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Original scientific paper

OBSERVATION OF GRAPEVINE PHYTOPLASMAS STATUS *(CANDIDATUS PHYTOPLASMA SOLANI)* IN THE REPUBLIC OF NORTH MACEDONIA

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Abstract

Phytoplasmas ("*Candidatus Phytoplasma*") are non-cultivable wall-less bacteria found in plant phloem and insect vector cells. Economically important Grapevine yellows (GY) diseases all over the world are caused by phytoplasmas belonging to the ribosomal subgroups 16SrV-C and -D (etiological agents of Flavescence dorée, FD) and 16SrXII-A ("*Candidatus Phytoplasma solani*", etiological agents of *Bois noir,* BN) which cause similar symptoms but in epidemiology considerably different.

The aim of this study was to identify and characterize the phytoplasmas associated with GY in vineyards in our country, through the molecular analysis of the genes *stamp* and *map*. The obtained results showed the presence of BN *Bois noir*, *stolbur*, economic important phytoplasma that cause serious losses on grapes. In detail, molecular characterization evidenced that BN phytoplasma strains belong to three distinct *stamp* genotypes (St1, St30, St37), while FD phytoplasma is not present in our country yet.

This data will be helpful for further analysis focused on investigating the epidemiology of BN and FD.

Key words: *grapevine yellows, Flavescence doree, Bois Noir, stamp and map genes*

INTRODUCTION

Grapevine yellows (GY), a group of diseases that were originally thought to be caused by viruses, are now known to have a phytoplasma etiology. The first disorder reported from Vitis vinifera, and the most widely known of the GY group, is certainly *Flavescence dorée* (FD), which appeared in South-West France in the 1950's, where from it spread to other viticultural districts of France, Northern Italy and neighbouring European countries. *Bois noir* (BN), which symptoms are indistinguishable from those of FD, was also firstly reported from France, then from the most important viticultural areas of Europe, including Italy (Belli *et al.,* 2010).

Phytoplasmas are plant pathogenic bacteria in the class *Mollicutes* and were formerly called "*mycoplasma - like organisms*" (MLOs) (Doi *et al.*, 1967). They are transmitted by insect vectors (leafhoppers and psyllids) and infect hundreds of plant species worldwide, including many economically important crops, fruit trees, and ornamental plants (Hogenhout et al., 2008; Oshima *et al.,* 2013). More than 700 plant species are affected by phytoplasma diseases and many of them show symptoms such as yellowing, witches broom (proliferating shoots), phyllody (leaf-like petals and sepals), virescence (greening of floral organs), and sometimes withering of plants (Lee *et al.,* 2000).

Phytoplasmal diseases are the primary factors limiting production of many important crops all over the world: since the risk to introduce these diseases by the movement of phytoplasma infected plants (mainly propagating material) precise and strict quarantine regulations are applied in all over the world.

The presence of BN phytoplasmas in North

Macedonia was reported for the first time in 2003, in a survey limited to a small viticultural region, i.e. Veles and Skopje areas (Šeruga *et al*., 2003).

The affected vines show downward rolling of the leaves accompanied by yellow or bright red discoloration of veins and blades, berry withering and uneven or total lack of cane lignification. GY, however, have different phytoplasma species as causal agent, as well as different insect vectors, which are either leafhoppers or planthoppers (Homoptera: Auchenorrhyncha) that feed either specifically or occasionally on the vines. (Belli *et al*., 2010). It seems, that the relationship between diseases and insect vectors is subject that needs to be more deeply investigated for a better understanding of GY epidemiology and for the hopeful development of new sustainable means for their containment. Damages caused by GY may be extensive and economically relevant, since the most of the diseased vines are lost.

The cycle of transmission of phytoplasmas depends on the life cycle (monovoltine, multivoltine) and feeding habit (monophagous, polyphagous) of their insects vectors, polyphagous vectors have the potential to inoculate a wide range of plant species, depending on the resistance to infection of each host plant. (Bosco *et al.*, 1997).

The transmission process consists of three steps, acquisition, latent period, and inoculation. Vectors acquire phytoplasmas by feeding on infected plants for some hours/ days and become inoculative after a latent period of two or more weeks during which the microorganisms multiply in their organs and hemolymph. A method to reduce alternative vector host plants of phytoplasma-infected crop plants and weeds is by roguing. The most effective means of insect vector control is through physical prevention – either by use of screening or by use of a mineral coating on the plant itself.

The primary means of controlling phytoplasma vectors is by insecticides; however, increasing pressure to find less toxic and more biologically based techniques to control, or at least manage, insect vectors necessitates an even greater reliance on solid understandings of the biology of insect vectors from the cellular to the ecological level. (Conti & Alma, 2002).

The activities carried out in this paper were laboratory analyses conducted on Vranec variety in Peshirovo locality (N. Macedonia).

MATERIAL AND METHODS

GY monitoring and sample collection in vineyards

In order to determine the GY associated phytoplasmas (check status of BN and FD phytoplasmas) in the examined vine regions, leaf samples of symptomatic grapevines (*Vitis vinifera* L.) were collected in September and October 2018 in N. Macedonia (Figure 1 a. and b.). In details, leaf samples were collected from several symptomatic plants from the variety Vranec in Peshirovo (Ovce Pole, N. Macedonia) (Table 1).

Figure 1. Collected plant samples from vineyards in Peshirovo, Ovce Pole, N. Macedonia

- Typical symptoms on leaves (triangle form, reddening);
- Mix infection of symptomatology (red spots on leaves and reddening – virus and phytoplasmas symptoms)

Table 1. Selected grapevine leaves samples from Peshirovo, North Macedonia for laboratory analyses.

Location	Variety	Lab code of plant samples
Pesirovo, Sv. Nikole	Vranec, local red variety	BN34
		BN35
		BN36
		BN37
		BN38

Laboratory analysis

Leaf samples collected in vineyards were stored at –20°C, for the following molecular analyses: (i) total nucleic acid extraction; (ii) identification of phytoplasmas by amplification of the genes *stamp* (BN) and *map* (FD); (iii) molecular characterization of phytoplasmas through analysis of nucleotide sequences of the genes stamp and map.

Extraction of total nucleic acids

Total nucleic acids (TNA) were extracted from 1g of plant material using a modification of the cethyltrimethyl-ammonium bromide (CTAB) procedure described by Angelini et al. (2001). Nucleic acids were diluted in sterile deionised water to a final concentration of 20 ng/µl.

Analysis of Nanodrop-Spectrophotometry

The NanoDrop is a spectrophotometer that allows us to quantify nucleic acids from different samples, using micro-volumes and freeing itself from the use of the classic cuvettes. It is composed of an instrumental part and software installed in a computer (Figure 2).

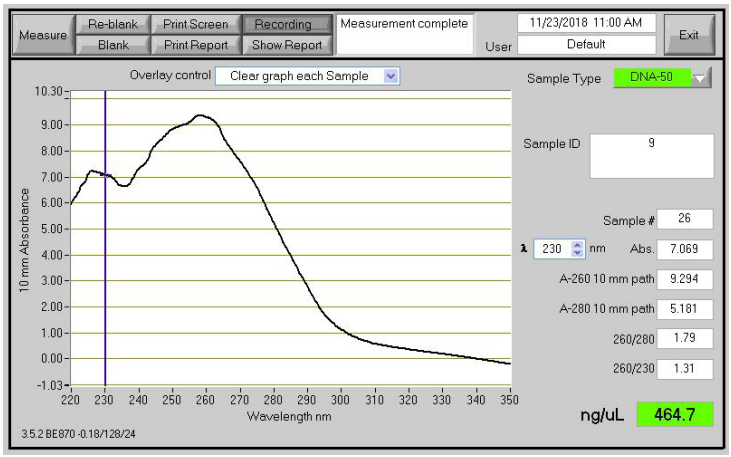

Figure 2. Example of DNA analysis by Nanodrop-spectrophotometer.

Molecular Identification - Polimerase chain reaction PCR

The molecular detection of BN phytoplasma was carried out on the stamp gene (coding the antigenic protein of 'Ca. P. solani' membrane). *Stamp* gene was amplified by nested-PCR using primer pairs: Stamp-F (5'-GTAGGTTTTGGATGTTTTAAG-3') / Stamp-R0 (5'-AAATAAAAGAACAAGTATAGACGA-3'), followed by primers Stamp-F1 (5'-TTCTTTAAACACACCAAGAC-3') / Stamp-R1 (5'- AAGCCAGAATTTAATCTAGC-3') (Fabre et al., 2011). The reaction conditions were as follows: (i) dPCR: 94°C x 4 min; 35 cycles consisting in: 94°C x 30 s, 56°C x 30 s and 72°C x 1 min 30 s; final extension at 72 °C x 7 min; (ii) nested-PCR: 94°C x 4 min; 35 cycles consisting in: 94°C x 30 s, 52°C x 30 s and 72°C x 30 s; final extension at 72 °C x 7 min.

The PCR mix was as follows: Taq 0.125 U / µl; Buffer 1 X; $MgCl_2$ 1.5 mM, Forward example 0.4 µM, Reverse example 0.4 µM, dNTPs 0.2 µM.

All PCR products were analysed by electrophoresis in 1% agarose gel and stained with Midori Green. Electrophoresis was performed using 100 V for 30 minutes. DNA was visualized under UV light on a transilluminator.

The molecular detection of FD phytoplasma was carried out on the *map* gene (coding methionine aminopeptidase). Plant total nucleic acids were employed as templates in nested PCR

assays performed using primer pair: FD9f5 (5'-CAAAAAATTACTTTTGGCGGGAC-3') and MAPr1 (5'-TGCTCAAAATGAGCGCTTAAAC-3'), followed by FD9f6 (5'-GTCGCTTTAGAATCGACACA-3') and MAPr2 (5'-TCGGAAGTAACAGCAGTCCA-3'). Direct and nested PCR conditions were: 1 min at 92°C and 35 cycles, with 1 cycle consisting of 30 s at 92°C, 30 s at 52°C, and 1 min 30 s at 66°C (Arnaud et al., 2007). The PCR mix was as follows: Taq 0.125 U/ml; Buffer 1 X; $MgCl_2$ 1.5 mM, Forward primer 0.4 mM, Reverse primer 0.4 mM, dNTPs 0.2 mM. All PCR products were analyzed by electrophoresis in 1% agarose gel and stained with Midori Green.

Nucleotide sequence analysis

The PCR products of *stamp* and *map* genes, amplified by vine plants, were sequenced at a commercial service (Eurofins Genomics, Germany) with a minimum coverage of 3X. The obtained nucleotide sequences, whose quality has been evaluated through electropherogram analysis, have been assembled with the CAP (Contig Assembly Program) function of the BioEdit software, version 7.2.5 (Hall, 1999). In order to characterize the phytoplasma strains, the nucleotide sequences of the *stamp* and *map* genes were inserted into a database containing the sequences of the representative strains of the stamp gene variants of *'Ca. P. solani'* and the map gene variants of phytoplasmas associated with FD, available in GenBank (Quaglino et al., 2016; Casati et al., 2017)

The sequences have been aligned through the "ClustalW Multiple Alignment" function of the Bioedit software. The alignments obtained were used to calculate the sequence identity with the "Sequence identity Matrix" function. Based on the sequence identity of the stamp and map genes the phytoplasmas were inserted into gene variants already known or proposed for the first time in this work. In addition, the alignments were used for the subsequent phylogenetic analysis conducted with the MEGA6 software (Tamura et al., 2013), using the 'neighbour-joining' algorithm (bootstrap 1000).

RESULTS AND DISCUSSION

Identification of GY phytoplasmas

PCR amplification showed that: (i) three (BN34, BN36, BN38) out of five Vranec plants from Macedonia were infected by BN phytoplasma, and FD phytoplasma was not detected in tested samples (Figure 3).

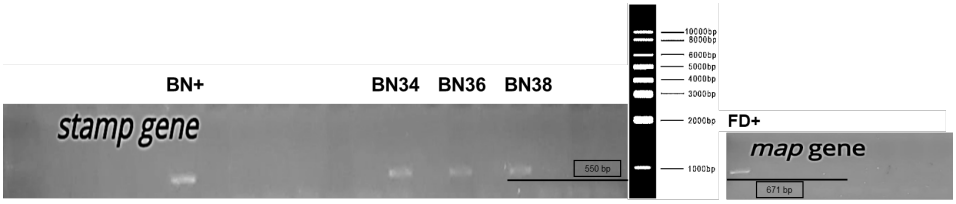

Figure 3. Visualization of PCR products obtained through the amplification of *stamp* and *map* genes.

Table 3. BN and FD phytoplasma identification in grapevines.

Locality	Variety	Sample	Date of sampling	PCR	
				stamp (BN)	map (FD)
Peshirovo, Ovce Pole	Vranec	BN34	IX.2018	+	-
		BN35	IX.2018	-	-
		BN36	IX.2018	+	-
		BN37	IX.2018	-	-
		BN38	IX.2018	+	-

Molecular characterization

Analysis of the gene *stamp*

Sequence identity analysis of *stamp* gene nucleotide sequences showed that BN phytoplasma strains identified in grapevine BN34, BN36 and BN38 are characterized by distinct *stamp* gene variants. In detail, strains identified in plants BN34 and BN38 have stamp variants undistinguishable from the variants St1 and St30, respectively. The strain infecting the plant BN36 showed best identity with sequence variants St4, St15 and St37 (Table 4, 5).

Table 4. Sequence identity matrix of the *stamp* gene. Comparison between the gene variants was found. The identical gene variants are indicated by the same colour.

	Seq->	1	2	3	4	5	6	7	8	18	19	32	33	34	39	40
1	BN34	ID	0.964	0.997	1	0.987	0.952	0.962	0.979	0.962	0.956	0.927	0.997	0.985	0.952	0.964
2	BN36	0.964	ID	0.966	0.964	0.976	0.987	0.995	0.968	0.995	0.978	0.946	0.966	0.978	0.985	0.995
3	BN38	0.997	0.966	ID	0.997	0.985	0.954	0.964	0.977	0.964	0.954	0.925	1	0.983	0.954	0.966
4	Rqg50_(KC703019)_St1	1	0.964	0.997	ID	0.987	0.952	0.962	0.979	0.962	0.956	0.927	0.997	0.985	0.952	0.964
5	Rqg31_(KC703017)_St2	0.987	0.976	0.985	0.987	ID	0.964	0.974	0.991	0.974	0.968	0.938	0.985	0.997	0.964	0.976
6	Rpm35_(KC703015)_St3	0.952	0.987	0.954	0.952	0.964	ID	0.983	0.955	0.983	0.974	0.938	0.954	0.966	0.985	0.983
7	STOL_(FN813261)_St4	0.962	0.995	0.964	0.962	0.974	0.983	ID	0.966	0.995	0.978	0.944	0.964	0.976	0.981	0.991
8	GGY_(FN813256)_St5	0.979	0.968	0.977	0.979	0.991	0.955	0.966	ID	0.97	0.964	0.942	0.977	0.989	0.955	0.972
18	P7_(FN813258)_St15	0.962	0.995	0.964	0.962	0.974	0.983	0.995	0.97	ID	0.974	0.948	0.964	0.976	0.981	0.995
19	L973_(FN813255)_St16	0.956	0.978	0.954	0.956	0.968	0.974	0.978	0.964	0.974	ID	0.942	0.954	0.968	0.976	0.974
32	Vv12_274_(KJ469717)_St29	0.927	0.946	0.925	0.927	0.938	0.938	0.944	0.942	0.948	0.942	ID	0.925	0.936	0.934	0.946
33	Vv24_(KC703022)_St30	0.997	0.966	1	0.997	0.985	0.954	0.964	0.977	0.964	0.954	0.925	ID	0.983	0.954	0.966
34	Rqg42_(KC703016)_St31	0.985	0.978	0.983	0.985	0.997	0.966	0.976	0.989	0.976	0.968	0.936	0.983	ID	0.964	0.978
39	Carv2_(KT184880)_St36	0.952	0.985	0.954	0.952	0.964	0.985	0.981	0.955	0.981	0.976	0.934	0.954	0.964	ID	0.981
40	Char7_(KT184881)_St37	0.964	0.995	0.966	0.964	0.976	0.983	0.991	0.972	0.995	0.974	0.946	0.966	0.978	0.981	ID

Table 5. *Stamp* and *map* sequence variants identified in BN and FD phytoplasma strains identified in this study.

		PCR		Variety of sequence	
	samples	*stamp*	*map*	*stamp*	*map*
North Macedonia Pesirovo	BN34	+	-	St1	-
	BN35	-	-	-	-
	BN36	+	-	St4, St15, St37	-
	BN37	-	-	-	-
	BN38	+	-	St30	-

Phylogenetic analysis

Phylogenetic analysis of *stamp* gene alignment showed the presence of four main clusters: cluster a, related to nettle epidemiology (nettle-related), and clusters b-I, b-II and b-III, related to bindweed epidemiology (bindweed-related). The BN phytoplasma strains identified in Vranec variety from North Macedonia grouped in the clusters b-II and b-III (Figure 4).

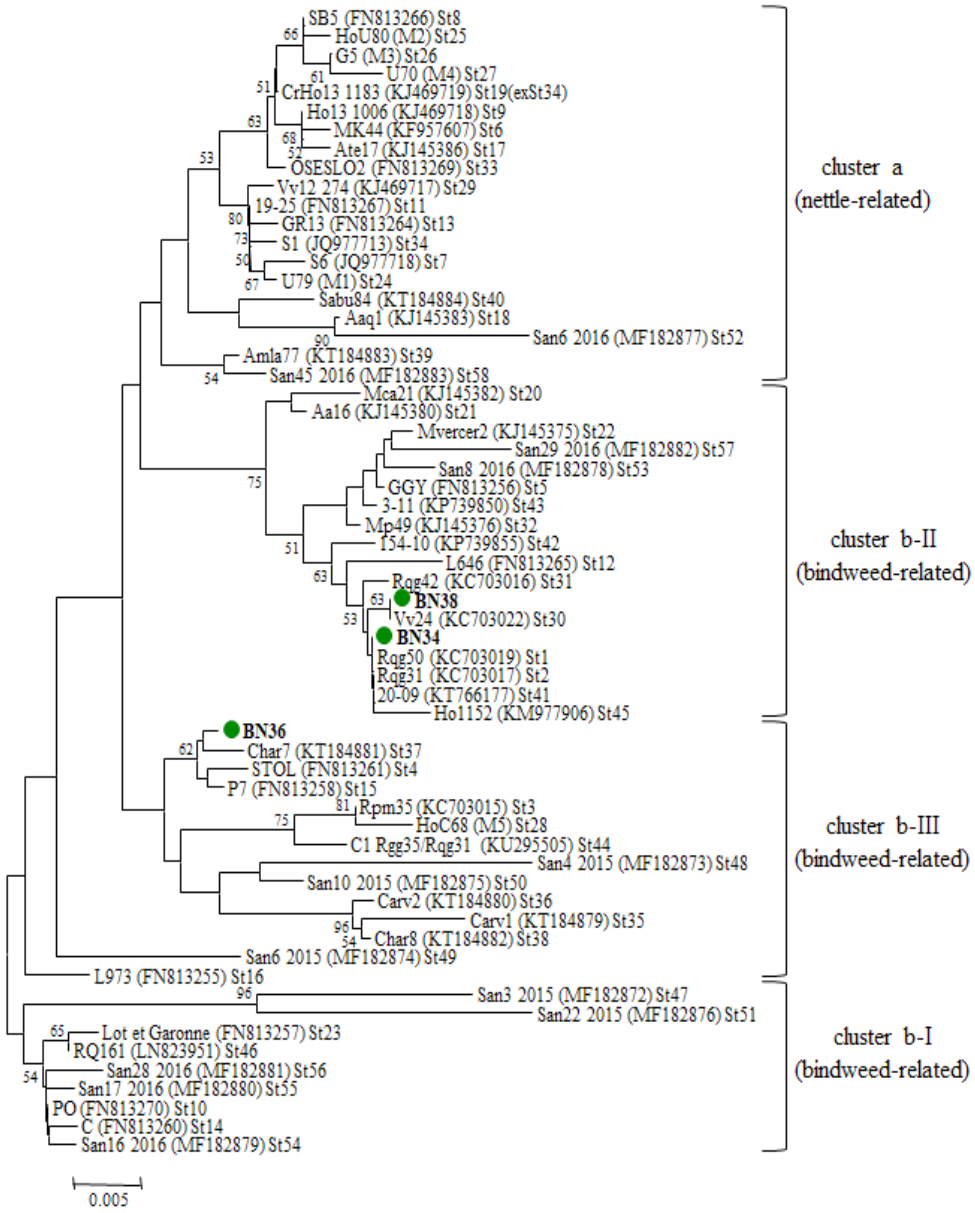

Figure 4. Phylogenetic tree built on the basis of the nucleotide sequences of the stamp gene.

CONCLUSION

The results obtained in this thesis have confirmed the prevalence of *'Ca. P. solani'*, the etiological agent of BN, in N. Macedonia (Kostadinovska et al., 2014), and the absence of FD. This evidence could suggest that such varieties could be less susceptible to phytoplasmas associated with GY.

Molecular characterization by the analysis of *stamp* gene nucleotide sequences showed the presence of distinct variants (St1, St30, St37) among BN phytoplasmas identified in North Macedonia. These variants were largely identified, in previous studies, in Macedonia, Serbia, Croatia and Georgia (Mitrev et al., 2008, Cvrkovic et al., 2014; Kostadinovska et al., 2014; Quaglino et al., 2016). BN epidemiology involves a broad range of plant hosts and insect vectors (Marcone et al., 1997; Schneider et al., 1997; Mori et al., 2015).

This study, for the first time observed a complete laboratory analyzes for BN and FD phytoplasmas, including molecular identification, nucleotide sequence analysis and phylogenetic analyzes which group BN phytoplasma in clusters b-II and b-III.

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ИСПИТУВАЊЕ НА ФИТОПЛАЗМАТСКИОТ СТАТУС КАЈ ВИНОВАТА ЛОЗА (*CANDIDATUS PHYTOPLASMA SOLANI*) ВО РЕПУБЛИКА СЕВЕРНА МАКЕДОНИЈА

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Резиме

Фитоплазмите (*Candidatus Phytoplasma*) се безклеточни форми на бактерии кои не се изолираат на хранлива подлога, и кои се наоѓаат во растителниот флоем и во клетките на инсектите вектори. Економското значење на болестите предизвикани од „жолтилата кај виновата лоза" *Grapevine yellows* (GY) е големо насекаде во светот и најчесто распространетите фитоплазми припаѓаат на рибозоналните подгрупи 16SrV-C и -D (етиолошки причинител *Flavescence dorée*, FD фитоплазмата) и 16SrXII-A (*Candidatus Phytoplasma solani,* етиолошки причинител *Bois noir*, BN фитоплазмата). Овие две групи на фитоплазми предизвикуваат слични симптоми, но разликите се забележуваат во епидемиологијата.

Целта на ова истражување беше идентификација и карактеризација на фитоплазмите кои припаѓаат на GY групата („жолтила") во лозовите насади во нашата земја, со помош на молекуларна анализа на геновите *stamp* и *map*. Добиените резултати од истражувањето го докажаа присуството на BN *Bois noir*, *stolbur*, економски значајна фитоплазма која предизвикува значителни економски загуби кај грозјето. Од деталните истражувања, преку молекуларна карактеризација на видовите од BN фитоплазмата го потврдивме присуството на три различни *stamp* генотипови (St1, St30, St37), додека FD фитоплазмата сè уште не е потврдена во нашата земја.

Оваа дата база на податоци добиени од нашето истражување може да биде корисна за идни анализи фокусирани на испитување на епидемиологијата на BN и FD.

Клучни зборови: *„жолтила кај виновата лоза", Flavescence doree, Bois Noir, stamp и map гени*