Identification and Epidemic Distribution of Two *Flavescence Dorée*-Related Phytoplasmas in Veneto (Italy)

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ABSTRACT

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Grapevine yellows associated with phytoplasmas of the elm yellows group (16SrV), better known as *flavescence dorée* (FD), is a serious quarantine problem in some important grapevine growing regions in the European Union. A survey was carried out in 1997 to 1998 in Veneto region (Italy) where a serious outbreak of FD was in progress. Phytoplasma identification by nested polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) analyses on 275 grapevine samples and on batches of *Scaphoideus titanus* was carried out. RFLP analyses of the 16S rDNA/spacer region with *TaqI* detected the presence of two distinct elm yellows phytoplasma subgroups designated 16SrV-C and 16SrV-D in 77 FD-infected grapevine samples. Only phytoplasmas of the 16SrV-D subgroup were detected in *S. titanus*. In 1997, the two phytoplasma subgroups appeared to be located in two diverse geographic areas; but in 1998, the 16SrV-D type also was detected in the provinces where in 1997 only 16SrV-C type was identified. The sequencing of a 400-bp fragment at the 3' end of 16S rDNA plus spacer region allowed a specific primer construction that was successfully employed for detection of both FD types in grapevine by direct PCR.

Grapevine yellows diseases occur in various regions of the world. They exhibit nearly identical symptoms but are believed to be caused by molecularly distinguishable phytoplasmas (3,8,12,14,17,24,29). Among grapevine yellows diseases, the *flavescence dorée* (FD) was the first one described in France (11) and, with "Bois Noir" (BN, or in Germany, "Vergilbungs-krankheit," VK), is one of the most important yellows diseases for quarantine purposes. FD is transmitted by the nearctic leafhopper *Scaphoideus titanus*.

The etiological agents of grapevine yellows diseases have been attributed to phytoplasmas of different 16S rRNA restriction fragment length polymorphism (RFLP) groups, namely 16SrI (aster yellows and related strains), 16SrIII (X-disease and related strains), 16SrV (elm yellows and related strains), and 16SrXII (stolbur and related strains) (22,33).

In Italy, preliminary molecular identification tests showed phytoplasmas belonging to 16S rDNA groups XII-A (stolbur and related strains) and V (elm yellows and related strains) were associated, respectively, with BN and FD, and frequently

occurred in mixed infections (7,9). More recently, a strong epidemic of FD was reported in the Veneto region (4,5,28,30) that continued to spread in spite of efforts to control *S. titanus*. Over the past 3 years, molecular detection tests were utilized to assure that propagative cuttings were not taken from FD-infected vineyards. Since recent reports showed the presence of FD phytoplasmas diverse for chromosomal (10,13) and in one case for ribosomal sequences (25), a verification of FD phytoplasmas identity was performed using nested polymerase chain reaction (PCR)/RFLP analyses on 16S rDNA/spacer region. Sequencing of some of the FD strains was also performed on a portion of this region, and primers for specific direct PCR were designed. A short account of part of this research was presented elsewhere (26).

MATERIALS AND METHODS

Grapevine and insect samples. Samples belonging to about 40 diverse grapevine cultivars were collected in Veneto vineyards during 1997 and 1998. A total of 275 samples from five different provinces of Veneto (Verona, Treviso, Vicenza, Venezia, and Padova) were collected.

Batches containing 1 to 10 *S. titanus* collected in vineyards located in Verona, Vicenza, Padova, and Treviso provinces were also obtained.

Reference phytoplasma strains. Control samples used in PCR/RFLP analyses for group 16SrV phytoplasmas in naturally infected plants were rubus infected with

stunt disease (20), eucalyptus little leaf, hornbeam with witches'-broom collected in diverse regions of central and south Italy (26), and grapevine FD strain B1872 (kindly provided by E. Boudon-Padieu, INRA, Dijon, France). Further reference phytoplasma strains (in periwinkle) used to represent the majority of phytoplasma groups reported in the literature were: North American aster yellows from tomato and Maryland aster yellows (BB and AY-1; I.-M. Lee, USDA, Beltsville, MD); clover phyllody (CPh; L. N. Chiykowski, Agriculture Canada, Ottawa, Ontario, Canada [via I.-M. Lee]) for the subgroups 16SrI-A, 16SrI-B, and 16SrI-C respectively; Italian periwinkle virescence (IPVR) for the group 16SrXII-A (formerly subgroup 16SrI-G); peach X disease for the 16SrIII-A (CX; B. C. Kirkpatrick, University of California, Davis, [via I.-M. Lee]); ash yellows for the 16SrVII-A (ASHY; W. A. Sinclair, Cornell University, Ithaca, NY [via E. Seemüller]); Australian tomato big bud for 16SrII (TBB; M. F. Clark, HRI, East Malling, Kent, UK [via E. Seemüller]); beet leafhopper transmitted virescence for 16SrVI (BLTVA; D. Golino, University of California, Davis, [via E. Seemüller]); apple proliferation for the 16SrX-A (AP; L. Carraro, University of Udine; Italy); annual blue grass white leaf for 16SrXIV-A (ABGWL) (23); elm yellows (EY; H. Griffith and W. A. Sinclair, Cornell University, Ithaca, NY); and elm witches'-broom (ULW; E. Seemüller, BBA, Dossenheim, Germany) for 16SrV-A group.

Phytoplasma detection. Total nucleic acids were extracted from about 1 g of fresh plant tissue (leaf midribs) ground in liquid nitrogen. The DNA extraction protocol employed has already been described by Prince et al. (29).

Plant nucleic acid was diluted in sterile deionized water to a final concentration of 20 ng/μl, and 1 μl of this solution was used for PCR assays.

Following a modified DNA extraction procedure previously described (34), *S. titanus* adults were ground in Eppendorf tubes with 200 μl of sterile CTAB buffer (2% wt/vol CTAB, 1.4 M NaCl, 0.2% [vol/vol] 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0). The suspension was then incubated 20 min at 60°C and mixed at regular intervals. An equal volume of chloroform-octanol was added to the suspension, mixed carefully,

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and centrifuged 8 min at 14,000 rpm. After precipitation with cold isopropanol and centrifugation for 30 min at 14,000 rpm, the pellet was washed with 70% ethanol, centrifuged, and suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). One microliter of sterile-water-diluted supernatant (1:50) was used in PCR assays.

Total DNAs extracted were processed with nested PCR using two phytoplasma universal primer pairs, R16F1/R0 (1,400 bp) and R16F2n/R2 (1,200 bp) (19).

Each reaction was performed in a total volume of 25 µl containing 2.5 µl of the 10× buffer (31), 200 µM of dNTP, 0.625 U of *Taq* polymerase (Roche, Branchburg, NY), and 0.4 µM of primer pair. Tubes with water instead of the template DNA were included in each reaction series as negative PCR controls.

The PCR conditions for all the above-listed primers were 35 cycles in a Perkin-Elmer Cetus thermal cycler: each cycle was of 1 min (2 min for the first cycle)

denaturation step at 94°C, 2 min for annealing at 50°C, and 3 min (10 min for the last cycle) at 72°C for primer extension. Six microliters of each sample were subjected to electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination.

Eight microliters of the PCR products amplified by using the R16F2n/R2 were digested with 2 U of *MseI* (New England Biolabs, Beverly, MA) following the instruction of the manufacturer to identify

1264

FD 5'-CAACTCGACT TCATGAAGCT GGAATCGCTA GTAATCGCGA CACCGCCCGT CAAACCACGA AAGTTAGCAA TACCCG--AAAG
Co 5'-CAANTINGANT TCATGAAGCT GGAATCGNTA GTAATCGCGA ATCAGCATGT CGCGGTGAAT ACGTTCTCGG GGTTTGTA--
Ga 5'-
Pe 5'-
RS 5'-
RuS 5'-CAACTCGACT TCATGAAGCT GGAATCGCTA GTAATCGCGA ATCAGCATGT CGCGGTGAAT ACGTTCTCGG GGTTTGTA--
EY 5'-CAACTCGACT TCATGAAGCT GGAATCGCTA GTAATCGCGA ATCAGCATGT CGCGGTGAAT ACGTTCTCGG GGTTTGTA--

1344

FD CACCGCCCGT CAAACCACGA AAGTTAGCAA TACCCG--AAAG CAGTGGCTTA ACTTCGAAAG AAGAGGGAGC TGTCTAAGGT
Co CACCGCCCGT CAAACCACGA AAGTTAGCAA TACCCG--AAAG CAGTGGCTTA ACTTCGAAAG AAGAGGGAGC TGTCTAAGGT
Ga
Pe
RS CACCGCCCGT CAAACCACGN AAGTNAGCAA TACCCGAAAG CAGTGGCTTA ANTTNGAAG AAGAGGGAGC NGTNTAAGGT
RuS CACCGCCCGT CAAACCACGA AAGTTAGCAA TACCCG--AAAG CAGTGGCTTA ACTTCGAAAG AAGAGGGAGC TGTCTAAGGT
EY CACCGCCCGT CAAACCACGA AAGTTAGCAA TACCCG--ANAG CAGTGGCTTA ACTTCGAAAG AAGAGGGAGC TGTCTAAGGT

1425

FD AGGGTTGATG ATTGGGGTTA AGTCGTAACA AGGTATCCTT ACCGGAAGGT GAGGAT--GGAT CACCTCC--TTT CTAAGGACAT
Co AGGGTTGATG ATTGGGGTTA AGTCGTAACA AGGTATCCTT ACCGGAAGGT GAGGAT--GGAT CACCTCC--TTT CTAAGGACAT
Ga
Pe
RS AGGGTTGANG ATTGGGGTTNA AGTCGTNACA AGGTATCCTT ACCGGAAGGT GAGGAT--GGAT CACCTCCNTTT CTAAGGACAT
RuS AGGGTTGATG ATTGGGGTTA AGTCGTAACA AGGTATCCTT ACCGGAAGGT GAGGAT--GGAT CACCTCC--TTT CTAAGGACAT
EY AGGGTTGATG ATTGGGGTTA AGTCGTAACA AGGTATCCTT ACCGGAAGGT GAGGAT--GGAT CACCTCC--TTT CTAAGGACAT

1507

FD ACATAT--NAAA ATCATCATCT TCAGTTTGA AAGACTTAGG TTAATAATATA AGTTTTCTT TTTATAAANA AAGTGTTCCT
Co ACATAT--NAAA ATCATCATCT TCAGTTTGA AAGACTTAGG TTAATAATATA AGTTTTCTT TTTATAA--AA AAGTGTTCCT
Ga ACATAT--NAAA ATCATCATCT TCAGTTTGA AAGACTTAGG TTAATAATATA AGTTTTCTT TTTATAA--AA AAGTGTTCCT
Pe ACATATGGCAA ATCATCATCT TCAGTTTGA AAGACTTAGG TTAATAATATA AGTTTTCTT TTTATAA--AA AAGTGTTCCT
RS ACATAT--NAAA ATCATCATCT TCAGTTTGA AAGACTTAGG TTAATAATATA AGTTTTCTT TTTATAA--AA AAGTGTTCCT
RuS ACATAT--NAAA ATCATCATCT TCAGTTTGA AAGACTTAGG TTAATAATATA AGTTTTCTT TTTATAA--AA AAGTGTTCCT
EY --CATATNAAA ATCATCATCT TCAGTTTGA AAGACTTAGG TTAATAATATA AGTTTTCTT TTTATAA--AA AAGTGTTCCT

1589

FD CTTATATNAA AGACCAA--AGG GCCTATAGCT CAGTTGGTTA GAGCACACGC CTGATAAGCG TGAGGT--CGGT GGTCAAGTC
Co CTTATATNAA AGACCAA--AGG GCCTATAGCT CAGTTGGTTA GAGCACACGC CTGATAAGCG TGAGGT--CGGT GGTCAAGTC
Ga CTTATATNAA AGACCAA--AGG GCCTATAGCT CAGTTGGTTA GAGCACACGC CTGATAAGCG TGAGGT--CGGT GGTCAAGTC
Pe CTTATATNAA AGACCAA--AGG GCCTATAGCT CAGTTGGTTA GAGCACACGC CTGATAAGCG TGAGGT--CGGT GGTCAAGTC
RS CTTATATNAA AGACCAA--AGG GCCTATAGCT CAGTTGGTTA GAGCACACGC CTGATAAGCG TGAGGT--CGGT GGTCAAGTC
RuS CTTATATNAA AGACCAA--AGG GCCTATAGCT CAGTTGGTTA GAGCACACGC CTGATAAGCG TGAGGT--CGGT GGTCAAGTC
EY CTTATATNAA AGACCAA--AGG GCCTATAGCT CAGTTGGTTA GAGCACACGC CTGATAAGCG TGAGGT--CGGT GGTCAAGTC

1672

FD CACTTAGGCC C-ACC--AATTTT ATATCAGGAA AATATTTACT--TCGAAGAAAG TT--CTTTCAA AAGTAGATAA ACATGA TT
Co CACTTAGGCC C-ACC--AATTTT ATATCAGGAA AATATTTACT--TCGAAGAAAG TTTCTTTGAA AAGTAGATAA ACATGA TT
Ga CACTTAGGCC CACCTAATTTT ATATCAGGAA AATATTTACT--TCGAAGAAAG TT--CTTTGAA AAGTAGATAA ACA-GA TN
Pe CACTTAGGCC C-ACC--AATTTT ATATCAGGAA AATATTTACT--TCGAAGAAAG TTNCTTTGAN AANTAGATAA ACATGANN
RS CACTTAGGCC C-ACC--AATTTT ATATCAGGAA AATATTTACT--TCGAAGNAAG TTNNTTNGA NAGTAGATAA ACATGA TT
RuS CACTTAGGCC C-ACC--AATTTT ATATCAGGAA AATATTTACT--TCGAAGAAAG TT--CTTTGAA AAGTAGATAA ACATGA TT
EY CACTTAGGCC C-ACC--AATTTT ATATCAGGAA AATATTTACT--TCGAAGAAAG

Fig. 1. Alignment of the phytoplasma sequences from three grapevine *flavescence dorée* (FD) infected and the rubus stunt (RS) control with FD, rubus stunt in periwinkle (RuS), and elm yellows (EY) strains from GenBank (accession numbers, respectively, X76560, Y16395, and L33763). Boxes indicate differences in sequences; primer V1731 is underlined. Co, grapevine cv. Corvina; Ga, grapevine cv. Garganega; Pe, grapevine cv. Perera.

phytoplasmas. The RFLP fragments were visualized by electrophoresis in a 5% polyacrylamide gel in 1× TBE buffer (0.09 M Tris-borate, 0.002 M EDTA) followed by staining with ethidium bromide and visualization under a UV transilluminator.

Differentiation of phytoplasmas associated with FD. FD phytoplasma identification was preliminarily carried out with group V specific primers R16(V)F1/R1 (1,100 bp) (21). On samples positive for EY-type phytoplasmas, a new nested PCR procedure was adopted using primers 16R_{723f}/P7 (1,100 bp) (27,32) followed by primers 16R_{758f}/M23SR_{1804r} (1,050 bp) (18,27) in order to amplify the 3' end of the 16S region and most of the spacer region.

The PCR products amplified by using the specific primers R16(V)F1/R1 were digested overnight (16 h) at 37°C with *Bfa*I. Further RFLP of amplified products with primers 16R_{758f}/M23SR_{1804r} were carried out with *Taq*I and incubated overnight at 65°C. The digestions were performed in buffers supplied by the manufacturer (New England Biolabs) according to instructions.

Further direct PCR analyses were carried out on selected FD-infected samples using nonribosomal primers FD9f/r in the conditions described by Daire et al. (13), followed by RFLP with *Mse*I and *Alu*I. Primers 16R_{758f}/V_{1731r} (see below) were then employed for direct detection and classification of FD phytoplasmas in grapevine.

Sequencing and primer construction. Amplified PCR products with primers 16R_{758f}/M23SR_{1804r} from three FD-infected grapevine cultivars (Corvina and Garganega from Verona and Perera from Treviso province [6]) plus a rubus stunt strain (20) (Fig. 1) were sequenced in the 3' end of 16S rDNA and in the 16Sr-23S spacer region (from base 1,264 to base 1,754) in order to study variability for specific primer design.

The sequencing was performed in an "ABI Prism DNA sequencing" Model 377 (PE Applied Biosystem, Foster City, CA) using primer P3 (32) and dichlororhodamine dye terminators. After sequencing, a primer was designed (V_{1731r}) (Fig. 1) and tested in seminested PCR with primer 16R_{758f} after a first PCR run with primers 16R_{758f}/M23SR_{1804r} on all the control samples described above for specificity.

RESULTS

Phytoplasma detection. Nested PCR using universal primers followed by RFLP analyses detected the presence of phytoplasmas in 165 grapevine samples: 77 were infected by a phytoplasma of the EY group after PCR with specific primers R16(V)F1/R1 and/or RFLP analyses with *Mse*I. The EY-infected plants were distributed in all the provinces examined and on different grapevine cultivars with the highest percentage of infected plants in Verona prov-

ince (Table 1). The other positive samples were infected by a BN-type (16SrXII-A) phytoplasma or in some cases by 16SrI-B or 16SrI-C type phytoplasmas (Fig. 2).

Differentiation of phytoplasmas associated with FD. Further RFLP analyses with *Bfa*I on R16(V)F1/R1-amplified products allow the distinction of FD- from EY-infected samples: none of the grapevines were infected by 16SrV-A type of phytoplasmas (EY) (Fig. 3A).

Similar analyses on 16R_{758f}/M23SR_{1804r}-amplified products with *Taq*I showed the

presence of two diverse FD types of phytoplasmas in the grapevines (Fig. 3C). The samples of *S. titanus* tested showed the presence of FD phytoplasmas (16SrV group) when collected in Verona and Vicenza provinces, and no phytoplasma presence when collected in Treviso and Padova areas (Fig. 3B). In some of the samples from Verona, a BN-type phytoplasma (16SrXII-A group) also was present (Fig. 3C). The two subgroups detected were designated 16SrV-C and 16SrV-D, respectively, and *S. titanus*

Table 1. Distribution of the two phytoplasma *flavescence dorée* (FD) types identified in three provinces of the Veneto region during 1997 and 1998 (tests were carried out on selected samples)

Geographic locations	Cultivars	1997	1998	Phytoplasmas
Verona (VR)				
1. Affi	Pinot grigio		September	16SrV-D
2. Affi	Sangiovese		September	16SrV-D
3. Affi	Chardonnay		September	16SrV-D
4. Bardolino	Ribolla		October	16SrV-D
5. Bussolengo	Marzemino		September	16SrV-D
6. Calmasino	Corvina		October	16SrV-D
7. Castelnuovo	Trebbiano	June		16SrV-D
8. Cavaion	Trebbiano Toscano		September	16SrV-D
9. Cavaion	Corvina		October	16SrV-D
10. Cavaion	Corvina		October	16SrV-D
11. Costermano	Garganega		September	16SrV-D
12. Lazise	Chardonnay	June		16SrV-D
13. Lazise	Chardonnay	June		16SrV-D
14. Lazise	Trebbiano	June		16SrV-D
15. Lazise	Garganega	June		16SrV-D
16. Lazise	Corvina		October	16SrV-D
17. Negrar	Corvinone		October	16SrV-D
18. Negrar	Croatina		October	16SrV-D
19. Peschiera	Trebbiano		October	16SrV-D
Treviso (TV)				
1. Caerano	Cabernet	June		16SrV-C
2. Castelcucco	Prosecco	June		16SrV-C
3. Castello di Godego	Cabernet-Sauvignon	June		16SrV-C
4. Cimadolmo	Chardonnay	July		16SrV-C
5. Cordignano	Cabernet-Sauvignon		September	16SrV-C
6. Fontanelle	Prosecco		September	16SrV-C
7. Fontanelle	Cabernet		September	16SrV-C
8. Maser	Chardonnay	June		16SrV-C
9. Mussolente	Unknown hybrid		October	16SrV-D
10. Povegliano	Cabernet-Sauvignon	June		16SrV-C
11. Preganziol	Verduzzo		September	16SrV-D
12. San Fior	Merlot	July		16SrV-C
13. San Pietro di F.	Marzemino		September	16SrV-C
14. Spresiano	Chardonnay		September	16SrV-C
15. Spresiano	Cabernet-Sauvignon		September	16SrV-C
16. Spresiano	Prosecco	February		16SrV-C
17. Spresiano	Corvina	February		16SrV-C
18. Spresiano	Chardonnay	February		16SrV-C
19. Susegana	Perera		September	16SrV-C
20. Susegana	Prosecco		September	16SrV-D
21. Susegana	Bianco Manzoni		September	16SrV-C
22. Susegana	Bianco Manzoni		September	16SrV-D
23. Volpago	Riesling	June		16SrV-C
Vicenza (VI)				
1. Breganze	Marzemino		October	16SrV-D
2. Gambellara	Garganega	September		16SrV-D
3. Rosà	Unknown hybrid		October	16SrV-D
4. San Giorgio	Malvasia		October	16SrV-D
5. San Giorgio	Malvasia		October	16SrV-D
6. Sarcedo	Pinot grigio		October	16SrV-D
Padova (PD)				
1. Dossi Terrassa	Trebbiano Toscano		September	16SrV-D
2. Torreglia	Merlot		September	16SrV-D
3. Torreglia	Cabernet-Sauvignon		October	16SrV-D
4. Villa di Teolo	Bianco Manzoni		September	16SrV-D
5. Villa di Teolo	Merlot		September	16SrV-D

only contained 16SrV-D type phytoplasmas.

The existence of these two FD subgroups also was confirmed by RFLP on DNA fragments amplified with chromosomal primers FD9f/r. The control FD strain (B1872) had an identical pattern after restriction digest with *MseI* and *AluI* to the samples containing 16SrV-D type phytoplasmas, and these were distinguishable from those containing the 16SrV-C type phytoplasmas as well (Fig. 4).

The manual alignment of 400 bp from Corvina, Garganega, Perera, and Rubus

with EY, RuS (a rubus stunt isolate in periwinkle), and FD strain sequences from GenBank showed that there is a differential restriction site for *TaqI* in Corvina located in position 1,398 in the 16S ribosomal sequence (Fig. 1). The alignment shows a high degree of conservation in sequences at the 5' end of the spacer region, where a reverse primer of 20 bases specific for the studied strains was designed (Fig. 1).

The comparative tests carried out in seminested PCR with primer V_{1731r} show the specificity of the system for 16SrV-group phytoplasmas (Fig. 5A to C). More

specificity tests carried out with controls from the other 16S rRNA phytoplasma groups listed in Materials and Methods confirm this results (data not shown). The RFLP analyses with *TaqI* confirmed the suitability of the system to distinguish the two FD types present in the material examined (Fig. 5D). The subgroup 16SrV-C is not discriminated from subgroup 16SrV-A, since the reverse primer contains the restriction site for *TaqI* located in position 1,714 of the spacer region. Direct PCR with primers 16R_{758f}/V_{1731r} allowed the detection of FD phytoplasma from infected plants (Fig. 6).

From the epidemiological survey in 1997, it appears that the two types of FD phytoplasmas were distributed in geographically distinct provinces (i.e., 16SrV-D in Verona and Vicenza and 16SrV-C in Treviso). In 1998, the 16SrV-D phytoplasmas were present also in samples from Treviso province, sometimes in the same localities; in one case, the same cultivar showed plants infected with one or the other type of phytoplasma, i.e., Bianco Manzoni located at Susegana (Table 1).

DISCUSSION

The tests carried out in different periods of the year (Table 1) on different grapevine tissues showed that phytoplasma detection was possible in vegetative and in dormant materials (i.e., leaf midribs or phloem from canes and trunk).

Two molecularly diverse phytoplasmas related to FD were present in the Veneto region, but only one of the two types appeared to spread. A survey on the geographical location of these strains in the 2-year period indicated a rapid diffusion of the 16SrV-D type in areas around the original epidemic center (Verona), al-

Fig. 2. Polyacrylamide gels (5%) showing the *MseI* restriction fragment length polymorphism (RFLP) patterns of phytoplasma 16SrDNA fragments of 1,200 bp obtained with primers R16F2n/R2 from grapevine samples and from phytoplasma reference strains. Aster yellows, AY-1; clover phyllody, CPh; Italian periwinkle virescence, IPVR; Elm yellows, EY. Grapevine samples are indicated following Table 1 or by cultivar name. VR, Verona; TV, Treviso; VE, Venezia; VI, Vicenza; and PD, Padova. P, marker ϕ X174 *HaeIII* digested; fragment sizes in base pairs from top to bottom: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72. S, marker pBR322 *MspI* digested; fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, 9.

Fig. 3. Polyacrylamide gels (5%) showing (A) *BfaI* restriction fragment length polymorphism (RFLP) patterns of phytoplasma 16S rDNA fragments of 1,100 bp obtained with primers R16(V)F1/R1 and (B) *TaqI* RFLP patterns of 1,050-bp fragments obtained with primers 16R758f/M23SR1804. EY, elm yellows (USA); ULW, elm yellows (EU); HOR, hornbeam; RS, rubus stunt; BB, North American aster yellows from tomato; AY-1, aster yellows; CPh, clover phyllody; IPVR, Italian periwinkle virescence. VR, Verona; TV, Treviso. Grapevine samples are indicated following numbers in Table 1. P, marker ϕ X174 *HaeIII* digested; S, marker pBR322 *MspI* digested.

though treatments to contain *S. titanus* were applied by growers.

16SrV-D was the only phytoplasma associated with FD detected in *S. titanus*;

some leafhoppers appeared to carry BN phytoplasmas, but no involvement of this vector in the spread of BN was reported. However, phytoplasmas of this group

(stolbur or 16SrXII-A ex 16SrI-G) were detected in eggs of *S. titanus*, together with other aster yellows-related phytoplasmas (2,15).

The differences demonstrated in the 16S ribosomal DNA region of FD-related phytoplasmas allowed the establishment of a new 16SrV subgroup; these data were also in agreement with the presence in France of two FD-type phytoplasmas identified by RFLP on randomly cloned FD chromosomal DNA (13).

RFLP with *AluI* on products amplified with primers FD9f/r phytoplasmas showed a 16SrV-D pattern indistinguishable from the one of B1872 (French FD control) and also from pattern FD88 described by Daire et al. (13); strain 16SrV-C appeared to be identical to FD70 (13); patterns with *MseI* showed identity between 16SrV-D and B1872 and differentiated them from 16SrV-C.

Among the samples employed as 16SrV group controls from naturally infected plants, the rubus stunt strain employed was tentatively assigned to the 16SrV-C subgroup (22) in agreement with the literature. The *TaqI* difference from the 16SrV-C subgroup observed on 16R_{758f}/M23SR_{1804r} amplified sequence was not supported by the partial sequence carried out. The hornbeam strain was enclosed in the 16SrV-A subgroup, while the examined strain of eucalyptus little leaf from southern Italy appeared to belong to subgroup 16SrV-D. The latter was the first report of 16SrV-D group phytoplasmas in a host different from grapevine in southern Italy, where only yellows-related phytoplasmas were reported in this host (1,16).

The detection system developed will facilitate FD-related phytoplasma detection: direct PCR on the ribosomal region for routine testing associated with *TaqI* distinction of the two FD-strains increases possibilities for larger epidemiological studies to monitor the FD outbreaks. Further research is necessary to verify the existence of alternative vectors that could

Fig. 4. Polyacrylamide gels (5%) showing the restriction fragment length polymorphism (RFLP) patterns of phytoplasma chromosomal fragments amplified with primers FD9f/r from grapevine samples and from reference strain B1872 using *MseI* (A) and *AluI* (B) enzymes. EY, elm yellows (USA); ULW, elm yellows (EU); VR, Verona; TV, Treviso. Grapevine samples are indicated following numbers in Table 1. P, marker ϕ X174 *HaeIII* digested; S, marker pBR322 *MspI* digested.

Fig. 5. Agarose gel electrophoresis photographs of polymerase chain reaction (PCR) products of phytoplasma DNA amplified with primers 16R723f/P7 in direct PCR (A) and in nested PCR with primers 16R758f/V1731r in (B) and in (C). CX, peach X disease; AP, apple proliferation; EY, elm yellows; RS, rubus stunt; BB, North American aster yellows from tomato; AY-1, aster yellows; CPh, clover phyllody; IPVR, Italian periwinkle virescence. VR, Verona; TV, Treviso. Grapevine samples are indicated following numbers in Table 1 or by cultivar name. P, marker ϕ X174 *HaeIII* digested; S, marker pBR322 *MspI* digested; M, marker 1-kb DNA ladder; fragment sizes in kilobase pairs from top to bottom: 10.0, 8.0, 6.0, 5.0, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, 1.0, 0.5. (D) restriction fragment length polymorphism (RFLP) analyses with *TaqI* of the PCR products in (C).

Fig. 6. Agarose gel of direct polymerase chain reaction (PCR) with primer pair 16R758f/V1731r on grapevine samples indicated following numbers in Table 1. ULW, elm yellows (EU); PD, Padova; VR, Verona; TV, Treviso. M, marker 1-kb DNA ladder.

contribute to the spread of one or both FD strains involved in outbreaks of this quarantine disease.

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