TAGUNGSBEITRÄGE ANLÄSSLICH DES 4. EUROPEAN BOIS NOIR WORKSHOP 9.-11. MÄRZ, 2016, KLOSTERNEUBURG ÖSTERREICH

PROCEEDINGS OF THE 4TH EUROPEAN BOIS NOIR WORKSHOP 9-11 MARCH 2016, KLOSTERNEUBURG, AUSTRIA

organized by

Reinhard Eder, Monika Riedle-Bauer, Franz G. Rosner
(Federal College and Research Institute for Viticulture and Pomology, Austria)

CHARACTERIZATION OF '*CANDIDATUS* PHYTOPLASMA SOLANI' STRAINS FROM GRAPEVINES, *HYALESTHES OBSOLETUS* , REFERENCE STRAINS IN PERIWINKLE AND IN COLONIES OF STRAIN STOL

Nicoletta Contaldo[1], Yuri Zambon[1], Samanta Paltrinieri[1], Nicola Mori[2], Jelena Mitrović[3], Bojan Duduk[3] and Assunta Bertaccini[1]

[1] Alma Mater Studiorum, University of Bologna, Plant Pathology
IT-40127, Bologna, Viale Fanin 42
[2] Department of Agronomy, Food, Natural resources, Animals and Environment, University of Padua
IT-35020 Legnaro (Padua), Viale dell'Università 16
[3] Institute of Pesticides and Environmental Protection, Department of Plant Pests
RS-11080 Zemun, Banatska 33

E-Mail: nicoletta.contaldo2@unibo.it

Multilocus typing (MLT) was performed on 72 phytoplasma strains obtained from grapevine samples and *Hyalesthes* obsoletus specimens collected in different Bois noir-infected vineyards, on six phytoplasma strains maintained in the periwinkle reference collections and on a colony obtained from cultivation of a '*Candidatus* Phytoplasma solani' strain derived from the STOL periwinkle reference collection sample. RFLP analyses of *tuf*, *sec*Y, *groel* and *Stamp* genes allowed to differentiate 8 lineages in the 72 field-collected grapevine and *H. obsoletus* samples. In some cases there was a good consistency in the MLT profiles of lineages detected in the grapevine and those identified in *H. obsoletus* indicating the presence of phytoplasma populations shared between the two hosts. On the other hand, the lineages identified in the six "stolbur" strains in collection in periwinkle were all different from those in the vineyard collected samples. The partial molecular characterization of the colonies obtained from the STOL strain after its isolation in a chemically defined medium shows *Stamp* gene congruence with those of the original strain maintained in micropropagation.

Bois noir (BN) phytoplasmas are transmitted by cuttings and predominantly by Cixiidae (Auchenorrhyncha), therefore the study of the epidemiology of the disease is difficult. Disease management of outbreaks and the differentiation of phytoplasma populations in vineyards is of utmost importance. The characterization of BN phytoplasmas was performed in several grapevine growing areas by multilocus sequence analysis on diverse genes. However, very often a consistent differentiation of phytoplasma populations in specific geographic areas was difficult to achieve. This problem could be linked to the molecular markers selected for the differentiation studies, as some of the employed molecular markers are subjected to a high environmental selection pressure and can therefore be problematic for epidemiologic research. Due to the absence of markers directly linked to BN pathogenicity, the use of diverse marker combinations might allow the following of epidemics. Thus, in the present study a multigene characterisation of '*Ca.* P. solani' strains from

different sources was carried out by collective RFLP typing of *tuf*, *sec*Y, *groel* and *Stamp* genes in order to detect their possible usefulness as combined markers in the study of BN epidemiology.

MATERIAL AND METHODS

Seventy-three '*Candidatus* Phytoplasma solani' strains from grapevine and *Hyalesthes obsoletus* samples collected in vineyards located in Northern Italy and Serbia (Table 1) were employed. "Stolbur" reference strains maintained in periwinkle were STOL (from Serbia), ASLO (from Slovenia) STOLC, STOL-PO, STOL-CH, MOL (from France). DNA from colonies grown in chemically defined medium after isolation from STOL periwinkle in micropropagation (Contaldo et al., 2012) was also used. Total nucleic acids were extracted from 1 g of midribs and phloem scrapes of infected samples maintained at -20 °C with a chloroform/phenol method, while *H. obsoletus* samples maintained in -20 °C were extracted by a CTAB procedure (Angelini et al., 2001). Amplicons produced on 16S rDNA (Gundersen and Lee, 1996), *Stamp* (Fabre et al., 2011), and *sec*Y (Lee et al., 2010) genes were subjected to RFLP analyses with *Tru1*I. RFLP on *groEL* gene was performed on nested amplicons obtained with STOLgroesF/STOLstampR followed by AYgroelF/STOLgroelR2 primers (Mitrović et al., 2011, 2012 and 2013) with *Tru1*I. The *tuf* gene was amplified in a nested PCR procedure using primer pairs tuf1f/r/TufAYf/r and TufINT1f/TufINT4r (Langer and Maixner, 2004) and amplicons were subjected to the RFLP analyses with *Hpa*II.

RESULTS AND DISCUSSION

The overall RFLP profiles on four phytoplasma marker genes were obtained from 35 grapevine and 15 *H. obsoletus* samples collected in vineyards as shown in Table 1. Eight lineages were differentiated by RFLP analyses. In 22 field samples not all the four genes could be amplified even if '*Ca.* P. solani'-related phytoplasmas were identified by RFLP analyses on 16S ribosomal gene. The analysis of 5 cixiid and 17 grapevine samples resulted only in amplification of two to three genes, while *Stamp* was amplified in all samples (Fig. 1). Moreover the rate

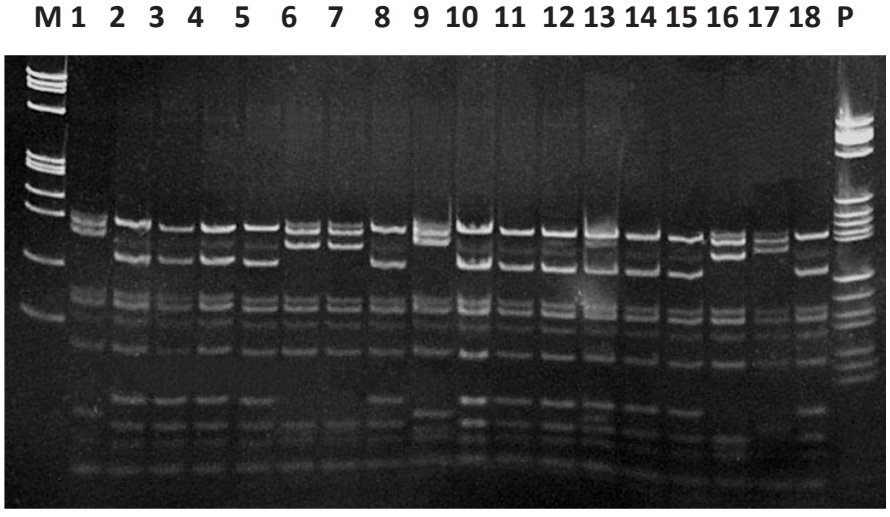

Fig. 1. Polyacrylamide (6.7 %) gel showing RFLP profiles of *Stamp* gene amplicons digested with *Tru*1I. M, Marker DNA phiX174 *Hind*III digest; P, Marker DNA pBR322 *MspI* digested. From 1 to 18 grapevine samples showing 3 of the differential profiles identified on the amplicon from this gene.

of amplification was decreasing from amplification of *tuf* to *sec*Y and *groEL* genes. In the latter, only 9 out of 22 samples were amplified, confirming the reported reduced sensitivity in detection of primers other than those amplifying 16S rDNA and is also in agreement with the expected amplicon length. While in the amplicons of the *Stamp* gene five different restriction profiles were differentiated by *Tru1*I, two profiles were distinguished in the *tuf* gene by *Hpa*II, two in the *groEL* gene by *Tru1*I and three profiles were shown by the same enzyme in *sec*Y gene (Table 1). Overall, 8 lineages could be obtained by the different combination of restriction profiles in the samples from vineyards. Three profiles were only detected in one sample from grapevine or *H. obsoletus* (lineages I, IV and XI); profile X was only detected in 2 grapevine samples from Serbia. The profile III was detected in *H. obsoletus* from Italy as well as in grapevine samples from different localities in Serbia, while the profile VI was present in 7 grapevine samples, two of which are from Serbia and the rest from different provinces of the same Italian region (Emilia-Romagna). On the other hand the profile V was found in both grapevine and *H. obsoletus* in the same Italian region at the time of an epidemic of BN. The results obtained are in agreement with previous results (Contaldo et al., 2009, 2011, 2012 and 2013; Mitrović et al., 2013) and indicate that the MLT of these 4 genes could be useful as combined markers in the study of BN epidemiology under field conditions.

It is important to underline that none of the tested reference strains maintained in periwinkle in micropropagation showed the profiles detected in the field collected samples, but the overall lineage profiles showed to be consistent with the geographic origin of the strains (i.e. France, Slovenia and Serbia). The STOL strain colonies grown on agar media after isolation in broth medium tested positive on both, 16S ribosomal and *Stamp* genes. The RFLP profiles and their sequences obtained after direct sequencing in both directions with primers used for amplification confirmed the presence of STOL DNA, in agreement with the previously reported cultivability of this phytoplasma strain (Contaldo et al., 2012 and 2015).

Table 1: Characteristics of the '*Ca*. P. solani' containing samples, RFLP profiles on the four phytoplasma genes employed and lineages obtained

Acronym	Sample from	ORIGIN					Lineages
			tuf	*Stamp*	*secY*	*groEL*	
CHSM1	grapevine	Veneto	B	B	B	B	I
CHSM2	grapevine	Veneto	B	A	A	A	II
2 E2	*H. obsoletus*	Veneto	B	A	A	A	II
H.o 23	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 7	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 9	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 3	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 13	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 19	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
H.o. 17	*H. obsoletus*	Emilia Romagna	B	A	A	A	II
2 E1	*H. obsoletus*	Veneto	B	B	B	A	III

Acronym	Sample from	ORIGIN					Lineages
			tuf	*Stamp*	*secY*	*groEL*	
Aleks. 122/10	grapevine	Serbia	B	B	B	A	III
Bela Cr. 132/10	grapevine	Serbia	B	B	B	A	III
Bela Cr. 134/10	grapevine	Serbia	B	B	B	A	III
Radmil. 191/09	grapevine	Serbia	B	B	B	A	III
Radmil. 192/09	grapevine	Serbia	B	B	B	A	III
3 E	*H. obsoletus*	Veneto	A	A	B	A	IV
Ra9818	grapevine	Emilia Romagna	A	A	A	A	V
Ra 9912	grapevine	Emilia Romagna	A	A	A	A	V
Ra9910	grapevine	Emilia Romagna	A	A	A	A	V
Ra 9830	grapevine	Emilia Romagna	A	A	A	A	V
Ra9801	grapevine	Emilia Romagna	A	A	A	A	V
Ra9804	grapevine	Emilia Romagna	A	A	A	A	V
Ra14486	grapevine	Emilia Romagna	A	A	A	A	V
Ra9709	grapevine	Emilia Romagna	A	A	A	A	V
Ra9707	grapevine	Emilia Romagna	A	A	A	A	V
Ra9802	grapevine	Emilia Romagna	A	A	A	A	V
REV8	grapevine	Emilia Romagna	A	A	A	A	V
REV10	grapevine	Emilia Romagna	A	A	A	A	V
REV13	grapevine	Emilia Romagna	A	A	A	A	V
MOV27	grapevine	Emilia Romagna	A	A	A	A	V
FE9805	grapevine	Emilia Romagna	A	A	A	A	V
FC10044	grapevine	Emilia Romagna	A	A	A	A	V
BO14394	grapevine	Emilia Romagna	A	A	A	A	V

Acronym	Sample from	ORIGIN					Lineages
			tuf	*Stamp*	*secY*	*groEL*	
BO9866	grapevine	Emilia Romagna	A	A	A	A	V
H.o. 2	*H. obsoletus*	Emilia Romagna	A	A	A	A	V
H.o. 16	*H. obsoletus*	Emilia Romagna	A	A	A	A	V
H.o. 5	*H. obsoletus*	Emilia Romagna	A	A	A	A	V
H.o. 4	*H. obsoletus*	Emilia Romagna	A	A	A	A	V
H.o. 7b	*H. obsoletus*	Emilia Romagna	A	A	A	A	V
Ra9827	grapevine	Emilia Romagna	B	B	A	A	VI
FE9806	grapevine	Emilia Romagna	B	B	A	A	VI
FE9810	grapevine	Emilia Romagna	B	B	A	A	VI
BO9870	grapevine	Emilia Romagna	B	B	A	A	VI
BO9867	grapevine	Emilia Romagna	B	B	A	A	VI
Aleks. 125/10	grapevine	Serbia	B	B	A	A	VI
Smeder. 66/11	grapevine	Serbia	B	B	A	A	VI
STOL-CH	periwinkle	France	B	C	C	A	VII
MOL	periwinkle	France	B	C	C	A	VII
STOL-C	periwinkle	France	B	C	C	A	VII
STOL-PO	periwinkle	France	B	C	C	A	VII
ASLO	periwinkle	Slovenia	B	D	C	A	VIII
STOL	periwinkle	Serbia	B	A/B	C/B	A	IX
STOL	Colony	Periwinkle-Serbia	-	A	-	-	*
Bela Cr. 144/10	grapevine	Serbia	B	E	B	A	X
Krčedin 93/10	grapevine	Serbia	B	E	B	A	X
Smeder. 69/11	grapevine	Serbia	B	B	A	B	XI
CHCA1	grapevine	Veneto	-	A	A	A	n.d.
5A	*H. obsoletus*	Veneto	-	A	A	A	n.d.
4 A1	*H. obsoletus*	Veneto	-	A	A	A	n.d.

Acronym	Sample from	ORIGIN					Lineages
			tuf	*Stamp*	*secY*	*groEL*	
H.o. 9	*H. obsoletus*	Emilia Romagna	-	B	A	A	n.d.
4 A7	*H. obsoletus*	Veneto	A	A	-	A	n.d.
CH2	grapevine	Veneto	B	A	-	-	n.d.
PiGru1	grapevine	Veneto	B	A	-	A	n.d.
PiGru2	grapevine	Veneto	B	A	-	A	n.d.
4 A12	*H. obsoletus*	Veneto	B	A	-	-	n.d.
Aleks. 123/10	grapevine	Serbia	B	A	-	A	n.d.
Aleks. 127/10	grapevine	Serbia	B	E	-	A	n.d.
Bela Cr. 130/10	grapevine	Serbia	B	A	B	-	n.d.
Bela Cr. 131/10	grapevine	Serbia	B	B	-	-	n.d.
Bela Cr. 140/10	grapevine	Serbia	B	B	-	-	n.d.
Bela Cr. 142/10	grapevine	Serbia	B	B	B	-	n.d.
Bela Cr. 143/10	grapevine	Serbia	B	B	B	-	n.d.
Bela Cr. 145/10	grapevine	Serbia	B	B	-	-	n.d.
Krčedin 95/10	grapevine	Serbia	B	A	B	-	n.d.
Smeder. 65/11	grapevine	Serbia	B	E	-	-	n.d.
Smeder. 67/11	grapevine	Serbia	B	B	B	-	n.d.
Smeder. 68/11	grapevine	Serbia	B	B	-	-	n.d.
Smeder. 70/11	grapevine	Serbia	B	B	-	-	n.d.

REFERENCES

Angelini, E., Clair, D., Borgo, M., Bertaccini, A. and 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(2): 79-86

Contaldo, N., Duduk, B., Paltrinieri, S., Kolber, M., Ember, I. and Bertaccini, A. 2009: Towards strain differentiation among grapevine bois noir phytoplasmas. Le Progrès agricole et viticole HS:184-5

Contaldo, N., Duduk, B., Paltrinieri, S., Dal Molin, F., Mitrović, J. and Bertaccini, A. 2011: Molecular variability on 16S rDNA of 'bois noir' phytoplasmas in grapevine from Italy and Serbia. Petria 21:136-137

Contaldo, N., Canel, A., Mitrović, J., Paltrinieri, S., Bertaccini, A. and Duduk, B. 2012: Multilocus analyses on grapevine 'bois noir' phytoplasmas from Italy and Serbia. 17th Meeting of ICVG, Davis California, USA, October 7th-14th: 226-227

Contaldo, N., Bertaccini, A., Paltrinieri, S., Windsor, H.M. and Windsor, D.G. 2012: Axenic culture of plant pathogenic phytoplasmas. Phytopathologia Mediterranea 51(3): 607−617

Contaldo, N., Paltrinieri, S., Mori, N., Canel, A., Zamora, L., Duduk, B. and Bertaccini, A. 2013: Are the "bois noir" - associated phytoplasmas molecularly differentiable from stolbur phytoplasmas infecting other species? 3rd European Bois Noir workshop, Barcelona, Spain March 20-21: 59-60

Contaldo, N., Zambon, Y., Satta, E., Paltrinieri, S. and Bertaccini, A. 2015: Cultivation in chemically defined media of phytoplasmas from field-infected grapevine plants showing yellows symptoms. 18th Congress of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG). September 7-11, Ankara, Turkey: 121-123

Fabre, A., Danet, JL. and Foissac, X. 2011: The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection. Gene 472: 37-41

Gundersen, D.E. and Lee, I-M. 1996: Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea 35:114-151

Langer, M. and Maixner, M. 2004: Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA. Vitis 43: 191–199

Lee, I-M., Bottner-Parker, K.D., Zhao, Y., Davis, R.E., Harrison, N.A. 2010: Phylogenetic analysis and delineation of phytoplasmas based on *sec*Y gene sequences. International Journal of Systematic and Evolutionary microbiology 60: 1703-1713

Mitrović, J., Kakizawa, S., Duduk, B., Oshima, K., Namba, S. and Bertaccini A. 2011: The *groEL* gene as an additional marker for finer differentiation of 'Candidatus Phytoplasma asteris' – related strains. Annals of Applied Biology 159: 41-48

Mitrović, J. and Duduk, B. 2012: Variability of groEL gene in stolbur phytoplasmas. Book of Abstracts of the 19th International Congress of the IOM, Toulouse, France: 157-158

Mitrović, J., Contaldo, N., Avramov, Z., Smiljković, M., Bertaccini, A. and Duduk, B. 2013: *GroEL* gene characterization of "bois noir" phytoplasma from Serbia, Bulgaria and Italy. 3rd European Bois Noir workshop, Barcelona, Spain March 20-21: 63-64