191-199

Lee I-M, Gundersen-Rindal DE, Davis RE, Bottner KD, Marcone C (2004a) '*Candidatus* Phytoplasma asteris', a novel taxon associated with aster yellows and related diseases. Int J Syst Evol Microbiol 54:1037–1048. https://doi.org/10.1099/ijs.0.02843-0

Lee I-M, Martini M, Marcone C, Zhu SF (2004b) Classification of phytoplasma strains in the elm yellows group (16SrV) and proposal of '*Candidatus* Phytoplasma ulmi' for the phytoplasma associated with elm yellows. Int J Syst Evol Microbiol 54:337–347. https://doi.org/10.1099/ijs.0.02697-0

Malembic-Maher S, Desqué D, KhaliI D, Salar P, Bergey B, Danet J-L, Duret S, Dubrana-Ourabah M-P, Beven L, Ember I, Acs Z, Della Bartola M, Materazzi A, Filippin L, Krnjajic S, Krstić O, Toševski I, Lang F, Jarausch B, Kolber M, Jović J, Angelini E, Arricau-Bouvery N, Maixner M, Foissac X (2020) When a Palearctic bacterium meets a nearctic insect vector: genetic and ecological insights into the emergence of the grapevine flavescence dorée epidemics in Europe. PLOS Path 16:e1007967. https://doi.org/10.1371/journal.ppat.1007967

Mehle N, Seljak G, Rupar M, Ravnikar M, Dermastia M (2010) The first detection of a phytoplasma from the 16SrV (Elm yellows) group in the mosaic leafhopper *Orientus ishidae*. New Dis Rep 22:11. https://doi.org/10.5197/j.2044-0588.2010.022.011

Mori N, Passera A, Quaglino F, Posenato G, Bianco PA (2015a) Epidemiological role of spontaneous weeds in the spreading of "bois noir" phytoplasma. Phytopathogenic Mollicutes 5:105–S106

Mori N, Quaglino F, Tessari F, Pozzebon A, Bulgari D, Casati P, Bianco PA (2015b) Investigation on 'bois noir' epidemiology in northeastern italian vineyards through a multidisciplinary approach. Ann Appl Biol 166:75–89. https://doi.org/10.1111/aab.12165

Okur N, Kayikcioglu HH, Ates F, Yagmur BA (2016) Comparison of soil quality and yield parameters under organic and conventional vineyard systems in Mediterranean conditions (West Turkey). Biol Agric Hortic 32:73–84. https://doi.org/10.1080/01448765.2015.1033645

Parise G (2017) Notes on the biology of *Orientus ishidae* (Matsumura, 1902) in Piedmont (Italy) (Hemiptera: Cicadellidae: Deltocephalinae). Cicadina 17:19–28. https://doi.org/10.25673/92250

Pierro R, Passera A, Panattoni A, Casati P, Luvisi A, Rizzo D, Bianco PA, Quaglino F, Materazzi A (2018a) Molecular typing of 'bois noir' phytoplasma strains in the Chianti Classico area (Tuscany, central Italy) and their association with symptom severity in *Vitis vinifera* L. cv. Sangiovese Phytopathol 108:362–373. https://doi.org/10.1094/PHYTO-06-17-0215-R

Pierro R, Passera A, Panattoni A, Rizzo D, Stefani L, Bartolini L, Casati P, Luvisi A, Quaglino F, Materazzi A (2018b) Prevalence

of a '*Candidatus* Phytoplasma solani' strain, so far associated only with other hosts, in Bois noir-affected grapevines within Tuscan vineyards. Ann Appl Biol 173:202–212. https://doi.org/10.1111/aab.12453

Pierro R, Panattoni A, Passera A, Materazzi A, Luvisi A, Loni A, Ginanni M, Lucchi A, Bianco PA, Quaglino F (2020) Proposal of a new bois noir epidemiological pattern related to '*Candidatus* Phytoplasma solani' strains characterized by a possible moderate virulence in Tuscany. Pathogens 9:268. https://doi.org/10.3390/pathogens9040268

Quaglino F, Zhao Y, Casati P, Bulgari D, Bianco PA, Wei W, Davis RE (2013) '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur and bois noir related diseases of plants. Int J Syst Evol Microbiol 63:2879–2894. https://doi.org/10.1099/ijs.0.044750-0

Quaglino F, Maghradze D, Casati P, Chkhaidze N, Lobjanidze M, Ravasio A, Passera A, Venturini G, Failla O, Bianco PA (2016) Identification and characterization of new '*Candidatus* Phytoplasma solani' strains associated with Bois noir disease in *Vitis vinifera* L. cultivars showing a range of symptoms severity in Georgia, the Caucasus Region. Plant Dis 100:904–915. https://doi.org/10.1094/PDIS-09-15-0978-RE

Quaglino F, Sanna F, Moussa A, Faccincani M, Passera A, Casati P, Bianco PA, Mori N (2019) Identification and ecology of alternative insect vectors of '*Candidatus* Phytoplasma solani' to grapevine. Sci Rep 9:19522. https://doi.org/10.1038/s41598-019-56076-9

Quaglino F, Passera A, Faccincani M, Moussa A, Pozzebon A, Sanna F, Casati P, Bianco PA, Mori N (2021) Molecular and spatial analyses reveal new insights on Bois noir epidemiology in Franciacorta vineyards. Ann Appl Biol 179:151–168. https://doi.org/10.1111/aab.12687

Rossi M, Pegoraro M, Ripamonti M, Abbà S, Beal D, Giraudo A, Veratti F, Malembic-Maher S, Salar P, Bosco D, Marzachì C (2019) Genetic diversity of flavescence dorée phytoplasmas at the vineyard scale. Appl Environ Microbiol 85:e03123–e03118. https://doi.org/10.1128/AEM.03123-18

Rotaru L, Stoleru V, Mustea M (2011) Fertilization with green manure on Chasselas Dore grapevine as an alternative for sustainable viticulture. J Food Agric Environ 9:236–243

Strauss G, Reisenzein H (2018) First detection of flavescence dorée phytoplasma in *Phlogotettix cyclops* (Hemiptera, Cicadellidae) and considerations on its possible role as vector in austrian vineyards. Integr Prot Viticulture IOBC-WPRS Bull 139:12–21

Weintraub PG, Beanland LA (2006) Insect vectors of phytoplasmas. Ann Rev Entomol 51:91–111. https://doi.org/10.1146/annurev.ento.51.110104.151039