

Incidence and molecular characterization of flavescence dorée and stolbur phytoplasmas in grapevine cultivars from different viticultural areas of Serbia

S. KUZMANOVIĆ¹), M. MARTINI²), P. ERMACORA²), F. FERRINI²), M. STAROVIĆ¹), M. TOŠIĆ³), L. CARRARO²) and R. OSLER²)

¹Institute of Plant Protection and Environment, Belgrade, Serbia

²Dipartimento di Biologia e Protezione delle Piante, Università di Udine, Udine, Italy

³Faculty of Agriculture, University of Belgrade, Belgrade-Zemun, Serbia

Summary

The presence and distribution of grapevine phytoplasmas was investigated from 2003 to 2005 in some of the most important viticultural areas of Serbia, considering in particular the susceptibility and sensitiveness of both local and imported grapevine cultivars. Both flavescence dorée (FD) and bois noir (BN) phytoplasmas were detected using molecular techniques. The presence of FD phytoplasma at the moment seems limited, while BN phytoplasma appears to be present in the majority of grape growing regions in Serbia. Field surveys demonstrate that grapevine yellows (GY) epidemics in the vineyards inspected in Serbia spread very fast, indeed the incidence of symptomatic plants increased considerably year by year. In particular, the average rate of FD diffusion increased from 45.5 to 93.0 % in the Sićevočko region, while the spread of BN resulted lower. The local cultivar 'Plovdina' appeared to be extremely sensitive to FD phytoplasma showing a percentage of infected plants ranging from 91 to 100 %. PCR-RFLP and phylogenetic analyses based on ribosomal protein (rp) and *secY* gene sequences performed on Serbian FD grapevine strains demonstrated their close relationship with the Italian FD-C strain present in north-east Italy. Based on both phylogenetic markers, Serbian FD strains represent a new distinct lineage and together with the FD-C strain form a major phylogenetic group within the elm yellows group.

Key words: Grapevine yellows, PCR/RFLP, phylogenetic analysis, ribosomal protein, *secY*.

Introduction

Grapevine yellows (GY) are diseases associated with phytoplasmas that occur in many grape growing areas over the world. Flavescence dorée (FD) and bois noir (BN) are the most important GY in Europe. FD is associated with different phytoplasma strains belonging to the elm yellows group (16SrV), subgroups 16SrV-C and -D (MARTINI *et al.* 2002; LEE *et al.* 2004). It is a quarantine disease within the European community and causes severe damage particularly in France, Italy and Spain. It is mainly spread by the vector *Scaphoideus titanus* Ball, whose presence has also been demonstrated in Serbia (MAGUD and TOŠEVSKI,

2003). BN is a GY associated with a stolbur phytoplasma (16SrXII-A) present in Asia Minor, the Mediterranean and European countries (MAIXNER *et al.* 2006).

Symptoms of GY were observed in Serbia for the first time in 1986 (KUZMANOVIĆ, unpubl.). Redness and downward rolling of leaves were noticed at that time on a few vines of the local cv. 'Plovdina' in the viticultural region of Župa (Aleksandrovac). In the same region, during the late 90s, the presence of symptomatic plants belonging to different cultivars became more frequent (IVANOVIĆ and Ivanović 2000). The phytoplasma infection in vines showing GY symptoms was proved by electron microscopy observations (KUZMANOVIĆ *et al.* 2002, 2003), as well as by PCR methods (DUDUK *et al.* 2003 a, b). Molecular analyses demonstrated the presence of FD phytoplasma (16SrV-C) in the viticultural region of Župa (DUDUK *et al.* 2003 a, b; DUDUK *et al.* 2004). KUZMANOVIĆ *et al.* (2004) confirmed the presence of FD phytoplasma in the Župa region, and found the same pathogen in the viticultural area of Vrelo-Jasenovik. The same authors (DUDUK *et al.* 2004; KUZMANOVIĆ *et al.* 2004) also proved the presence of BN phytoplasma (16SrXII-A) in some areas of northern Serbia. The presence of BN phytoplasma has recently been shown in other viticultural regions of Serbia (DUDUK *et al.* 2006). It is interesting to note that in some neighboring countries such as Croatia (ĆURKOVIC-PERICA *et al.* 2003; ŠERUGA *et al.* 2003), Hungary (KÖLBER *et al.* 2003) and Bosnia-Herzegovina (DELIĆ *et al.* 2006) stolbur phytoplasma has been found to be associated with symptomatic grapevines.

The purpose of our work was to thoroughly investigate the presence and diffusion of grapevine phytoplasmas in some of the most important viticultural regions of Serbia, especially the susceptibility and sensitiveness of both local and imported grapevine cultivars. A further aim was to perform sequence analyses and infer phylogenetic relationships of Serbian FD grapevine strains with other members of the EY phytoplasma group based on 16S rDNA, ribosomal protein (rp) and *secY* gene sequences.

Material and Methods

Presence and diffusion of grapevine yellows in grape-growing regions of Serbia: Seventeen vineyards located in five different viticultural regions of Serbia - Župsko, Sićevočko,

Kutinsko, Vršačko and Deliblatska peščara (Table) - were systematically inspected visually for three years (2003-2005) in order to register the type of symptoms referable to GY present on diseased grapevines, and the increasing percentage of symptomatic grapevines in each vineyard. Surveys were carried out three times a year in July, August and September.

The most important symptoms were: discoloration and necrosis of leaves, downward curling of leaves, abortion of inflorescence, shrinking of berries, incomplete lignification of canes and stunting and necrosis of shoots.

Sources of infected plant material for molecular analyses: A total of 52 samples of symptomatic grapevines were collected in October 2003 and September 2004 from the 17 vineyards inspected (Table). For molecular investigations grapevine shoots and leaves were collected from five domestic grapevine cultivars - 'Plovdina', 'Smederevka', 'Prokupac', 'Župljanka' and 'Župski bojadiser' - as well as from five imported cultivars - 'Frankovka' (from Croatia), 'Chardonnay' (from France), and 'Italian Riesling', 'Rhine Riesling' and 'Black Burgundy', whose propagative material came from domestic nurseries.

Phytoplasma reference strains maintained in periwinkle were: stolbur of tomato (P-TV; isolated by L. CARRARO, Univ. of Udine, Italy) belonging to the 16SrXII-A subgroup; elm yellows (EY1; kindly provided by H. GRIFFITHS and W. A. SINCLAIR, Cornell Univ., Ithaca, NY, USA) belonging to the 16SrV-A subgroup; alder yellows (ALY) and rubus stunt (RuS) (kindly provided by C. MARCONE, Italy) belonging to the 16SrV-C and 16SrV-E subgroups, respectively. Phytoplasma strains from natural hosts used as reference strains were FD-C and FD-D (kindly provided by A. BERTACCINI, Univ. of Bologna, Italy) belonging to the 16SrV-C and 16SrV-D subgroups, respectively.

DNA extraction and PCR/RFLP analyses for phytoplasma identification and characterization: Total nucleic acids were extracted from 1 g of leaf midribs following the phytoplasma enrichment procedure (AHRENS and SEEMÜLLER 1992) modified by MALISANO *et al.* (1996). The extracted DNA was diluted 1:50 in sterile water. Two pairs of phytoplasma universal primers were used in nested-PCR assays. The first PCR was done with primer pair P1/P7 (DENG and HIRUKI 1991, SCHNEIDER *et al.* 1995); after a 1:30 dilution of the PCR products nested-PCR was performed with primer pair R16F2n/R16R2 (GUNDERSEN and LEE 1996). Amplifications were performed with an automated thermal cycler (MJ Research DNA Thermal Cycler PTC-100) in 25 µl reactions containing 200 µM each of the four dNTPs, 0.4 µM of each primer, 1.5 mM MgCl₂, 0.625 unit of DNA polymerase, POLYTAQ (Polymed, Florence, Italy) and 1 µl of 1:50 diluted DNA. The PCR program consisted of 38 cycles: denaturation at 94 °C for 1 min (2 min for the first cycle), annealing at 55 °C for 1 min, and extension at 72 °C for 2 min (10 min for the last cycle).

Five µl of the amplified products were electrophoresed through a 1 % agarose gel, stained in ethidium bromide, and visualized on a UV transilluminator.

RFLP analysis of final products was carried out with *TruI* enzyme (Fermentas, Vilnius, Lithuania). The restriction products were then separated by electrophoresis through 5 % polyacrylamide gel stained and visualized as described above. RFLP patterns were compared with those of reference strains P-TV (16SrXII-A) and EY1 (16SrV-A).

The FD-infected samples were further analyzed using primers P1/P7 followed by primers P1A/P7A (LEE *et al.* 2003) in nested-PCR.

The final products were digested with *TaqI* restriction enzyme (Fermentas, Vilnius, Lithuania) to determine the 16SrV subgroup affiliation of flavescence dorée phytoplasmas (MARTINI *et al.* 2002). Electrophoresis, gel staining and visualization were performed as described above. RFLP patterns were compared with those of reference strains EY1, ALY, RuS, FD-C and FD-D.

Fourteen grapevine samples representing each grape-growing region and cultivar considered in this study, were selected for further characterization of FD strains based on ribosomal protein (rp) and *secY* genes. To prepare rp genes, nested PCR was performed using the rp primer pair rp(V)F1/rpR1 specific for the EY phytoplasma group (LEE *et al.* 1998 a; LIM and SEARS 1992), followed by the second EY group-specific primer pair rp(V)F1A/rp(V)R1A (LEE *et al.* 2004). The rp(V)F1A/rp(V)R1A nested PCR products yielded a DNA fragment (about 1.2 kb) covering the region with rp genes *l22* and *s3*. In this case, RFLP analyses of final products were carried out with restriction enzymes *TruI* and *Tsp509I*. The fragments were then separated by electrophoresis through a 12 % polyacrylamide gel.

To prepare the *secY* gene, nested PCR was performed using primer pair FD9f/FD9r (DAIRE *et al.* 1997), followed by nested PCR with primer pair FD9f3/FD9r2 (ANGELINI *et al.* 2001) encoding almost the complete *secY* gene. Nested PCR products were digested with restriction enzyme *TruI*. The restriction fragments were then separated by electrophoresis through a 12 % polyacrylamide gel.

RFLP patterns were compared with those of reference strains EY1, ALY, RuS, FD-C, FD-D and with those previously published (LEE *et al.* 2004).

Cloning of PCR products and sequencing of DNA: PCR amplified products of 16S rRNA, rp and *secY* genes of two selected Serbian grapevine FD strains, FD57 and FD68, (respectively from grape samples 57 and 68) were cloned and sequenced. To obtain nearly full length 16S rDNA, P1A/16S-SR PCR products (about 1.55 kb and extends from the 5'-end of 16S rRNA to 40 nucleotides inside the spacer region) were cloned. P1A/16S-SR DNA fragments were amplified by PCR using diluted P1/P7-PCR products as templates and the universal primer pair P1A/16S-SR (LEE *et al.* 2003, 2004). The P1A/16S-SR PCR products, rp(V)F1A/rp(V)R1A PCR products (about 1.2 kb, containing *rpl22-rps3* gene sequence) and FD9f2/FD9r3 (about 1.16 kb, containing *secY* gene sequence) were purified using Wizard® SV Gel and the PCR Clean-Up System Kit (Promega, USA) and cloned into *Escherichia coli* by using the pGEM®-T Easy Vector System (Promega, USA) according to the manu-

Table

Incidence (%) of GY symptomatic vines during three year observations 2003-2005 and phytoplasma identification by nested-PCR/RFLP analyses in symptomatic grapevines collected in 2003 (samples no. 1-23, 25-30) and 2004 (samples no. 51-73) in seventeen vineyards from some of the most important viticultural regions of Serbia

Region	Vineyard locality	Grapevine variety / no. of plants	Symptomatic grapevines no. and (%)			PCR/RFLP tested sample no.	Phytoplasma	
			2003	2004	2005			
Župsko	1) Tuleš	Black Burgundy/ 1426	105 (7)	127 (9)	153 (11)	9 60-61	- FD	
		Italian Riesling/ 3173	72 (2)	92 (3)	113 (4)	8 58-59	- FD	
	2) Tuleš	Plovdina/ 23	18 (78)	22 (96)	23 (100)	11	FD	
		Smederevka/ 193	26 (13)	52 (27)	81 (42)	10 56-57	- FD	
	3) Tuleš	Župski bojadiser/ 125	24 (19)	43 (34)	74 (59)	12-14, 51-53	FD	
		Frankovka/ 974	48 (5)	117 (12)	282 (29)		54 55	FD BN
	4) Tuleš	Plovdina/ 428	68 (16)	244 (57)	398 (93)	15-16	FD	
	5) Tuleš	Plovdina/ 159	112 (70)	146 (92)	159 (100)	17-18	FD	
	6) Tuleš	Plovdina/ 648	78 (12)	546 (84)	648 (100)	62	FD	
		Prokupac/ 275	11 (4)	25 (9)	44 (16)	63 64	- FD	
	7) Tuleš	Rhine Riesling/ 877	53 (6)	158 (18)	324 (37)	65-66	BN	
	Sićevačko	8) Vrelo	Plovdina/ 501	452 (90)	487 (97)	501 (100)	1-2	FD
		9) Vrelo	Frankovka/ 79	13 (16)	28 (35)	33 (42)	5 67-68	- FD
		10) Vrelo	Plovdina/ 126	3 (2)	18 (14)	123 (98)	3-4	FD
11) Jasenovik		Plovdina/ 64	2 (3)	13 (20)	58 (91)	6-7	FD	
12) Jasenovik		Plovdina/ 144	3 (2)	19 (13)	133 (93)	25-26	FD	
13) Jasenovik		Plovdina/ 67	3 (4)	22 (33)	62 (92)	27-28	FD	
14) Jasenovik		Plovdina/ 71	3 (4)	19 (27)	69 (97)	29-30	FD	
Kutinsko	15) Niš	Chardonnay/ 105	8 (8)	13 (12)	16 (15)	19 20	- BN	
		Vršačko	Chardonnay/ 102	9 (9)	12 (12)	18 (18)	21-22, 71	BN
Frankovka/ 134	39 (29)		45 (34)	47 (35)	23, 72	BN		
Župljanka/ 98	5 (5)		7 (7)	11 (11)	73	BN		
Deliblatska pešćara	17) Banatski Karlovac	Župljanka/ 103	6 (6)	8 (8)	14 (14)	69-70	BN	

facturers' instructions. Sequencing was performed with an automated DNA sequencer (ABI Prism Model 3730) at the Genelab (ENEA Casaccia, Rome, Italy). The cloned nucleotide sequences were deposited in GenBank and accession numbers are given below and shown in Fig. 3.

Sequence homologies and phylogenetic analyses: Alignments of 16S rRNA (1.5 kb), and almost complete gene sequences of *rpl22-s3* (1.2 kb) and *secY* (1.16 kb) genes of the two selected Serbian FD strains and members of EY phytoplasma group available in GenBank (Fig. 3) were carried out and the sequence similarities calculated using CLUSTAL, version 5, and DNASTAR's Laser Gene software MegAlign program (DNASTAR, Madison, WI, USA). Phylogenetic interrelationships between the Serbian grapevine strains and representative phytoplasma strains of the EY group were assessed based on *rp* and *secY* gene sequences that revealed greater phylogenetic divergence than the 16S rRNA gene in the EY phytoplasma group (LEE *et al.* 2004). Cladistic analyses were performed with PAUP (phylogenetic analysis using parsimony), version 4.0 (SWOFFORD 1998). Uninformative characters were excluded from analyses. Two phylogenetic trees were constructed by a heuristic search via random stepwise addition implementing the tree bisection and reconnection branch-swapping algorithm to find the optimal tree(s) (Gundersen *et al.* 1994). Potato witches'-broom PWB (16SrVI-A) and aster yellows AV2192 (16SrI-L) phytoplasma strains were selected as the outgroups (Fig. 3) to root the *rp* and *secY* gene-based trees respectively. The analyses were replicated 1000 times. Bootstrappings were performed to estimate the stability and support for the inferred clades.

Results

Presence and diffusion of grapevine yellows in grape-growing regions of Serbia: During field surveys, symptoms referable to GY, were usually observed either on the whole plant or, sometimes, only on a few shoots. They included: delayed budburst in the spring, reddening (red cvs) or yellowing (white cvs) of leaves, downward rolling of the leaf edges to give a triangular shape to the leaf. Other symptoms were: partial or complete necrosis of flowers; wrinkling and wilting of berries; stunting and incomplete or uneven lignification of shoots; dark pustules on canes. Sometimes diseased grapevines died completely. All the above mentioned symptoms of GY were in agreement with those previously described for diseased vines in Serbia (IVANOVIĆ and IVANOVIĆ 2000; KUZMANOVIĆ *et al.* 2002, 2003; DUDUK *et al.* 2003 b, 2004) and elsewhere (CAUDWELL 1988).

The data on disease incidence, obtained from field surveys, are summarized in the Table. Symptomatic plants were observed in all 17 inspected vineyards located in five grape-growing regions of Serbia. All the 10 local and imported grapevine cultivars showed GY symptoms.

The average incidence (%) of GY symptomatic plants was 11.7 % in 2003, 22.8 % in 2004 and 34.2 % in 2005. GY in the surveyed regions increased 1.95 times

from 2003 to 2004, and 1.5 times from 2004 to 2005. In Sićevečko, where molecular assays demonstrated the presence of FD phytoplasma only, the amount of symptomatic plants increased from 45.5 % to 93.0 % during the period 2003-2005; whereas in Kutinsko, Vršačko and Deliblatska peščara (where stolbur phytoplasma was the only one present), the incidence varied from 12.4 % to 19.6 % in the same three-year period.

In 2005, 10 vineyards of the cultivar 'Plovdina', infected by FD only, had a disease incidence over 90 %. At the beginning of our observations in 2003, in five of these 10 vineyards less than 5 % of plants were diseased.

Among the imported grapevine cultivars, 'Frankovka' showed the highest GY incidence varying from 29 to 35 %.

PCR/RFLP analyses for phytoplasma identification and characterization: The results obtained with nested-PCR/RFLP analyses based on the 16S rRNA gene from Serbian grapevine samples are summarized in the Table. Forty-six out of 52 samples (88.5 %) collected during the two year survey 2003-2004 in the five viticultural regions of Župsko, Sićevečko, Kutinsko, Vršačko and Deliblatska peščara, were phytoplasma positive in nested-PCR experiments using universal primer pairs: P1/P7 followed by R16F2n/R16R2 (Table).

RFLP analyses of nested-PCR products R16F2n/R16R2 using the enzyme *TruI* allowed to identify two different phytoplasmas, belonging to the EY and stolbur phytoplasma groups, associated with GY symptomatic grapevines (Fig. 1a). In particular digestion with the enzyme *TruI* demonstrated that the phytoplasma involved in GY in Sićevečko, where 14/15 (93.3%) samples were positive, belonged to the elm yellows group (16SrV). In contrast phytoplasma involved in grapevine yellows in Vršačko, Kutinsko and Deliblatska peščara, where 9/10 (90.0 %) samples resulted positive, belonged to the stolbur phytoplasma group, subgroup-A (16SrXII-A). In the samples originating from Župsko both phytoplasmas were found to be present. In this region 23/27 (85.2%) samples tested positive: 20/23 (87.0%) positive samples belonged to the EY group and 3/23 (13.0%) positive samples belonged to the stolbur group (Table).

Restriction analysis with *TaqI* endonuclease of nested-PCR products obtained using primer pair P1/P7 followed by primer set P1A/P7A permitted the further identification of the FD phytoplasma strains present in Serbia as belonging to the 16SrV-C subgroup (Fig. 1 b), confirming previous investigations (DUDUK *et al.* 2003 a, b).

The molecular characterization based on *rp* and *secY* genes, carried out on fourteen grapevine FD-infected samples chosen as representative of the above mentioned cultivars from Župsko and Sićevečko demonstrated that FD phytoplasmas infecting Serbian grapevines are indistinguishable from the FD-C phytoplasma reference strain (Fig. 2) previously found in the Veneto region, Italy (MARTINI *et al.* 2002, LEE *et al.* 2004).

Sequence similarities and phylogenetic analyses: 16S rRNA, *rp* and *secY* genes of two Serbian grapevine FD strains, FD57 and FD68, rep-

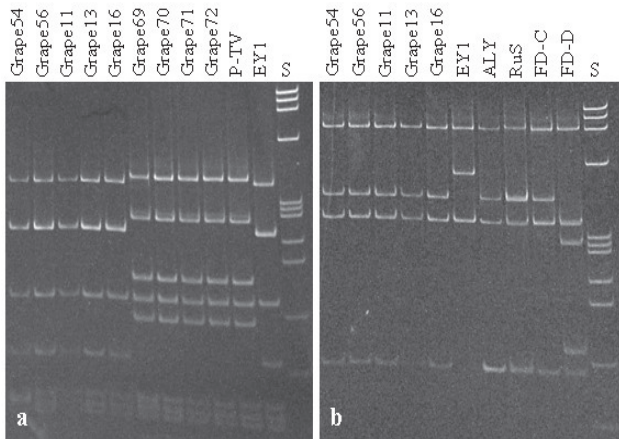


Fig. 1: RFLP analyses with enzyme *TruII* (a) of nested-PCR products amplified with primer pair P1/P7 followed by primer pair R16F2n/R16R2, and with enzyme *TaqI* (b) of nested-PCR products amplified with primer pair P1/P7 followed by primer pair P1A/P7A from Serbian grapevine samples (Table), phytoplasma reference strain P-TV (16SrXII-A) and representative phytoplasma strains in the EY group (16SrV). RFLP products were separated by electrophoresis through 5% polyacrylamide gel. Lane S, Φ 174 *HaeIII* digested (New England BioLabs, USA); fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72).

representing the two viticultural regions in which FD phytoplasmas have been identified, were sequenced and aligned with sequences of representative phytoplasma strains belonging to the EY group present in GenBank. Based on the 16S rRNA gene the highest sequence similarity was 99.9 % among the two Serbian grapevine strains FD57 (EF581166) and FD68 (EF581165), FD-C (AY197645), ALY (AY197646) and ALY882 (AY197642) phytoplasma strains. Based on *rpl22-rps3* genes Serbian grapevine FD57 (EF581167) and FD68 (EF581168) phytoplasmas strains shared 100 % sequence similarity between themselves and among representative strains of the EY group they shared 99.9 % sequence similarity with FD-C strain, confirming results obtained by RFLP analyses of the same genes. The

rp gene-based alignment helped identify a single nucleotide variation (T instead of C) at base 108, between the two Serbian grapevine strains and the FD-C strain. The two Serbian grapevine strains have this sequence variation in common with other representative phytoplasma strains belonging to the EY group such as CLY5 (AY197679), PYIn (AY197680), JWB (AY197681) and also with PWB (EF183487) belonging to the 16SrVI-A group. These results showed that FD strains associated with grapevine yellows in Serbia are not identical to the FD-C strain from Italy.

Based on the *secY* gene Serbian grapevine FD57 (EF581170) and FD68 (EF581169) strains shared 99.5 % sequence similarity. Among the representative strains of the EY group they shared the highest similarity of 99.4 and 99.7 % respectively, with the FD-C strain, confirming results obtained with RFLP analyses of the same gene. The sequence alignment showed a single sequence variation (T instead of G at position 1322 of the FD-C *secY* gene sequence deposited in GenBank with accession number AY197688) that the two Serbian strains have in common and that distinguished them from the Italian FD-C strain, confirming that the Serbian FD strains are slightly different from the Italian FD-C one also in this gene sequence.

Phylogenetic analyses based on the more variable rp gene using two Serbian grapevine strains, 13 representative phytoplasmas of the EY group, and PWB as outgroup resulted in 7 equally parsimonious trees, one of which is presented in Fig. 3 a. Phylogeny based on the *secY* gene of the same strains and AV2192 used as outgroup resulted in 1 most parsimonious tree (Fig. 3 b). The phylogenetic relationships among the analyzed strains based on both rp and the *secY* gene are in agreement with those previously reported in which 12 distinct lineages were resolved (LEE *et al.* 2004). In this study it was possible to resolve another distinct lineage, though supported by low bootstrap values, represented by Serbian grapevine strains that clustered together with the FD-C strain into a major phylogenetic group supported by high bootstrap values (Fig. 3).

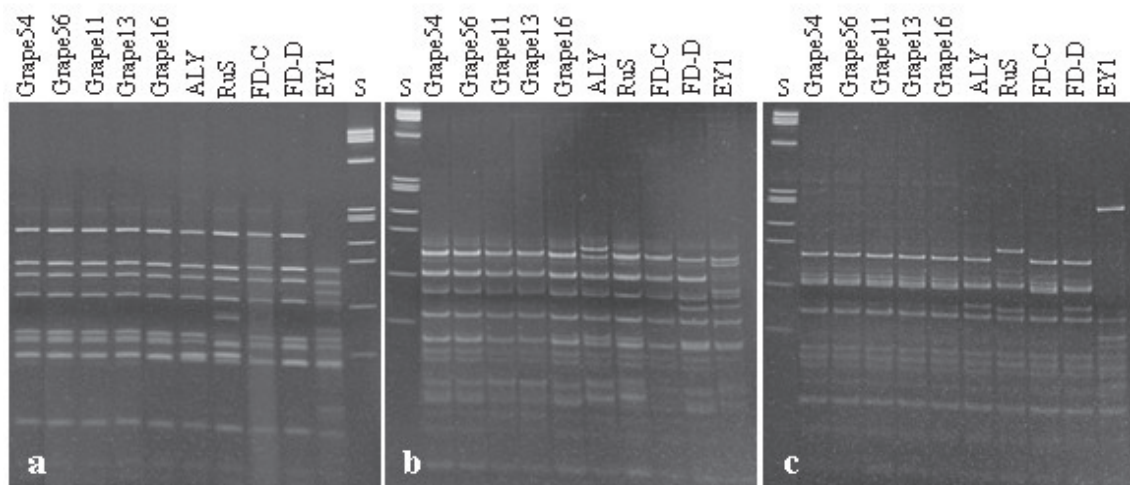


Fig. 2: RFLP analyses with enzymes *Tsp509I* (a) and *TruII* (b) of nested-PCR products amplified with primer pair rp(V)F1/rpR1 followed by primer pair rp(V)F1A/rp(V)R1A, and with enzyme *TruII* (c) of nested-PCR products amplified with primer pair FD9f/FD9r followed by primer pair FD9f3/FD9r2 from Serbian grapevine samples (Table) and representative phytoplasma strains in the EY group (16SrV). RFLP products were separated by electrophoresis through 12% polyacrylamide gel. Lane S, Φ 174 *HaeIII* digested (New England BioLabs, USA); fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72).

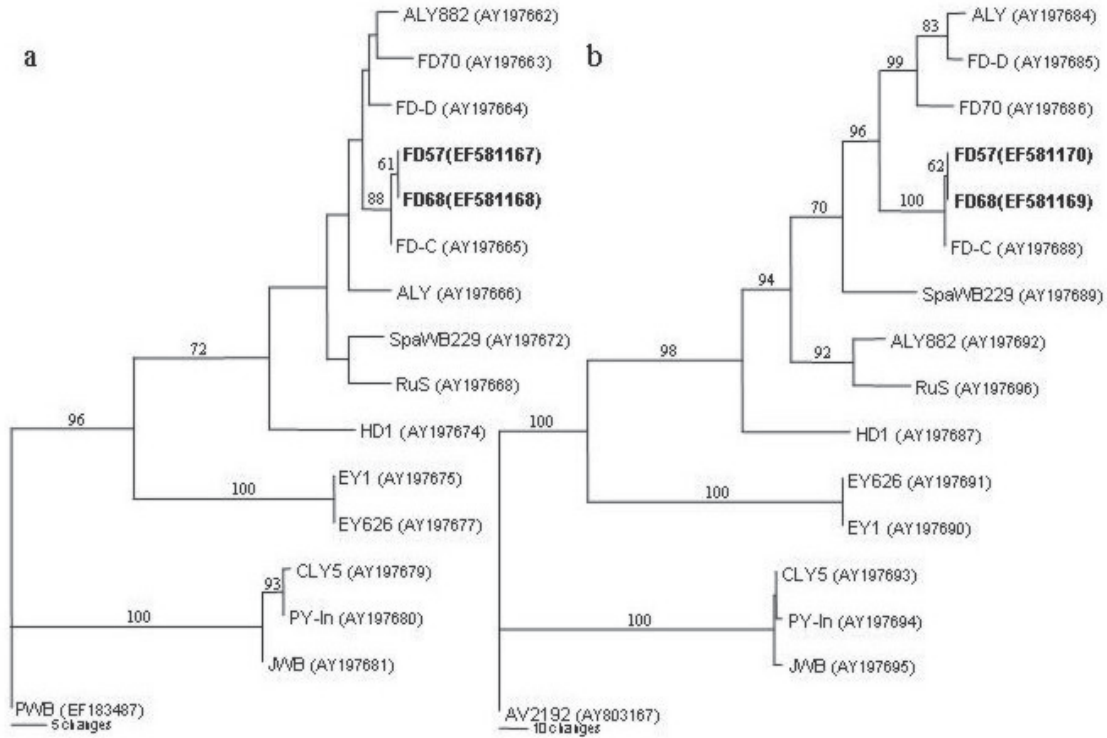


Fig. 3: Phylogenetic trees constructed by parsimony analyses of ribosomal protein genes (*rpl22* and *rps3*) (a) and the *secY* gene (b) from two Serbian grapevine FD57 and FD68 phytoplasma strains and from representative phytoplasma strains in the EY group (16SrV). Sequences were aligned with Clustal version 5 (DNASTAR Lasergene software, Madison, WI). Outgroups were described in Materials and Methods. Bar length represents inferred character state changes. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap values are shown on branches.

Discussion

This work confirmed that phytoplasmas are widespread in all the surveyed viticultural regions in Serbia, such as Župsko, Sićevačko, Kutinsko, Vršačko and Deliblatska peščara and that two phytoplasma types, FD and BN, are associated with grapevine yellows in Serbia.

Considering this and previous works (DUDUK *et al.* 2004, 2006; KUZMANOVIĆ *et al.* 2004) FD phytoplasma at the moment is present in a restricted area (Župsko and Sićevačko) in southern Serbia; while BN is present in all the most important grape growing regions except Sićevačko.

Field surveys carried out during this study demonstrated that the GY epidemics in Serbia are spreading very fast, indeed the incidence of symptomatic plants increased significantly from one year to the next (1.95 times from 2003 to 2004, and 1.5 times from 2004 to 2005) in the examined vineyards. Thus, suitable control strategies must urgently be adopted and adapted to the local epidemiological situation.

It seems that FD phytoplasma has been spreading more quickly than stolbur phytoplasma, given that in Sićevačko the average rate of FD diffusion increased from 45.5 to 93.0 %, while in Kutinsko, Vršačko and Deliblatska peščara the rate of stolbur diffusion ranged from 12.4 to 19.6 %. According to our investigations the grape-growing regions that present the highest incidence of GY are Sićevačko and Župsko, where FD phytoplasma is widespread and 'Plovdina' is the predominant cultivar.

Considering all the surveyed vineyards, a total of 10 different grapevine varieties has been monitored and all

were shown to be affected by GY (Table). In fact, the FD phytoplasma was found in grapevines of 'Plovdina', 'Italian Riesling', 'Black Burgundy', 'Smederevka', 'Župski bojadiser', 'Prokupac' and 'Frankovka' cvs. in Župsko, as well as in samples of 'Plovdina' and 'Frankovka' originating from Sićevačko. Stolbur phytoplasma was detected in grapevine samples of 'Chardonnay', 'Frankovka', 'Župljanka' and 'Rhine Riesling' cvs. growing in Vršačko, Deliblatska peščara, Kutinsko and Župsko.

'Plovdina' appeared to be extremely sensitive to FD since in 2005 the percentage of infected plants in the inspected vineyards ranged from 91 to 100 %, thus confirming previous investigations (KUZMANOVIĆ *et al.* 2006). In some 'Plovdina' vineyards the incidence of symptomatic plants was very high during all three years, but in others the incidence increased dramatically from 2-4 to 91-98 % during the same period. In Kutinsko only BN phytoplasma has been detected so far, no symptomatic 'Plovdina' plants were found.

All 18 symptomatic samples of 'Plovdina' collected in Župsko and Sićevačko, were positive for FD, 16SrV-C, phytoplasma. The symptoms on 'Plovdina' were in some cases different from those on other varieties, showing also red colored leaves with yellowish and greenish main veins. These symptoms are considered to be due to the co-presence of GLRaVs, which have been detected in mixed infections in some samples (KATIS, pers. comm.).

It has been confirmed that 'Chardonnay' is sensitive to BN. However, in the regions where FD phytoplasma was present, symptomatic 'Chardonnay' plants were not found. 'Frankovka', a cultivar imported from Croatia and of minor

importance in several regions of Serbia, was shown to be sensitive and susceptible to both phytoplasmas.

Fine molecular analyses based on nested-PCR/RFLP, sequencing and phylogenetic analyses of *rp* and *secY* genes, being more variable than 16S rDNA (LEE *et al.* 2004), established that FD phytoplasma infecting Serbian grapevines is very similar to the Italian FD-C phytoplasma strain, present in north-east Italy (MARTINI *et al.* 2002). In particular RFLP patterns obtained from Serbian grapevine samples are indistinguishable from FD-C phytoplasma strains, only sequencing and phylogenetic analyses showed that Serbian strains are slightly different from FD-C phytoplasma, resulting in a new distinct lineage, that together with FD-C represents a major phylogenetic group within the EY group. It would be interesting to analyze Serbian FD infected grapevines using the new genetic markers (ARNAUD *et al.* 2006), which proved to be a useful tool for differentiating between phytoplasma strains in the EY group.

References

- AHRENS, U.; SEEMÜLLER, E.; 1992: Detection of DNA of plant pathogenic mycoplasma-like organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytopathology* **82**, 828-832.
- ANGELINI, E.; CLAIR, D.; BORGIO, 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.
- ARNAUD, G.; MALEMBIC-MAHER, S.; SALAR, P.; FOISSAC, X.; BOUDON-PADIEU, E.; 2006: Map gene as a new non ribosomal marker to study the molecular variability of grapevine yellows and related phytoplasmas from group 16SrV in Europe, 165-167. Ext. Abstr. 15th Meeting ICVG, 3-7th April, Stellenbosch, South Africa.
- CAUDWELL, A.; 1988: Grapevine yellows diseases. In: R. C. PEARSON; A. C. GOHEEN (Eds): *Compendium of Grape Diseases*, 45-47. Am. Phytopathol. Soc. Press, St. Paul, Minnesota, USA.
- ČURKOVIC-PERICA, M.; ŠERUGA, M.; KOZINA, B.; KRAJAČIĆ, M.; ŠKORIĆ, D.; 2003: Grapevine yellows - spread of the disease in Croatia, 95-96. Ext. Abstr. 14th Meeting ICVG, 12-17th September, Locorotondo (Bari), Italy.
- DAIRE, X.; CLAIR, D.; REINERT, W.; BOUDON-PADIEU, E.; 1997: Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to stolbur subgroup by PCR amplification of non-ribosomal DNA. *Eur. J. Plant Pathol.* **103**, 507-513.
- DELIĆ, D.; MARTINI, M.; ERMACORA, P.; CARRARO, L.; MYRTA, A.; 2006: First report of grapevine bois noir in Bosnia and Herzegovina. *J. Plant Pathol.* **88**, 226.
- DENG, S.; HIRUKI, C.; 1991: Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *J. Microbiol. Meth.* **14**, 53-61.
- DUDUK, B.; BOTTI, S.; IVANOVIĆ, M.; BERTACCINI, A.; 2006: Status of grapevine yellows in Serbia, 193-194. Ext. Abstr. 15th Meeting ICVG, 3-7th April, Stellenbosch, South Africa.
- DUDUK, B.; BOTTI, S.; IVANOVIĆ, M.; DUKIĆ, N.; BERTACCINI, A.; 2003 b: Molecular characterization of a flavescence dorée phytoplasma infecting grapevine in Serbia, 91-93. Ext. Abstr. 14th Meeting ICVG, 12-17th September, Locorotondo (Bari), Italy.
- DUDUK, B.; BOTTI, S.; IVANOVIĆ, M.; KRSTIĆ, B.; DUKIĆ, N.; BERTACCINI, A.; 2004: Identification of phytoplasmas associated with grapevine yellows in Serbia. *J. Phytopathol.* **152**, 575-579.
- DUDUK, B.; IVANOVIĆ, M.; DUKIĆ, N.; BOTTI, S.; BERTACCINI, A.; 2003 a: First report of an elm yellows, subgroup 16SrV-C phytoplasma infecting grapevine in Serbia. *Plant Dis.* **87**, 599.
- GUNDERSEN, D. E.; LEE, I. M.; 1996: Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathol. Mediterr.* **35**, 144-151.
- GUNDERSEN, D. E.; LEE, I. M.; REHNER, S. A.; DAVIS, R. E.; KINGSBURY, D. T.; 1994: Phylogeny of mycoplasma-like organisms (phytoplasmas): a basis for their classification. *J. Bacteriol.* **176**, 5244-5254.
- IVANOVIĆ, M.; IVANOVIĆ, D.; 2000: Pojava simptoma sličnih fitoplazmozama na vinovoj lozi u kruševačkom vinogorju. XI jugoslovenski simpozijum o zaštiti bilja i savetovanje o primeni pesticida. Zlatibor, 4-9. decembar. Zbornik rezimeja, 42.
- KÖLBER, M.; EMBER, I.; VARGA, K.; BOTTI, S.; MARTINI, M.; LAZAR, J.; BERTACCINI, A.; 2003: Six-year survey of grapevine yellows distribution on Hungary, 99-101. Ext. Abstr. 14th Meeting ICVG, 12-17th September, Locorotondo (Bari), Italy.
- KUZMANOVIĆ, S.; STAROVIĆ, M.; TOŠIĆ, M.; STOJANOVIĆ, S.; TOMIĆ, T.; 2002: Detection of phytoplasma disease on grapevine in Serbia by electron microscopy. *Plant Protection*, Belgrade **53** (2-3), No. 240-241, 75-86 (in Serbian).
- KUZMANOVIĆ, S.; STAROVIĆ, M.; TOŠIĆ, M.; STOJANOVIĆ, S.; TOMIĆ, T.; 2003: Phytoplasmas on grapevine in Serbia, 93-95. Ext. Abstr. 14th Meeting ICVG, 12-17th September, Locorotondo (Bari), Italy.
- KUZMANOVIĆ, S.; MARTINI, M.; FERRINI, F.; ERMACORA, P.; STAROVIĆ, M.; TOŠIĆ, M.; OSLER, R.; 2004: Stolbur and Flavescence dorée phytoplasmas present in grapevine in Serbia, 138-139. V. Congress of Plant Protection, 22-26th November, Zlatibor, Serbia.
- KUZMANOVIĆ, S.; OSLER, R.; TOŠIĆ, M.; MARTINI, M.; STAROVIĆ, M.; STOJANOVIĆ, S.; ALEKSIĆ, G.; 2006: Grapevine cv. Plovdina as indicator of Flavescence dorée. Ext. Abstr. 15th Meeting ICVG, 3-7th April, Stellenbosch, South Africa.
- LEE, I. M.; GUNDERSEN-RINDAL, D. E.; DAVIS, R. E.; BARTOSZYK, I. M.; 1998: Revised classification scheme of phytoplasmas based on RFLP analyses of 16SrRNA and ribosomal protein gene sequences. *Int. J. Syst. Bacteriol.* **48**, 1153-1169.
- LEE, I. M.; MARTINI, M.; BOTTNER, K. D.; DANE, R. A.; BLACK, M. C.; TROXCLAIR, N.; 2003: Ecological implications from a molecular analysis of phytoplasmas involved in an aster yellows epidemic in various crops in Texas. *Phytopathology* **93**, 1368-1377.
- LEE, I. M.; MARTINI, M.; MARCONE, C.; ZHU, S. F.; 2004: Classification of phytoplasma strains in the elm yellows group (16SrV) and proposition of '*Candidatus Phytoplasma ulmi*' for the phytoplasma associated with elm yellows. *Int. J. Syst. Evol. Microbiol.* **54**, 337-347.
- LIM, P. O.; SEARS, B. B.; 1992: Evolutionary relationships of a plant-pathogenic mycoplasma-like organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. *J. Bacteriol.* **174**, 2606-2611.
- MAGUD, B.; TOŠEVIKI, I.; 2003: *Scaphoideus titanus* Ball. (Homoptera, Cicadellidae) Nova tetoina na teritoriji Srbije. VI. savetovanje o zaštiti bilja, p. 96. Zbornik rezimeja, Zlatibor, November 24-28, 2003.
- MAIXNER, M.; 2006: Grapevine yellows - current developments and unsolved questions. Ext. Abstr. 15th Meeting ICVG, 3-7th April, Stellenbosch, South Africa.
- MALISANO, G.; FIRRAO, G.; LOCCI, R.; 1996: 16S rDNA-derived oligonucleotide probes for the differential diagnosis of plum leptonecrosis and apple proliferation phytoplasmas. *EPPO Bull.* **26**, 421-428.
- MARTINI, M.; BOTTI, S.; MARCONE, C.; MARZACHI, C.; CASATI, P.; BIANCO, P. A.; BENEDETTI, R.; BERTACCINI, A.; 2002: Genetic variability among Flavescence dorée phytoplasmas from different origins in Italy and France. *Mol. Cell. Probes* **16**, 197-208.
- SCHNEIDER, B.; SEEMÜLLER, E.; SMART, C. D.; KIRKPATRICK, B. C.; 1995: Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: S. RAZIN; J. G. TULLY (Eds.): *Molecular and Diagnostic Procedures in Mycoplasma*. Vol. 2, 369-380. Academic Press, New York.
- ŠERUGA, M.; ŠKORIĆ, D.; KOZINA, B.; ČURKOVIC-PERICA, M.; KRAJAČIĆ, M.; 2003: A comparison of Stolbur phytoplasma isolates from Croatian grapevine by analyses of ribosomal and non-ribosomal gene regions. Ext. Abstr. 14th Meeting ICVG, 12-17th September, Locorotondo (Bari), Italy.
- SWOFFORD, D. L.; 1998: PAUP*. Phylogenetic Analysis using Parsimony (*and other Methods), version 4. Sunderland, MA: Sinauer.

