

# Phytoplasma disease “Bois noir” in Crimea: diagnosis of the pathogen

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**Abstract.** In 2018-2019, grape leaf samples with symptoms of yellowing (reddening) and leaf rolling collected from grapevines of different regions of Crimea were tested for the presence of phytoplasma using the “nested”-PCR with primer pairs P1/16S-Sr and R16F2n/R16R2. Phytoplasmas were detected in 91% grape samples of 2018 and 46% samples of 2019. Genotyping performed by restriction fragment length polymorphism analysis for three genes: ribosomal (16S rRNA), the *tuf* gene encoding the elongation factor EF-Tu, and the *vmp1* gene encoding a membrane protein, showed that the phytoplasmas isolated from all samples were related to the species *Candidatus* Phytoplasma solani, type “b”. Polymorphism was observed only for the *vmp1* gene. The electrophoretic profiles of grape phytoplasma of 2018 (‘Chardonnay’ variety) differed from those of grape phytoplasma of 2019 (‘Pinot Noir’ and ‘Verdelho’ varieties), as well as from the profiles published in well-known works, which indicates the genetic diversity.

## Introduction

Yellows caused by phytoplasmas are among the most common and harmful diseases of grapes. Phytoplasmas are bacteria without a cell wall. They parasitize in cells of plant phloem and are carried by insects of *Hemiptera* order, *Auchenorrhyncha* and *Sternorrhyncha* suborders. Some of species within the family are vectors, others are not. Not all of individuals and species with identified phytoplasma can be the competent vectors [1, 2].

The most dangerous phytoplasma diseases in the main grape growing areas of Europe are the Grapevine Flavescence Dorée (FD) and the Bois Noir Phytoplasma (BN). Both diseases are common in the same European countries of viticulture, but FD is a quarantine object. This disease caused by phytoplasma of the group 16SrV has not been identified in Russia yet. BN is associated with phytoplasma of the stolbur group, subgroup 16SrXII-A - *Candidatus* Phytoplasma solani, the symptoms of which are yellowing or reddening of leaves and leaf blade rolling inwards are similar with FD. In nature the BN is transferred to

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grapes from field bindweed (*Convolvulus arvensis* L.), hedge bindweed (*Calystegia sepium* (L.) R.BR.), greater nettle (*Urtica dioica* L.) or planting material infected with planthoppers *Hyalesthes obsoletus* Sign., *Reptalus panzeri* Löw and others that are distributed in all viticultural areas around the world [2-9].

Stolbur is a dangerous disease of tomatoes and other crops of the Solanaceae family known since the 1930s in the South of Russia, but it has never been registered on grapevine cultivars before. However, since the second decade of the XXI century, grapevine Bois Noir Phytoplasma has become a real danger to the vineyards of Russia [3, 4, 10]. The reason of its distribution may be the introduced planting material, since the pathogen was first-ever discovered in such material in Crimea [3]. Therefore, it is so important to conduct a detailed study of phytoplasma genotypes peculiar for the regional flora and the corresponding local vectors.

The aim of the research was to determine the taxonomic inhering of the pathogen causing yellowing and rolling of grape leaves in Crimea.

Commonly used for detection of phytoplasma of a particular group highly conservative 16S rRNA gene, is not suitable for a detailed study of genetic diversity and phylogenetic relationships of isolates, as well as the relationships between isolates, host and geographical distribution, since the genetic variability within the stolbur subgroup, which is detected based on RFLP analysis of this gene, is very low. To obtain information on the molecular variability of stolbur phytoplasma isolates, causing diseases of grape, other crops and wildlife plant species, the researchers began to use nonribosomal genes, more suitable to differ and classify closely related phytoplasmas due to their greater variability, including the constitutive *tuf* gene – a gene that codes elongation factor EF-Tu (elongation factor Tu), which, like the 16S rRNA, is a highly conservative gene, often used to differ and classify phytoplasmas. In 1997 Schneider et al. designed two pairs of primers that could be used in the direct reaction of sequence amplification of the *tuf* gene of group majority of phytoplasmas fTuf1 / rTuf1, and several internal primers for the “nested” PCR. With the aid of one of these pairs, namely: ftufAY / rtufAY, it was possible to distinguish three strains (a, b and c) inside the 16SrXII-A stolbur subgroup after RFLP analysis using *HpaII* for endonuclease restriction.

The parameter of stolbur genetic diversity significantly increases if the analysis of encoding a membrane protein gene *vmp1* is carried out. It is considered that the variability of the *vmp1* may be the result of protein location on the cell surface, therefore it can play a certain role in the interaction of phytoplasma with the host. Genes encoding the surface proteins show greater variability than the rest of the genome, and therefore they can present suitable markers for molecular epidemiology. Based on PCR RFLP analysis using *RsaI* endonuclease restriction in the area of the European Mediterranean, 23 *vmp1* genotypes were identified on different cultures and wild-growing grass plants [11].

## Materials and methods

Studies were carried out during 2018-2019. Grapevine leaf samples with Bois Noir disease symptoms were collected by staff scientists of the Laboratory of Plant Protection of FSBSI Institute Magarach of the RAS on 25<sup>th</sup> and 30<sup>th</sup> of July, 2018; 8<sup>th</sup> of July, 2<sup>nd</sup> and 9<sup>th</sup> of August, 2019.

The leaves were flattened out and dried under a small press between layers of filter paper at a temperature of 27-30 °C. Central and lateral leaf ribs were deleted, dry material was finely chopped with scissors to the homogeneous condition (about 0.5 mm) and stored in paper bags at a room temperature.

The total DNA was isolated from 50-100 µg of dry material according to the method of Maixner et al., described in detail by Marcone [12]. Phytoplasma DNA was detected using

«nested» PCR with a pair of primers P1/16S-SR in the first amplification and R16F2n/R16R2 – in the second. The resulting products were treated with restriction endonucleases, DNA fragments were separated using electrophoresis, and RFLP-profiles were used to identify a group (subgroup) of phytoplasmas [13, 14]. Samples that gave a positive result for the presence of 16Sr gene, being a part of 16SrXII-A subgroup, were genotyped for nonribosomal *tuf* and *vmp1* genes. To amplify the *tuf* gene, we completed a PCR with primers fTuf1 and rTuf1 in direct PCR and with primers fTufAY and rTufAY- in the “nested” one. Restriction was performed with endonucleases *AluI*, *HpaII*, and *Sau3AI*. A fragment of *vmp1* gene was amplified using PCR with primers Stol H10F1 and Stol H10R1 in direct PCR and TYPH10F and TYPH10R in the “nested” one [15]. The final PCR product (1.4 Kb) was subject to *RsaI* endonuclease restriction.

## Results and discussion

In 2018, 23 samples of the most sensitive to phytoplasma infection ‘Chardonnay’ grape variety were tested for the presence of phytoplasma. Grape leaves with symptoms of disease were collected from different parts of vineyards of the South Coast of Crimea (SCC). In DNA isolated from 19 samples, the 1.2 Kb amplicon was detected directly in the reaction mixture obtained after the “nested” PCR. Four samples gave negative result. Considering that the reason could be the inhibition of PCR with admixtures contained in DNA solution, these samples were re-amplified using ten-fold diluted total DNA as a templet for direct PCR. Two samples were positive, in other two the target product was not detected (Table 1).

**Table 1.** Efficiency of sequence amplification of phytoplasma DNA from different grape varieties with primers, specific to *16Sr*, *tuf* and *vmp1* genes

S No.	Grape variety	Quantity of samples infected/tested		
		<i>16Sr</i> gene	<i>Tuf</i> gene	<i>Vmp1</i> gene
1	‘Chardonnay’	21/23	19/19	16/19 (63%)
2	‘Pinot Noir’	4/6	3/4	¼
3	‘Aligote’	0/2	NT	NT
4	‘Merlot’	½	0/1	0/1
5	‘Bastardo’	0/1	NT	NT
6	‘Verdelho’	½	1/1	1/1

Remark: NT — not tested

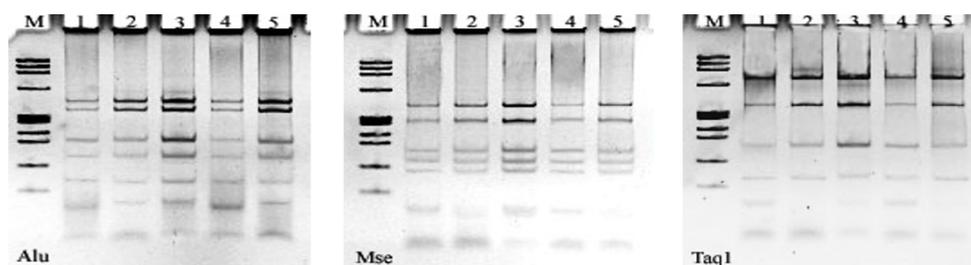
In 2019, 13 grape samples of five varieties were tested for the presence of phytoplasma DNA, collected from three regions of Crimea: ‘Pinot Noir’, ‘Aligote’ and ‘Merlot’ (Bakhchisaray district of Crimea), ‘Bastardo’ and ‘Pinot Noir’ (Sevastopol city) and ‘Verdelho’ (SCC, Yalta). Two samples of weed plants were also tested – field bindweed (*Convolvulus arvensis* L.) and clematis (*Clematis vitalba* L.), collected in a vineyard (SCC, Yalta). Only 6 out of 13 samples were positive (Table 1). Of the 4 tested samples of ‘Pinot Noir’ grape variety (Bakhchisaray district of Crimea), phytoplasma was found in 3 samples, as well as in both 2 tested samples of ‘Merlot’ variety. DNA of phytoplasma was not found in 2 samples of ‘Aligote’ grapes from the same region.

Phytoplasma was not detected in a sample of ‘Bastardo’ grapes, presence of pathogen was also seen in only one asymptomatic specimen out of 2 samples of ‘Pinot Noir’ grapes (Sevastopol city). Among 2 samples of ‘Verdelho’ grapes, one contained phytoplasma DNA, detected only after additional purification: dissolution in a citrate buffer containing 4M guanidine thiocyanate and 0.5% lauryl sarcosinate, followed by precipitation with

isopropanol. Phytoplasma was not found in samples of weed plants from this vineyard (field bindweed and grape-leaved clematis).

An attempt to use ten-fold diluted DNA in direct PCR for samples in which it was not possible to detect the presence of phytoplasma DNA was unsuccessful.

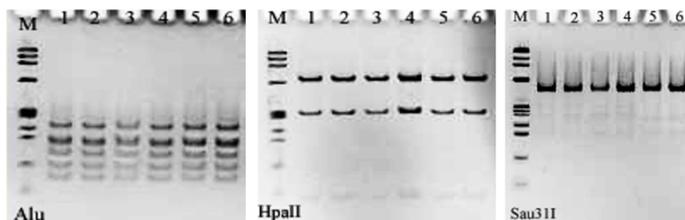
As a rule, up to 17 endonucleases are used to identify the group pertain of phytoplasmas using RFLP analysis. Since it is known that the Bois Noir disease is caused by phytoplasma of a stolbur group, in order to identify phytoplasma found in samples, it was enough to analyze the electrophoregrams obtained after amplicon restriction of the *16Sr* gene (1.2 kb) by three restriction endonucleases – *AluI*, *MseI* (*TruII*) and *TaqI*. Restriction of DNA by the endonuclease *AluI* allows the phytoplasma found in grapes to be attributed to the subgroup 16SrI-A or subgroup 16SrXII-A. The profile of fragments obtained with endonuclease restriction *MseI* (*TruII*) and *TaqI* excludes the presence of 16SrI-A subgroup and confirms the belonging of phytoplasma to the stolbur group, the 16SrXII-A subgroup. Figure 1 shows typical profiles of DNA fragments obtained as a result of restriction by three endonucleases, specific for all grape samples of 2018 (21 samples) and 2019 (6 samples).



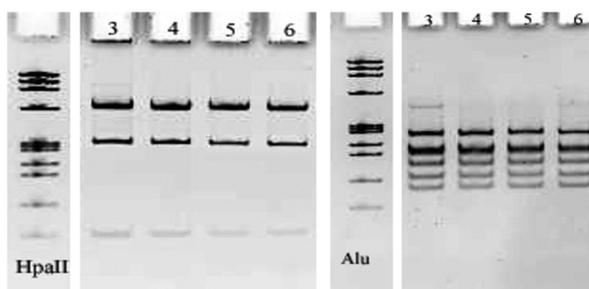
**Fig. 1.** Typical electrophoretic profiles of DNA amplicon fragments of the *16Sr* phytoplasma gene isolated from grape leaves and obtained under the influence of restriction endonucleases *AluI*, *MseI* and *TaqI* in 5% PAGE. M - marker of molecular weight ØX174 DNA / *BsuRI* (*HaeIII*) (Fermentas, Lithuania), fragment size (bp): 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72

To check the presence of the *tuf* gene, we analyzed 19 samples of ‘Chardonnay’ grape variety of 2018 and 6 samples of different varieties of 2019 crop year, tested positive for the presence of *16Sr* gene. All 19 samples of 2018 and 4 samples of 2019 gave positive result.

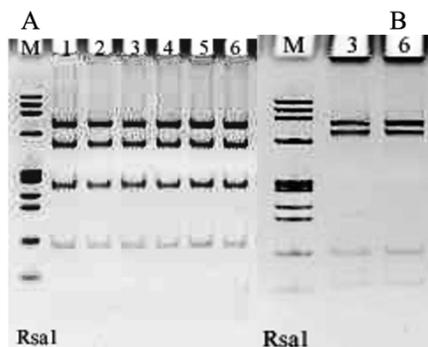
Restriction analysis of the *tuf* gene amplicons by endonucleases *AluI*, *HpaII* and *Sau3AI* in 5% PAGE of phytoplasma from grape varieties ‘Chardonnay’ of 2018, ‘Pinot Noir’ and ‘Verdelho’ of 2019 is presented in Figures 2 and 3 (in Fig. 3 the *Sau3AI* restricts are not shown, as similar to those in Fig. 2). It is known that electrophoregrams obtained with endonuclease restriction *HpaII* make it possible to distinguish three types of the *tuf* gene – a, b, and c. Comparison of the profiles shown in Figures 2 and 3 with the published electrophoregrams allows to include the *tuf* phytoplasma gene of Crimean grapes in the group of “b” type [15].



**Fig. 2.** Typical electrophoretic profiles of amplicon fragments of the *tuf* gene of phytoplasma DNA isolated from grape leaves of ‘Chardonnay’ variety in 2018, as a result of action of restriction endonucleases *AluI*, *HpaII* and *Sau3AI*. 1-6 – samples (a total of 19 samples with similar profiles). M - marker of molecular weight ØX174 DNA / *BsuRI* (*HaeIII*) (Fermentas, Lithuania), fragment size (bp): 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72



**Fig. 3.** Electrophoretic profiles of amplicon fragments of the *tuf* gene of phytoplasma DNA isolated from grape leaves of different varieties in 2019, after processing with restriction endonucleases *AluI* and *HpaII*: 3 and 4 – samples of ‘Pinot Noir’ grape variety (Crimea, Bakhchisaray district); 5 - sample of ‘Pinot Noir’ grape variety (Crimea, Sevastopol); 6 - sample of ‘Verdelho’ grape variety (Crimea, Yalta). M - marker of molecular weight is the same as in Figures 1 and 2



**Fig. 4.** Electrophoretic profiles of amplicon fragments of the *vmp* gene of phytoplasma DNA isolated from: A grape leaf samples of ‘Chardonnay’ variety of 2018, B – samples of ‘Pinot Noir’(3) and ‘Verdelho’ (6) varieties after processing with restriction endonuclease *RsaI*. M - marker of molecular weight is the same as in Figures 1 and 2

The number of sixteen out of nineteen samples of 2018, infected with *Ca. Phytoplasma solani* (‘Chardonnay’ variety) tested positive with primers for the *vmp* gene, and only two samples from six of 2019 crop year (‘Pinot Noir’ and ‘Verdelho’ cultivars) were also positive. However, the electrophoretic profiles obtained as a result of RFLP analysis using endonuclease restriction *RsaI* of *vmp1* phytoplasma genes from ‘Chardonnay’ grapes in 2018 differed significantly from the profiles of ‘Pinot Noir’ and ‘Verdelho’ grapes of 2019 crop year (Figure 4).

Thus, the phytoplasmas found in the Crimean vineyards in 2018 and 2019 belonged to the *Ca. Phytoplasma solani* species. Genotyping for the *tuf* gene, which included RFLP analysis using the restriction endonuclease *HpaII*, showed that they belong to the same type of this gene – type 5 “b”, which is a characteristic of phytoplasma genotypes found in the countries of Eastern Europe. Analysis of the *vmp1* gene revealed two different genotypes: the first is a characteristic of all samples collected in 2018, the second – for samples of 2019. The efficiency of amplifying the DNA sequence with primers specific for the *tuf* and *vmp1* genes was generally lower than with primers for ribosomal *16S*r genes, as was also confirmed by other researchers [16]. In the case of the *vmp1* gene, this difference was even more significant. We failed to find absolutely identical profiles of the *RsaI* gene endonuclease restriction identified by us in the works of other authors.

## Conclusion

Samples of grape leaves, with symptoms of yellowing or reddening and leaf rolling, collected from grapevine cultivars in various regions of Crimea in 2018 and 2019, were analyzed for the presence of phytoplasma. Phytoplasmas were detected in 91% grape samples of 2018 and in 46% of 2019. Genotyping performed using polymorphism analysis of restriction fragment length for three genes: ribosomal (*16S* rRNA), the *tuf* gene encoding the elongation factor EF-Tu, and the *vmp1* gene encoding a membrane protein, showed that phytoplasmas isolated from all samples belonged to the species *Candidatus Phytoplasma solani*, type “b”. Polymorphism was observed only for the *vmp1* gene. The electrophoretic phytoplasma profiles of 2018 (‘Chardonnay’ variety) differed from those of 2019 (‘Pinot Noir’ and ‘Verdelho’ varieties), and also differed from the profiles published in well-known works, indicating the genetic diversity. At the same time, only two types of nucleotide sequence of the *vmp1* gene have been identified in Crimea so far based on the length polymorphism of restriction fragments during the *RsaI* endonuclease restriction.

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