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STSM title: **Comparative multi gene characterisation of phytoplasmas from grapevine and apple in Serbia and Italy**

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Introduction

The aim of the stage was to verify the possibility to use multi gene analyses approach to differentiate strains of phytoplasmas infecting field collected samples of grapevine infected with 'Flavescence dorée' and 'Bois noir' and of apple infected with apple proliferation diseases in some geographic areas where the disease are either epidemic or endemic with stronger interest to sample collected in Serbia.

Grapevine – 'Flavescence dorée'

Selected samples (18) collected in three areas of Serbia, and in two areas of Italy were used to study variability in the translocase (Sec Y) gene. As reference strains TV-54, Liguria 3, FD-70, and FD88 (Martini *et al*., 2002) obtained from infected grapevines in Italy and in France respectively were employed.

Total nucleic acid extracted either with a chloroform/phenol or a CTAB based procedure; PCR/RFLP analyses on 16SrDNA confirmed that all phytoplasmas belonged to 16SrV-C group. Molecular characterization was performed by RFLP analyses on *SecY* (traslocase) gene using primers FD9f2/FD9r followed by FD9f3/FD9r2 in nested PCR, amplifying the entire *SecY* gene and a portion of *rpl15* gene (1,150 bp). The examined strains showed RFLP polymorphisms with *Tru*I and *Taq*I restriction enzymes that resulted partially related to their geographic origin. In particular strain differentiation was achieved from samples from Serbia and Italy and their tentative grouping showed identity between strains from Aleksandrovac (RS) and Tuscany (I) (Table 1). RFLP profiles identity was present also among samples from Niš, Irig, and Veneto. Further RFLP profiles differentiable from each other and from all the previous were also identified in samples from Niš and Irig (Table 1).

Grapevine – 'Bois noir'

Samples collected in grapevine growing areas of Hungary, Serbia, and Italy in which the presence of 'Bois noir' (BN) phytoplasmas was previously demonstrated by RFLP analyses with *Tru*I restriction enzyme on R16F2/R2 amplicons were used for multigene characterization in the nearly full 16Sr DNA gene plus spacer region, the tuf gene, and the

rpS3 gene (Table 2). Reference strains employed maintained in periwinkle were STOL (from Serbia), STOLC and STOL-PO (from France).

Total nucleic acids were extracted from midribs and petioles as described above and direct PCR amplification with P1/P7 universal phytoplasma primer pair amplifying 16S rDNA, the spacer region between 16S and 23S rDNA and the 5' portion of 23S rDNA, was performed for the phytoplasma detection. Nested PCR amplification was also carried out with primers listed in Table 2. RFLP analyses with *Hpy8*I, *Mbo*I, *Mbo*II, *Tru*I, *Rsa*I, *Alu*I, and *Tsp509*I restriction enzymes were carried out on the majority of amplicons produced (Table 2). Different profiles were observed in several of the examined samples, according with amplicon employed; overall restriction profiles indicate the possible presence of interoperon heterogeneity and/or of mixed phytoplasma population in some of the samples (Table 2).

Strains STOL, STOLC, and STOL-PO plus 7 field collected strains showing polymorphisms in the 16S plus spacer region amplicons were sequenced (Table 2). The sequences were assembled using DNA STAR software, and compared with selected sequences of phytoplasmas in GenBank database using Blast N 2.2.18. Virtual RFLP analyses on R16F2/R2 amplicons were carried out, using pDRAW32 program (AcaClone Software). Obtained aligned sequences ranged from 1,405 to 1,737 bp, all showing 99% homology among them self and with several of the 16SrXII-related strains deposited in Genbank. However it was possible to distinguish diversity in BN strains after virtual RFLP analyses on R16F2/R2 amplicons. The comparison between real and virtual RFLP analyses showed in some cases differences between real and virtual RFLP profiles (Table 2) confirming the possible presence of interoperon heterogeneity and/or of mixed phytoplasma population in some of the samples.

Further nested-PCR/RFLP characterization was carried out on *tuf* gene for all samples using nested-PCR procedures, and restriction analyses with *Hpa*II enzyme showed that all the samples belong to tuf-type II. RFLP analyses with *Ssp*I, *Hpy8*I and *Tai*I on samples positive for *rpS3* gene showed clear polymorphism in sample BC1 from Serbia and possible polymorphisms in some of the strains from Hungary and Italy with *Ssp*I restriction enzyme.

Apple – apple proliferation

Symptomatic apple samples collected in diverse areas of Italy and Serbia were employed (Table 3). As reference strains the three AP strains reported in literature as AP-15, AT-1 and AT-2 were used. To evaluate the possible presence of molecularly differentiable strains PCR/RFLP analyses were carried out on three diverse regions of the AP genome such as 16S rDNA plus spacer region and beginning of 23S; ribosomal protein (rp) gene sequences *rpl22* and *rps3,* and nitroreductase genes. DNA was extracted from fresh leaf midribs and phloem tissues as described above. Nested PCR amplification with R16(X)F1/R1 primers followed by RFLP analyses with *Rsa*I and *Ssp*I allow to confirm that all samples analyzed were infected with '*Candidatus* Phytoplasma mali'. Also primers F1/B6 amplified all tested samples and RFLP analyses on these amplicons distinguished two phytoplasma profiles (P-I and P-II) (Table 3).

Rp gene sequences *rpl22* and *rps3*. were amplified in direct PCR reactions using primers rpAP15f/rpAP15r. The RFLP analyses allow to identify in all the samples showing P-I profile the presence of phytoplasmas belonging to rpX-A subgroup, while in samples showing P-II profile it was possible to distinguish other described rp subgroups. The rpX-B and X-C

subgroups were identified in one of the TN samples and in the two VE samples respectively (Table 3). For the amplification of the nitroreductase gene a semi-nested PCR was employed using 1:30 diluted products of the direct amplification as templates with primers AP8/AP10 followed by primers AP13/AP10; enzymes *Rca*I and *Hinc*I were then employed for RFLP analyses that allow to differentiate among strains belonging to rpX-A subgroup: the two Serbian samples (RS) show AP profiles, while TN samples show AT-2 profiles (Table 3).

Table 1. RFLP results on FD-C amplicons obtained with primers FD9f3/r2 in nested PCR on FD9f2/r products. Identical letter = identical profile.

Location (Country)/sample number	Restriction enzyme		Profiles
	*Tru*I	*Taq*I	
Niš (RS)/154	A	A	I
Niš (RS)/155	A	A	I
Niš (RS)/156	B	B	II
Niš (RS)/157	C	A	III
Niš (RS)/158	C	A	III
Aleksandrovac (RS)/ARR5	E	C	IV
Aleksandrovac (RS)/AS2	E	C	IV
Aleksandrovac (RS)/AP1	E	C	IV
Irig (RS)/64	C	A	III
Irig (RS)/66	C	A	III
Irig (RS)/67	A	B	VI
Veneto (I)/38	C	A	III
Veneto (I)/53	C	A	III
Veneto (I)/87	C	A	III
Veneto (I)/107	C	A	III
Veneto (I)/108	C	A	III
Tuscany (I)/1	E	C	IV
Tuscany (I)/66	E	C	IV
TV-54 (I)	C	A	III
FD-70 (F)	D	A	V
Liguria 3 (I)	C	A	III

Table 2. RFLP results on BN infected samples from Hungary, Serbia and Italy and from reference strains. Identical letter = identical profile; *, almost identical profiles; bold, phytoplasma strains sequenced; small characters, results of virtual RFLP.

Samples (Country) acronym	Primers P1A/P7A°			Primers F1/B6 bp)		Primers M1/B6			Primers R16F2/R2				
	*Hpy8*I	*Mbo*I	*Mbo*II	*Tru*I	*Mbo*II	*Rsa*I	*Mbo*II	*Alu*I	*Tru*I	*Mbo*II	*Tsp509*I	*Hpy8*I	*Hpy188*I
Langeveildeground (H) L1	C	A	B	A	B	A	A	A	A	A	A	A	
Langeveildeground (H) L2	C	A	B	A	A	A	A	A	Aa	Aa	Aa	Aa	a
Brandmajor (H) B1	C	B	C	A*	C	A	A	A	A	C	A	A	
Brandmajor (H) B2	B	B	C	A	C	-	-	-	A	C	A	A	
Brandmajor (H) B3	C	B	C	A	C	-	-	-	A	C	A	A	
Brandmajor (H) B4	C	-	C	A*	C	-	-	-	Aa	Ca	Aa	Aa	b
Bela Crkva (RS) BC1	D	A	A	A	A	B	A	A	A	C	A	A	
Bela Crkva (RS) BC2	D	B	B	-	-	-	-	-	Aa	A*a	Aa	Aa	a
Bela Crkva (RS) BC3	E	B	A	-	-	-	-	-	A	A	A	A	
Bela Crkva (RS) BC4	D	B	C	-	-	-	-	-	A	A	A	A	
Radmilovac (RS) R1	D	B	B	-	-	-	-	-	Aa	Ac	Aa	Aa	a
Radmilovac (RS) R2	D	B	B	-	-	-	-	-	A	A	A	A	
Radmilovac (RS) R3	D	A	A	A	A	A	A	A	A	C	A	A	

Čoka (RS) C1	D	B	B	A	A	A	A	A	Aa	Aa	Aa	Aa	a
Veneto (I) 39	D	B	B	A	C	A*	B	B	Aa	Ca	Bb	Aa	a
Veneto (I) 41	D	A	B	A	C	A*	B	B	A	C	A	A	
Tuscany (I) 2	D	A	B	-	C	A*	A	A	A	C	A	A	
Tuscany (I) 3	D	A	A	-	C	A*	A	A	Aa	Ba	Aa	A	a
STOL (RS)	A	A	A	A*	B	B	A	A	Ab	Aa	Aa	Ab	a
STOLC (F)	A	A	B	-	B	A	B	B	Aa	Ba	Aa	Aa	a
STOL-PO (F)	A	A	A	-	-	A	A	A	Aa	Ba	Aa	Aa	a

°, Martini, 2004

Table 3. Results of molecular analyses to characterize AP strains.

Strain acronyms	Primers F1/B6		Group	Primers AP13/AP10		Group	Primers rpAP15f/rpAP15r	Group
	*Hpa*II	*Fau*I		*Rca*I	*Hinc*II		*Alu*I	
RS-135	A	A	PI	B	B	AP	A	rpX-A
RS-151	A	A	PI	B	B	AP	A	rpX-A
I-VE11	B	A	PII	-	-	nd	C	rpX-C
I-VE16	B	A	PII	B	B	AT1	C	rpX-C
I-TN1	A	A	PI	A	A	AT2	A	rpX-A
I-TN2	A	A	PI	-	-	nd	A	rpX-A
I-TN3	B	A	PII	?	B	nd	B	rpX-B
AP-15	A	A	PI	B	B	AP	A	rpX-A
AT-1	A	A	PI	B	A	AT1	B	rpX-B
AT-2	A	A	PI	A	A	AT2	A	rpX-A

Nd, group not determined