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Multilocus sequence typing of phytoplasmas associated with *Flavescence dorée* disease in Tuscany vineyards identifies a highly homogeneous lineage in the subgroup 16SrV–C

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ABSTRACT

Flavescence dorée (FD) is the most threatening grapevine yellows (GY) disease in Europe. Despite strict control measures, alarming signs of the spread of the disease in viticultural areas continue to be detected. FD is attributed to infection by phytoplasma strains of an incidentally cited species, '*Candidatus* Phytoplasma vitis'. In 2017, a GY field survey was carried out in traditional viticulture areas of Tuscany, central Italy. FD phytoplasma (FDp) was detected in 85 GY symptomatic vines, accounting for 17% of a total of 500 symptomatic samples screened. The FDp-positive vines were scattered in 50 vineyards across seven Tuscan provinces, indicating the distribution of FDp has further extended to central and southwestern parts of Tuscany including Florence and Livorno. Multilocus sequence typing of 15 representative FDp strains from six affected vineyards revealed that the Tuscan FDp strains constitute a highly homogeneous lineage within the subgroup 16SrV–C (FD-C). Single nucleotide polymorphisms (SNPs) were identified in the 16S rRNA, *rp*, and *secY* genes of the Tuscan FDp lineage. Such SNP markers provide clues to understanding the genetic relationships among different FDp lineages present in Europe and are useful for searching potential vectors and reservoirs involved in the spread of the FDp in the Tuscan region.

1. Introduction

Grapevine Yellows (GY) is a complex array of diseases in cultivated grapevines associated with phytoplasma infections. Characterized by symptoms including discoloration and downward curling of leaves, necrosis of leaf veins, uneven lignification of stems, abortion of inflorescences, and shriveling of grape clusters, GY has a profound negative economic impact on viticulture industry worldwide. GY diseases in different geographic regions are often attributed to infections by mutually distinct phytoplasmas affiliated with different '*Candidatus* Phytoplasma' species. In Europe, *Flavescence dorée* (FD) is the most threatening GY disease as severe FD outbreaks have occurred in major viticultural areas of the continent (EPPO 2017), affecting both vineyard productivity and landscape management (Rossi et al., 2019). FD symptoms are essentially indistinguishable from those of the other GY diseases. Typical FD symptoms consist of leaf yellowing (white berry varieties) or reddening (red berry varieties), desiccation of inflorescences, irregular ripening and shriveling of berries, and general decline. Plant death may occur in late infection stages (Belli et al., 2010).

The etiological agent of the FD disease is an incidentally cited phytoplasma species termed '*Candidatus* Phytoplasma vitis'. Being capable of infecting most grapevine (*Vitis vinifera* L.) cultivars and their interspecific hybrids, the FD phytoplasma (FDp) is transmitted from vine to vine mainly through phloem-feeding activities of the monophagous leafhopper *Scaphoideus titanus* Ball (Schvester et al., 1967; Mori et al., 2002). Previous studies on epidemiology of the FD disease revealed that some additional insects and plants may also play roles as potential FDp vectors and reservoirs, respectively (Maixner et al., 2000; Weintraub

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and Beanland, 2006; Filippin et al., 2009; Casati et al., 2017; Lessio et al., 2019), indicating the complexity of the FD pathosystem in the agro-ecosystem. Due to its epidemic potential, FDp is listed as a quarantine pathogen in the European Union.

Based on the phytoplasma classification scheme derived from RFLP analysis of the 16S rRNA gene (Lee et al., 1998), strains of known FDp were assigned into two subgroups of the elm yellows (EY) group, 16SrV–C (FD-C) and 16SrV-D (FD-D) (Lee et al., 2000; Davis and Dally, 2001). FDp strains belonging to the FD-D subgroup have been reported in Italy, France, Spain, and Switzerland (Arnaud et al., 2007), while strains associated with FD-C subgroup have been identified in Italy, France, Slovenia, and Serbia (Martini et al., 2002; Maixner 2006; Kuzmanović et al., 2008; Filippin et al., 2009; Rossi et al., 2019).

Considering significant genetic variability of phytoplasma strains within each 16Sr subgroup lineage, molecular characterization of phytoplasma strains is often carried out through multi-locus sequence typing (MLST) approach. Analyses of genes more variable than highly conserved 16S rRNA have provided additional informative molecular markers regarding the genotypes of diverse phytoplasma strains (Lee et al., 2010). Phylogenetic analyses of genes, such as *secY* (encoding the central subunit of a protein translocase channel), *uvrB-degV* (encoding a subunit of the exonuclease ABC) and *map* (encoding a methionine amino peptidase), allowed the identification of three consistent FDp phylogenetic clusters; each cluster differed in nucleotide sequence composition and geographic distribution (Arnaud et al., 2007; Malembic-Maher et al., 2020). While cluster FD1 strains were found exclusively in France and Italy, cluster FD3 strains were identified only in Italy and Serbia. Strains of cluster FD2 were present both in France and Italy, but more prevalent in the former (Arnaud et al., 2007; Plavec et al., 2019; Malembic-Maher et al., 2020). Additionally, an earlier study indicated that *rp* genes (encoding ribosomal proteins) were useful in differentiating closely related FDp strains as well, as a phylogenetic analysis of *rp* gene sequences separated FDp strains reported in Italy and France into three distinct clusters (Angelini et al., 2003). Recently, single nucleotide polymorphism (SNP) analysis has also been used to differentiate closely related FDp strains identified in various potential vector and reservoir plant species, gaining insights into ecological properties of FD epidemiological cycles in vineyards (Krstić et al., 2022).

The presence of FDp in the traditional viticulture areas of Tuscany, central Italy, was first reported nearly two decades ago (Bertaccini et al., 2003). In the ensuing years up to the 2015 survey, FDp was detected consistently in northwestern provinces and sporadically in southern provinces of Tuscany (Rizzo et al., 2018). Since in most GY surveys, FDp identification was achieved using a quantitative polymerase chain reaction (qPCR)-based diagnostic assay (Angelini et al., 2007), gene sequence information required for FDp strain typing was hardly available. Consequently, only very few FDp strains from Tuscany were characterized molecularly, mainly resulting as cluster FD1 strains (Arnaud et al., 2007; Malembic-Maher et al., 2011, 2020).

In 2017, a GY field survey was carried out in Tuscany's traditional viticulture areas. The main purpose of the survey was to i) assess the extent of FD disease in the region, especially in some provinces where FD phytoplasma was not detected in the 2015 survey, and ii) to determine the population and molecular features of the FDp strains present in Tuscany as such data was lacking from previous studies. Symptomatic grapevines were examined using qPCR assay for the detection of FD and Bois Noir (BN) phytoplasmas and enzyme-linked immunosorbent assay (ELISA) for the detection of grapevine leafroll viruses (GLRV). Among 500 samples screened, 85 were positive for FDp (17%), 147 were positive for BNp (29.4%), and 127 were positive for GLRV (unpublished data). Fifteen FDp-positive samples collected from six vineyards were used for FDp strain typing in the present study. These FDp-positive samples had no co-infection with BNp or GLRV. The 15 FDp strains were characterized using molecular markers present in the 16S rRNA, *rp* and *secY* genes. The study unveiled that the Tuscan FDp (designated as TusFDp) strains form a highly homogeneous lineage within subgroup FD-C. Strains of this lineage possess consistent single nucleotide polymorphism (SNP) markers in 16S rRNA, *rp*, and *secY* genes. The SNP markers not only provide a clue to understanding the genetic relationship among different FDp lineages but also to investigating whether the phytoplasma lineage is present in phloem-feeding insects (potential vectors) and other host plants (reservoirs) within and around the vineyards in the Tuscan region.

2. Materials and methods

2.1. Plant sampling and DNA extraction

Leaf samples exhibiting typical GY symptoms were collected from 50 *Vitis vinifera* cv. Sangiovese vineyards located in seven provinces of Tuscany, central Italy in September 2017. All grapevines in the surveyed vineyards had been trained as cordon and managed according to organic production standards. All sampled grapevines were positioned in the central parts of the surveyed vineyards. Midribs were dissected from fresh leaf samples and stored at −20 °C until DNA extraction. Total DNA was extracted from approximately 300 mg leaf midribs per sample using a modified cetyltrimethylammonium bromide (CTAB)-based protocol as described previously (Pierro et al., 2018a). The crude DNA was purified using DNeasy Plant Mini kit (Qiagen, USA). The symptomatic samples used for FD phytoplasma multilocus sequence typing were from six vineyards located in Lucca (Seravezza and Porcari), Siena (Montalcino and Montepulciano), and Florence (Barberino and Greve in Chianti) provinces.

2.2. Detection of FD phytoplasma

Presence of FD phytoplasma in GY symptomatic samples was screened using a TaqMan-based quantitative polymerase chain reaction (qPCR) protocol developed previously (Angelini et al., 2007). The detection target of the qPCR was a 103 bp 16S ribosomal RNA gene fragment specific to FD phytoplasmas. The forward/reverse primers and the probe sequences are 5′-AAGTCGAACGGAGACCCTTC-3′, 5′-TAGCA ACCGTTTCCGATTGT-3′, and 5′-AAAAGGTCTTAGTGGCGAACGGGT-3′ respectively.

2.3. Polymerase chain reaction (PCR) amplification of phytoplasma genes

A near full-length phytoplasma 16S rRNA gene was amplified by using semi-nested PCR with phytoplasma-universal primers P1/16S-SR followed by P1A/16S-SR. The PCR thermal cycling conditions were the same as described by Lee et al. (2004). Amplification of the *rp* locus (covering *rplV* and *rpsC* genes) was achieved using semi-nested PCR with primer pairs rpL2F3/rp(I)R1A followed by rpF1C/rp(I)R1A as previously described (Martini et al., 2007). Amplification of the full-length *secY* gene was achieved using semi-nested PCR with primers secYF1 (V)/secYR1(V) followed by secYF2(V)/secYR1(V) as previously described (Lee et al., 2010). All amplification reactions were performed using LA Taq DNA polymerase (TakaraBio, San Jose, CA), a high-fidelity polymerase mix with proofreading activities. At the end of each nested PCR, a small fraction of the amplification products was subjected to an electrophoresis on a 1% agarose gel in Tris-borate-EDTA, verifying the presence of the corresponding 16S rDNA, *rp*, and *secY* amplicons. Amplicons derived from DNA templates of previously characterized FD phytoplasmas were used as the size references of respective genes.

2.4. Cloning and DNA sequencing of phytoplasma genes

The PCR amplicons obtained above were purified using the QIAquick gel extraction kit (Qiagen, USA), inserted into pCR®**II** -TOPO cloning vector (Invitrogen, USA), and transformed into *Escherichia coli* competent cells (One Shot Top 10 electrocomp cells, Invitrogen, USA). For each

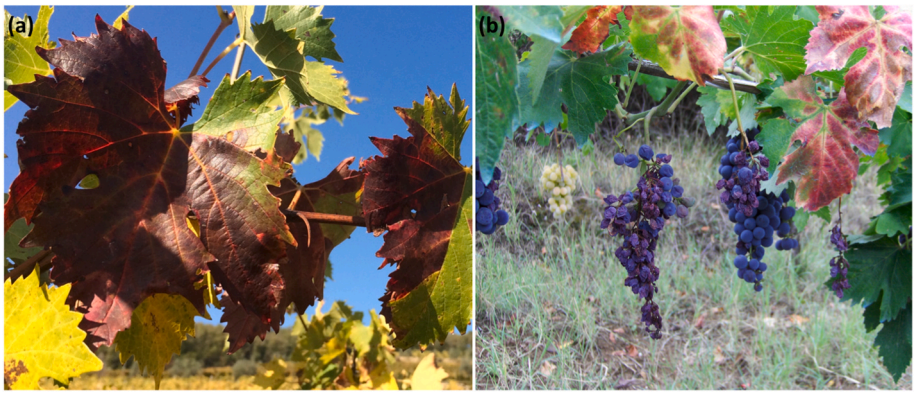

Fig. 1. Symptoms exhibited by *Flavescence dorée* diseased grapevine (*Vitis vinifera* cv. Sangiovese) plants. (a) Leaf and vein reddening and (b) berry shrivel.

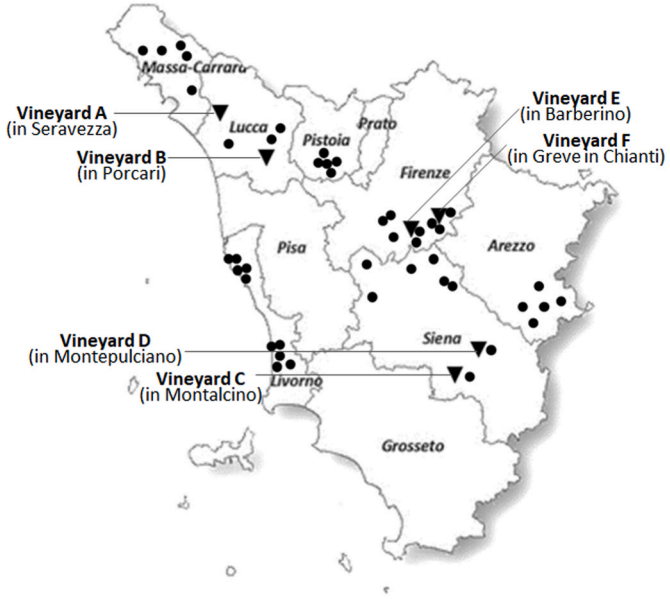

Fig. 2. A map of Tuscany, central Italy, showing the provinces where the *Flavescence dorée* (FD) disease was detected in the survey. Black dots indicate the locations of the vineyards where diseased grapevine plants were sampled. Solid triangles indicate the location of the vineyards (A–F) where the FD-positive vine samples were further analyzed for multilocus phytoplasma strain typing.

amplicon, three independent clones were randomly selected for DNA sequencing. Both strands of the cloned phytoplasma DNAs were sequenced to achieve at least 5X coverage per base position.

2.5. DNA sequence comparative and virtual RFLP analyses

DNA sequence reads were assembled using the Lasergene software (DNASTAR, USA). Multiple sequence alignment of 16S rRNA, rp and secY genes was carried out using the ClustalW algorithm and comparative analysis was performed using Sequence Identity Matrix program of the software BioEdit v. 7.0.5.3 (Hall, 1999). Single nucleotide polymorphisms (SNPs) were identified based on the alignment reported generated by the MegAlign program of the Lasergene software package. Virtual RFLP analysis of 16S rDNA and subgroup classification of phytoplasma strains were performed using the online classification tool *i*PhyClassifier (Zhao et al., 2009).

2.6. Phylogenetic and evolutionary divergence analyses

Phytoplasma gene sequence-based phylogenetic analysis was conducted using the Minimum Evolution method (Jukes-Cantor model) implemented in the software Molecular Evolutionary Genetics Analysis (MEGA-X, Kumar et al., 2018). The initial tree for the heuristic search was obtained by applying the Neighbor-Joining approach. The reliability of the analysis was subjected to a bootstrap test with 1000 replicates. Nucleotide sequence evolutionary divergence analysis was conducted using pairwise method with the *p*-distance model implemented in MEGA X (Kumar et al., 2018).

Table 1
Flavescence dorée phytoplasma strains and sequence types identified in the present study.

Strain	Location	Province	Sequence Type and GenBank Accession					
			16S rRNA		*rp*		*secY*	
TusFD315	Montalcino	Siena	16SrV–C_1	ON997106	Rp_1	ON997120	$SecY_2$	ON997132
TusFD318	Montalcino	Siena	16SrV–C_1	ON997107	Rp_1	ON997121	$SecY_1$	ON997133
TusFD219	Montepulciano	Siena	16SrV–C_1	ON997100	Rp_1	ON997115	$SecY_1$	ON997128
TusFD288	Montepulciano	Siena	16SrV–C_1	ON997104	Rp_1	ON997118	$SecY_1$	ON997130
TusFD295	Montepulciano	Siena	16SrV–C_1	ON997105	Rp_1	ON997119	$SecY_1$	ON997131
TusFD237	Seravezza	Lucca	16SrV–C_2	ON997102	Rp_2	ON997116	–	–
TusFD238	Seravezza	Lucca	16SrV–C_2	ON997103	Rp_2	ON997117	–	–
TusFD226	Porcari	Lucca	16SrV–C_1	ON997101	–	–	$SecY_1$	ON997129
TusFD389	Porcari	Lucca	16SrV–C_1	ON997110	Rp_1	ON997124	$SecY_4$	ON997136
TusFD390	Porcari	Lucca	16SrV–C_1	ON997111	Rp_1	ON997125	$SecY_5$	ON997137
TusFD189	Barberino Val D'Elsa	Florence	16SrV–C_1	ON997098	Rp_1	ON997113	$SecY_1$	ON997127
TusFD358	Barberino Val D'Elsa	Florence	16SrV–C_1	ON997109	Rp_1	ON997123	$SecY_1$	ON997135
TusFD196	Greve in Chianti	Florence	16SrV–C_3	ON997099	Rp_1	ON997114	–	–
TusFD329	Greve in Chianti	Florence	16SrV–C_3	ON997108	Rp_3	ON997122	$SecY_3$	ON997134
TusFD416	Greve in Chianti	Florence	16SrV–C_1	ON997112	Rp_1	ON997126	–	–

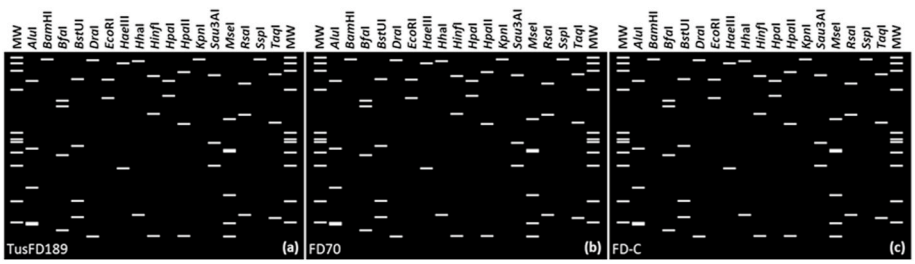

Fig. 3. Computer-simulated restriction fragment length polymorphism (RFLP) analysis of the phytoplasma 16S rDNA F2nR2 sequence by a set of 17 restriction enzymes. (a) Virtual RFLP profile of a representative strain, TusFD189 (GenBank accession no. ON997098), of the Tuscan Flavescence dorée phytoplasma lineage. (b) Virtual RFLP profile of the French 16SrV–C FDp reference strain FD70 (GenBank accession no. AF176319). (c) Virtual RFLP profile of the Italian 16SrV–C FDp reference strain FD-C (GenBank accession no. AY197645). The RFLP profiles exhibited by the three panels (a, b, and c) are identical. MW: φX174DNA *Hae*III digests.

Table 2
Estimated evolutionary divergence among FDp 16S rRNA, *rp*, and *secY* gene sequences.[a]

p-distance	Tuscany FDp lineage			All other 16SrV–C strains			Other grapevine FDp strains		
	16S rDNA	*rp*	*secY*	16S rDNA	*rp*	*secY*	16S rDNA	*rp*	*secY*
Minimum	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Maximum	0.00129	0.01264	0.00303	0.04327	0.00864	0.06474	0.04327	0.00727	0.05572
Mean	0.00017	0.00198	0.00088	0.00379	0.00441	0.02300	0.00343	0.00462	0.01996

[a] The divergence analysis was conducted using pairwise method with the *p*-distance model implemented in MEGA X (Kumar et al., 2018). *P*-distance value approximately equals to the number of nucleotide substitutions per site.

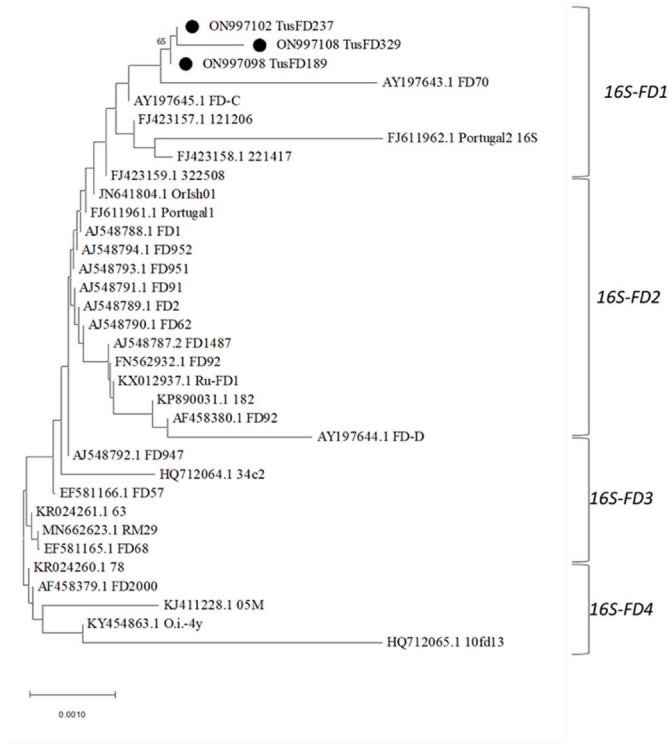

Fig. 4. Phylogenetic positions of Tuscan FDp strains as inferred from minimum evolution analysis of *16S rRNA* gene sequences. The initial tree for the heuristic search was obtained by applying the Neighbor-Joining method. The reliability of the analysis was subjected to a bootstrap test with 1000 replicates. The 16S rRNA gene sequences of three representative strains (indicated by black dots) were used in the analysis, representing the 15 Tuscan FDp strains identified in the present study (Table 1). 16S rRNA gene sequences of 31 previously characterized FDp and related strains were downloaded from the GenBank and used in the analysis. The numbers at the nodes of the branches indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test; only the values greater than 60% are displayed. The scale bar represents the number of nucleotide substitutions per site.

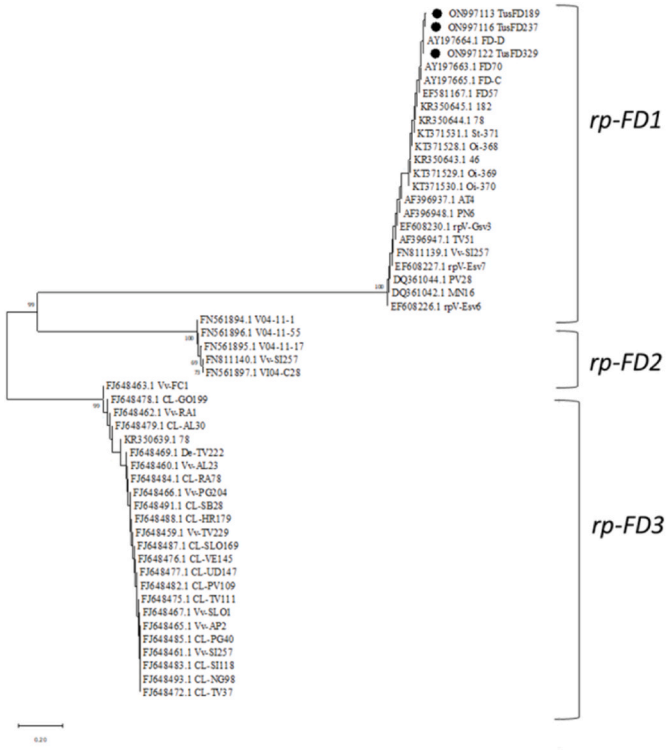

Fig. 5. Phylogenetic positions of Tuscan FDp strains as inferred from minimum evolution analysis of the *rp* locus. The nucleotide sequences cover the full length *rplV* and *rpsC* genes. The initial tree for the heuristic search was obtained by applying the Neighbor-Joining method. The reliability of the analysis was subjected to a bootstrap test with 1000 replicates. The *rp* gene sequences of three representative strains (indicated by black dots) were used in the analysis, representing the 14 Tuscan FDp strains identified in the present study (Table 1). The corresponding *rp* gene sequences of 49 previously characterized FDp and related strains were downloaded from the GenBank and used in the analysis. The numbers at the nodes of the branches indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test; only the values greater than 60% are displayed. The scale bar represents the number of nucleotide substitutions per site.

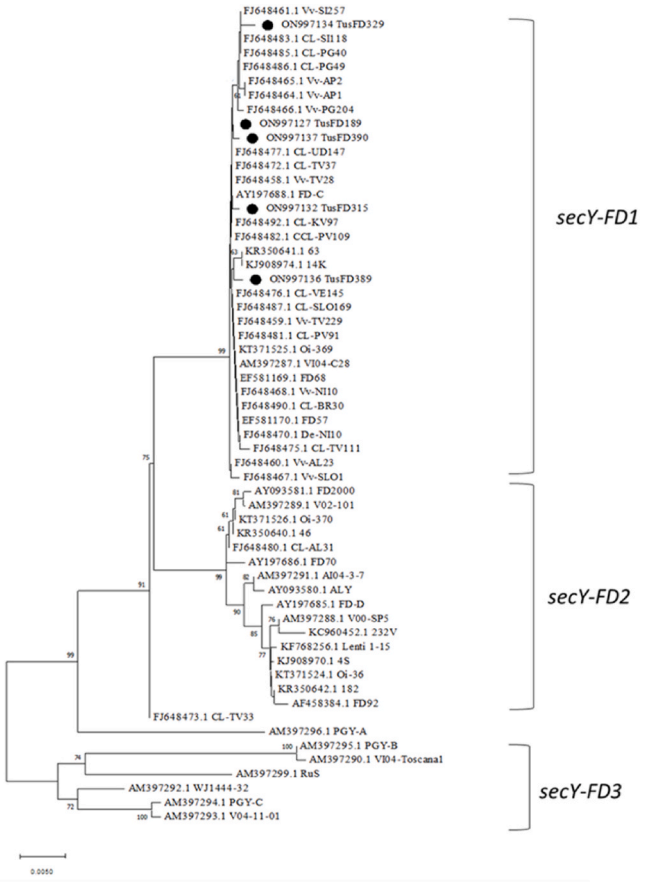

Fig. 6. Phylogenetic positions of Tuscan FDp strains as inferred from minimum evolution analysis of the *secY* gene. The initial tree for the heuristic search was obtained by applying the Neighbor-Joining method. The reliability of the analysis was subjected to a bootstrap test with 1000 replicates. The *secY* gene sequences of five representative strains (indicated by black dots) were used in the analysis, representing the 11 Tuscan FDp strains identified in the present study (Table 1). The *secY* gene sequences of 53 previously characterized FDp and related strains were downloaded from the GenBank and used in the analysis. The numbers at the nodes of the branches indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test; only the values greater than 60% are displayed. The scale bar represents the number of nucleotide substitutions per site.

3. Results and discussions

3.1. Distribution of FD phytoplasma has extended to central and southwestern parts of Tuscany

In September of 2017, a GY field survey was carried out in the traditional viticulture areas of Tuscany, central Italy. In the survey, a total of 500 GY symptomatic grapevines (*Vitis vinifera* cv. Sangiovese) were sampled. An initial screening with qPCR revealed that, of the symptomatic samples, 85 were qPCR positive for FDp. The most noticeable symptoms exhibited by these FDp qPCR-positive vines were leaf reddening and berry shrivel, and the leaf reddening also involved veins (Fig. 1). The degree of the symptoms varied from mild to severe. These FDp qPCR-positive grapevines were scattered in 50 vineyards across seven Tuscan provinces (Fig. 2). Among the affected vineyards, 10 are located in Florence Province and another 10 are in Livorno Province. This marks the first time that FDp has been detected in these two provinces. Such result indicates that, following the previous survey in 2015 (Rizzo et al., 2018), the distribution of FDp has further extended to central and southwestern parts of Tuscany.

3.2. Tuscan FD phytoplasma strains constitute a homogenous lineage belonging to subgroup 16SrV–C

Fifteen presumptive FDp-positive (qPCR-positive) samples from six vineyards were used for further confirmation of FDp infection and multilocus sequence typing of the FDp strains (Table 1, Fig. 2). Geographically, the six vineyards (A-F) belong to three different Tuscan provinces: Lucca, Siena, and Florence (Fig. 2). According to previous survey records, the three provinces differed in their FDp infection history. Vineyards A and B are in Seravezza and Porcari, respectively, of Lucca Province where FDp was consistently found in relatively high numbers. Vineyards C and D are in Montalcino and Montepulciano, respectively, of Siena Province where FDp was found sporadically. Vineyards E and F are in Barberino and Greve in Chianti, respectively, of Florence Province where FDp was never detected prior to the present study.

Confirmation of the presence of FDp in the 15 presumptive FDp-positive vine samples and molecular characterization of the FDp strains were first performed on the DNAs extracted from these samples using endpoint PCRs targeting phytoplasma-specific 16S rRNA genes. Semi-nested PCRs primed by primer pair P1/16S-SR followed by P1A/16S-SR resulted in amplicons of approximately 1.5 kb in all 15 qPCR-positive samples, while no amplification product was obtained in negative controls that used healthy plant DNAs as template. The result from DNA sequencing of the cloned amplicons revealed the presence of a signature sequence of 16S rRNA genes of phytoplasmas (5′-C_{243}AAGATTATGATGTGTAGCTGGACT$_{267}$-3’, IRPCM, 2004) and a sequence block unique to FDp (5′-A_{48}AAAGGTCTTAGTGGCGAACG GGT$_{71}$-3′, Angelini et al., 2007), confirming FDp infection in these symptomatic grapevine samples. The FDp strains were designated as TusFD189, TusFD196, etc. (Table 1). The 16S rRNA gene sequences of the TusFDp strains are highly homogeneous: 11 of the 15 strains have an identical sequence over the 1548 bp amplicon that covers a near-full-length 16S rRNA gene and a partial 16S–23S intergenic spacer (sequence type 1). Two strains (TusFD237 and TusFD238, mutually identical) have a single base insertion (sequence type 2) and the

Table 3
Single nucleotide polymorphisms (SNPs) in the 16S rRNA gene and the 16S–23S intergenic region that distinguish the Tuscan FDp lineage from FD reference strains and other 16SrV–C strains in Italy.

Strain	Country	Host	GenBank #	SNP position[a]				
				123	124	1247	1543	1548
TusFD189 & 10 more[b]	Italy	*V. vinifera*	ON997098	G	T	G	C	C
TusFD237 & 238[b]	Italy	*V. vinifera*	ON997102	G	T	G	C	C
TusFD329 & 196[b]	Italy	*V. vinifera*	ON997108	G	T	G	C	C
FD70	France	*V. vinifera*	AF176319	G	T	G	A	A
FD-C	Italy	*V. vinifera*	AF458378	–	–	G	A	A
Ls2MS & 10 more[c]	Italy	*L. spectabilis*	MT629816	A	C	A	–	–
Sj2MS & 11 more[c]	Italy	*S. junceum*	MT629806	A	C	A	–	–

[a] The numbering of the nucleotide position are based on the strain TusFD189 (ON997098).
[b] See Table 1 for the names and the corresponding GenBank accession numbers of the additional Tuscan FDp strains.
[c] See Supplementary Table 1 for the names and the corresponding GenBank accession numbers of the additional strains.

Table 4
The *rp* locus single nucleotide polymorphisms (SNPs) that distinguish the Tuscan FDp lineage from 16SrV–C reference strains FD70 and FD-C.

Strain	Country	Host	GenBank #	SNP position[a]									
				63	345	351	493	562	599	770	815	1186	1228
TusFD189 & 10 more[b]	Italy	*V. vinifera*	ON997113	T	A	A	A	C	T	A	G	T	G
TusFD237 & 238[b]	Italy	*V. vinifera*	ON997116	T	A	A	A	C	C	A	G	T	G
TusFD329	Italy	*V. vinifera*	ON997122	T	G	G	G	A	C	G	A	T	G
FD70	France	*V. vinifera*	AY197663	C	A	A	A	C	C	A	G	G	T
FD-C	Italy	*V. vinifera*	AY197665	C	G	G	G	A	C	G	A	T	G

[a] The numbering of the nucleotide position are based on the strain TusFD189 (ON997113).
[b] See Table 1 for the names and the corresponding GenBank accession numbers of the additional Tuscan FDp strains.

remaining two strains (TusFD196 and TusFD329) each have a single base variation over the entire amplicon (sequence type 3).

The result from an *i*PhyClassifier operation using the 16S rRNA gene sequences of the 15 TusFDp strains as queries revealed that all three sequence types exhibited an identical virtual RFLP profile (Fig. 3a), and the profile is the same as that of FD70 (AF176319, Fig. 3b) and that of FD-C (AY197645, Fig. 3c), the two FDp reference strains of the subgroup 16SrV–C. The strain FD70 (*map*-based cluster FD1) was originally identified in France from a diseased grapevine (*V. vinifera*) and from a grapevine leafhopper (*Scaphoideus titanus*) that fed on the diseased plants (Caudwell et al., 1970; Lee et al., 2004). The strain FD-C was originally identified in Italy from a diseased grapevine (Martini et al., 1999). A BLAST search of the GenBank nucleotide database also revealed that the TusFDp strains share the highest 16S rDNA sequence identity with that of FD70: 11 TusFDp strains scored 99.94% with FD70 and the other four TusFDp strains scored 99.87 with FD70. Thus, according to the current phytoplasma classification scheme, the TusFDp strains belong to subgroup 16SrV–C. The three 16S rDNA sequence types were designated as 16SrV–C_1, 16SrV–C_2, and 16SrV–C_3, respectively (Table 1).

Presence of FDp in Tuscan vineyards has been known for almost 20 years (Bertaccini et al., 2003). However, almost all previously reported Tuscan FDp strains were identified based on qPCR assays (Rizzo et al., 2018) and their 16S rDNA sequences were not determined. The 16S rDNA sequences used for comparative analysis in this study were FDp strains previously characterized and identified in other regions of Italy and other European countries (collectively termed as "other FDp strains"). The hosts from which these "other FDp strains" were identified include grapevines, other plant species, and phloem-feeding insects. A genetic divergence analysis that measures mean *p*-distance of the 16S rRNA gene sequences revealed that the Tuscan FDp strains are more homogenous (mean *p*-distance 0.00017) compared with the "other FDp strains" (mean *p*-distance 0.00379). For the latter, if excluding non-grapevine strains, the mean p-distance value drops slightly to 0.00343 (Table 2), indicating that the genetic divergence among the "other FDp strains" is mainly attributed to the grapevine-infecting strains.

The homogeneous nature of the Tuscan FDp strains is also evidenced by *rp* and *secY* gene sequences. A 1.2 kb *rp* locus that contains the *rplV* and *rpsC* genes were cloned from 14 out of the 15 Tuscan FDp strains and the nucleotide sequences of the cloned amplicons were determined. While three sequence variant types (Rp_1, Rp_2, and Rp_3) were observed, an overwhelming majority (11 strains) belongs to sequence type Rp_1 (Table 1). The mean *p*-distance value for the *rp* locus of the Tuscan FDp strains is 0.00198, less than that of previously characterized grapevine FDp strains (0.00462, Table 2).

Compared with the *rp* locus, the Tuscan FDp lineage has more sequence types in the *secY* locus, but the overall nucleotide substitution rate remains low (0.00088 vs 0.00198, Table 2). Five sequence types were observed among 11 Tuscan FDp strains sequenced; seven strains belong to type $SecY_1$ (Table 1). The mean *p*-distance value for the *secY* locus of the Tuscan FDp strains is 0.00088, far less than that of previously characterized grapevine FDp strains (0.01996, Table 2).

It is worth noting that while the Tuscan FDp lineage identified in the present study is highly homogeneous overall, there is an apparent "outlier", strain TusFD329. This is especially evidenced by its sequence variations in the *rp* loci: at 15 positions, strain TusFD329 has a nucleotide that is different from all other Tuscan FDp strains (168, T; 184, G; 345, G; 348, C; 351, G; 438, G; 493, G; 562, A; 655, C; 712, A; 815, A; 883, G; 1084, C; 1170, G). While all other Tuscan FDp strains share the highest *rp* locus sequence identity (ranging from 99.67% to 99.75%) with that of FD70 (AY197663), TusFD329 shares the highest sequence identity (99.33%) with FD57 (EF581167), a strain previously found in Serbia in diseased grapevine (Kuzmanović et al., 2008). In fact, within the Tuscan FDp lineage, the highest pairwise *p*-distance value for each of the three genetic loci (Table 2, 0.00129 for 16S rDNA, 0.01264 for *rp* locus, and 0.00303 for *secY* locus) was contributed by strain TusFD329.

3.3. Tuscan FD phytoplasma strains form a coherent phylogenetic subclade

The phylogenetic position of the TusFDp strains was first examined using the 16S rRNA gene sequences. Three TusFDp strains that represent three sequence types (16SrV–C_1, 16SrV–C_2 and 16SrV–C_3) and 31 previously identified FDp strains were included in the phylogenetic analysis. The Tuscan FDp strains formed a coherent subclade in the resulting Minimum Evolution tree (Fig. 4). The phylogenetic tree grouped known FDp strains into four major clades. Together with FD70 and FD-C, the Tuscan FDp subclade belonged to the clade *16S-FD1* (Fig. 4). However, as shown in the tree, the bootstrap values at the key nodes that separate the four clades were all below 60%. It is not a surprise as the 16S rRNA gene is more conserved than the *rp* and *secY* genes, therefore has less resolving power in distinguishing closely related phytoplasma lineages (Martini et al., 2007; Lee et al., 2010).

For the *rp* locus that encodes ribosomal proteins RplV and RpsC, the Tuscan FDp strains have three sequence variant types as well (Table 1). The *rp* gene sequences of three representative Tuscan FDp strains and those of 49 previously characterized FDp and related strains were used to construct a *rp* gene tree. The resulting phylogenetic tree grouped known FDp strains into three clades, *rp-FD1*, *rp-FD2*, and *rp-FD3*. The Tuscan FDp strains were tightly grouped together as they did in the 16S rDNA tree and were situated in the clade *rp-FD1*, with FD70, FD-C, and FD-D among their closest neighbor (Fig. 5). The other strains in the same cluster include those previously identified in Italy and Switzerland in grapevines and insects (*S. titanus* and *Orientus ishidae*) (Fig. 5). The topology of this *rp* gene tree is similar to the one previously constructed by Angelini et al. (2003). It is worth noting that the *rp* locus tree did not separate subgroup FD-C and subgroup FD-D lineages well.

The phylogenetic relationship inferred from the *secY* gene sequences is more complex. Among the Tuscan FDp strains, there are five sequence types for the *secY* locus (Table 1). In the phylogenetic tree, Tuscan FDp sequence types were suited in a single clade (*secY-FD1*) along with FDp strains previously reported in Italy, Macedonia, Slovenia, Croatia, and Serbia (Fig. 6). A previous study identified three FDp main genetic clusters (FD1, FD2, and FD3) based on phylogenetic relationships of diverse FDp *secY* variants; and the study found that the FDp strains in the

Table 5
The *secY* locus single nucleotide polymorphisms (SNPs) that distinguish the Tuscan FDp lineage from 16SrV–C French reference strain FD70.

Strain	Country	Host	GenBank #	SNP position[a]												
				435	441	456	571	615	632	680	726	837	885	886	1066	1111	1113	1131	1156	1306
TusFD189 & 6 more[b]	Italy	*V. vinifera*	ON997127	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
TusFD315	Italy	*V. vinifera*	ON997132	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
TusFD329	Italy	*V. vinifera*	ON997134	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
TusFD389	Italy	*V. vinifera*	ON997136	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
TusFD390	Italy	*V. vinifera*	ON997137	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
FD-C & 8 more[c]	Italy	*V. vinifera*	AY197688	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
CL-PV91 & 8 more[c]	Italy	*C. vitalba*	FJ648481	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
FD-3 & FD-4[c]	Croatia	*V. vinifera*	KP274908	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
FD-503 & 6 more[c]	Croatia	*S. titanus*	KJ908971	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
CL-KV97 & NG98[c]	Macedonia	*C. vitalba*	FJ648492	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
FD57 & FD68[c]	Serbia	*V. vinifera*	EF581170	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
CL-BR30 & 2 more[c]	Serbia	*C. vitalba*	FJ648490	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
Vv-SLO1	Slovenia	*V. vinifera*	FJ648467	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
CL-SLO169	Slovenia	*C. vitalba*	FJ648487	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
Oi-369	Switzerland	*O. ishidae*	KT371525	C	C	C	T	G	G	A	G	C	C	A	A	T	A	G	G	G
FD70	France	*V. vinifera*	AM397285	T	T	T	C	A	T	G	A	T	T	G	G	A	G	A	A	A
V00-SP5	France	*V. vinifera*	AM397288	T	T	T	C	A	T	G	A	T	T	G	G	A	G	A	A	A
V02-101	France	*V. vinifera*	AM397289	T	T	T	C	A	T	G	A	T	T	G	G	A	G	A	A	A
FD70-like	Slovenia	*O. ishidae*	HM367596	T	T	T	C	A	T	G	A	T	T	G	G	A	G	A	A	A

[a] The numbering of the nucleotide position are based on the strain TusFD189 (ON997127).
[b] See Table 1 for the names and the corresponding GenBank accession numbers of the additional Tuscan FDp strains.
[c] See Supplementary Table 1 for the names and the corresponding GenBank accession numbers of the additional strains.

different clusters differed in their geographic distributions (Arnaud et al., 2007). In the past 15 years, many more FDp strains have been discovered in several European countries. The updated *secY* phylogenetic tree produced in the present study still consists of three main phylogenetic clusters (Fig. 6). However, the correlation between the strain clustering and their geographic distribution has diminished as FDp strains identified from multiple countries/geographic locations were found in the same clades, especially clade *secY-FD1*.

3.4. Single nucleotide polymorphisms (SNPs) that distinguish the Tuscan FDp lineage

3.4.1. 16S rRNA gene and the 16S–23S intergenic region

Previous studies suggested that existent 16SrV–C FD phytoplasmas had two different origins, France and Italy (Caudwell et al., 1970; Martini et al., 2002; Lee et al., 2004). FD70 and FD-C have served the reference strains of the French and Italian FDp lineages, respectively. An alignment of the 16S rRNA gene sequences of the 15 Tuscan FDp strains identified in the present study with the counterparts of FD70 (AF176319) and FD-C (AY197645 and AF458378) revealed two SNPs that can distinguish the Tuscan FDp lineage from the two 16SrV–C reference strains. The SNPs are in the 16S–23S intergenic region at the nucleotide positions 1543 and 1548, respectively (Table 3).

Among previously characterized Italian 16SrV–C phytoplasmas, 11 strains were identified in *Livilla spectabilis* (a psyllid native to Sicily, Italy) and 12 strains were identified in *Spartium junceum* (a leguminous shrub widely cultivated in Italy). These 23 strains are highly homogenous in their 16S rRNA gene sequences. The present study found three consistent SNPs in the 16S rRNA gene that can reliably distinguish the Tuscan FDp lineage from the *L. spectabilis/S. junceum* lineage. The three nucleotide substitutions are located at positions 123, 124, and 1247, respectively (Table 3). *L. spectabilis* has long been considered a vector transmitting *Spartium* witches' broom (SpaWB) disease in Italy. The relationship between the SpaWB phytoplasma and FDp remains elusive and the risk of SpaWBp host-jumping to grapevine cannot be ruled out (Rizza et al., 2021). It would be interesting to learn whether these consistent SNPs reflect lineage-specific host adaptation as noted previously that closely related phytoplasma sequevars (referring to different '*Ca.* Phytoplasma pruni'-related strains infecting grapevines and peach trees) may have mutually distinct host specificity (Davis et al., 2015). The distinctions between the Tuscan FDp lineage and the *L. spectabilis/S. junceum* lineage are more striking in their *secY* gene sequences as they will be presented in the subsection below.

3.4.2. rp locus

For the *rp* locus, 13 out of the 14 Tuscan FDp strains share the highest sequence identity with French 16SrV–C reference strain FD70 (AY197663). However, there are three consistent SNPs (at positions 63, 1186, and 1228) that can distinguish all Tuscan FDp strains from the French 16SrV–C reference strain FD70 (Table 4). There are an additional SNP (at position 599) that can distinguish 11 out of 14 Tuscan FDp strains from FD70. On the other hand, there are seven SNPs (at positions 63, 345, 351, 493, 562, 770, and 815) that can distinguish the Tuscan FDp strains from the Italian 16SrV–C reference strain FD-C (Table 4) except for one strain, TusFD329. Overall, with regard to the SNP profiles at the *rp* locus, most of the Tuscan FDp strains are more similar to FD70, whereas strain TusFD329 is more similar to FD-C. In addition, since strain TusFD329 shares the highest *rp* sequence identity with FD57 (EF581167), we have also identified eight SNPs (at positions 63, 184, 348, 438, 655, 883, 1084, and 1170) that can distinguish TusFD329 from FD57.

3.4.3. secY locus

For the *secY* locus, the Tuscan FDp strains share the highest sequence identity (99.7%–99.8%) with the Italian 16SrV–C reference strain FD-C (AY197688); no consistent SNP is present between the Tuscan FDp

lineage and FD-C. On the other hand, the Tuscan FDp strains share 97.9%–98.0% sequence identity with the French 16SrV–C reference strain FD70 (AY197686); and there are 17 consistent SNPs that distinguish the Tuscan FDp lineage from FD70 (Table 5). It is noteworthy that several FDp strains previously reported in Croatia, Macedonia, Serbia, and Switzerland share the same SNP profile with the Tuscan FDp (FD-C) lineage, whereas several FDp strains previously identified in France share the same SNP profile with FD70 (Table 5). FDp strains of both FD-C and FD70 *rp* SNP types have been reported in Slovenia (Table 5).

An alignment of the *secY* gene sequences of the 11 Tuscan FDp strains identified in the present study with those of previously characterized FDp and related strains revealed a highly polymorphic sequence block that distinguishes the Tuscan FDp lineage from other FDp lineages (Supplementary Table 2). Within the polymorphic sequence block, four SNP alleles (α, β, γ, and δ) can be identified: the α allele includes 11 grapevine-infecting Tuscan FDp strains identified in the present study and previously characterized FD-C strains; the β allele includes 16SrV–C strains identified in psyllid *L. spectabilis* and in leguminous shrub *S. junceum* in Italy (Supplementary Table 2); the γ allele includes FDp strains identified in grapevine and *A. glutinosa* in France and Germany (Supplementary Table 2); the δ allele includes FDp strains identified in *S. titanus* and *O. ishidae* in Switzerland (Supplementary Table 2). The identification of such *secY* SNP alleles not only provides molecular markers for differentiation of mutually distinct FDp lineages but also raises an intriguing possibility whether such markers can be used to study the evolution, lineage-specific niche adaptation, and distribution of FD phytoplasmas.

In conclusion, the present study identified a highly homogenous FD phytoplasma lineage in the vineyards of Tuscan region, Central Italy. Results from multilocus sequence typing showed that the nucleotide sequences of individual genes share very high identity with the counterparts of numerous FDp strains previously identified in Italy, France, and other European countries, especially with those of 16SrV–C reference strains FD70 and FD-C. Nevertheless, the collective genotype (16S rDNA/*rp*/*secY*) of the Tuscan FDp strains is unique and constitute a new lineage within the 16SrV–C subgroup. The Tuscan FDp lineage can be distinguished from previously reported FDp lineages with a combination of SNPs.

Despite strict control measures, the spread of FD disease in Tuscan region has never ended. Identification of the unique Tuscan FDp lineage presents new challenges and opportunities to manage the disease in the region. According to the hypothesis presented by Pierro et al. (2018b), new FDp lineage (new collective genotypes) in central Italy may have emerged by two mutually complementary mechanisms: genetic recombination and niche adaptation. Co-infection in a plant host by two closely related strains from northern Italy and France would facilitate genetic recombination of homologues genes, generating a new lineage with intermediate genetic features. Changing environmental conditions and selective pressure may alter genetic population structures of FDp in Tuscan ecosystem. An in-depth study is warranted to extend the MLST typing of the Tuscan FDp lineage to other genetic loci and to examine the FDp population in other grapevine varieties, alternative plant hosts, and potential insect vectors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2022.106114.

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