

## Molecular identification of a phytoplasma infecting grapevine in the Republic of Macedonia

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### Summary

**Phytoplasmas from the ribosomal subgroup 16SrXII-A (stolbur) were identified by PCR and RFLP analyses of 16S rRNA gene and elongation factor-Tu phytoplasma gene (*tuf*) in symptomatic grapevines from the Veles and Skopje areas in Macedonia. Two methods were used for nucleic acid extraction. Amplification and RFLP results of 16S rDNA were not influenced by the method used, but RFLP patterns of *tuf* gene revealed additional bands if a less time consuming method was applied. The possible origin of these bands is discussed. This is the first report of the presence of the stolbur phytoplasma in cvs Chardonnay and Vranac in Macedonia.**

**Key words:** grapevine yellows, PCR, RFLP, stolbur, *tuf*, *Vitis vinifera* L., 16S rDNA.

Some other phytoplasma sequences, *e.g.* the gene for the elongation factor-Tu (*tuf*), other ribosomal genes, 23S rDNA and 16/23S rDNA intergenic region, as well as non-ribosomal DNA sequences are used as alternatives or are combined with 16S rDNA analyses to allow the phytoplasma distinction (GUNDERSEN *et al.* 1996; SMART *et al.* 1996; VIBIO *et al.* 1996; BOUDON-PADIEU *et al.* 1997; DAIRE *et al.* 1997a; SCHNEIDER *et al.* 1997; LEE *et al.* 1998; MARCONE *et al.* 2000).

In September 2001, symptoms resembling GY were observed in vineyards of the Skopje area (cv. Chardonnay) as well as in the Veles area (cvs Vranac and Chardonnay). The aim of this study was to present molecular evidence for the occurrence of phytoplasmoses in Macedonian vineyards and to characterize the pathogen by comparing RFLP fragments of its 16S rDNA and *tuf* gene to those of phytoplasma groups and subgroups which have already been described.

### Introduction

Phytoplasmas are endocellular bacteria from the class *Mollicutes* that cause numerous plant diseases (McCoy *et al.* 1989), including grapevine yellows (GY) diseases affecting various grapevine cultivars throughout the world (KUSZALA *et al.* 1993; PRINCE *et al.* 1993; MAIXNER *et al.* 1995; ALMA *et al.* 1996; PADOVAN *et al.* 1996; ŠERUGA *et al.* 2000, ČURKOVIĆ PERICA *et al.* 2001). The etiological agents have been attributed to phytoplasmas of different 16S rRNA RFLP groups, namely: 16SrI (aster yellows and related strains), 16SrIII (X-disease and related strains), 16SrV (elm yellows and related strains) and 16SrXII (stolbur and related strains). A number of authors have demonstrated mixed infections in GY-affected vines with phytoplasmas from different groups or subgroups (BERTACCINI *et al.* 1995; ALMA *et al.* 1996).

Nowadays, phytoplasmas are mainly identified by means of polymerase chain reaction (PCR) with the application of phytoplasma generic primers for 16S rRNA gene and/or primers specific for phytoplasma groups and subgroups in nested or direct PCR experiments (LEE *et al.* 2000). The latter primers are also useful for the classification of phytoplasmas. The approach that uses RFLP analysis of the 16S rRNA amplicons forms a basis for the latest phytoplasma classification scheme.

### Material and Methods

**Plant samples and phytoplasma strains:** Leaf and cane samples were taken from symptomatic vines in October 2001 and September 2002 (3 Chardonnay and 4 Vranac vines), 14 samples in total. Chardonnay samples were collected in the Skopje area while Vranac samples were collected in the Veles area.

Standard phytoplasma strains that were included in all PCR and RFLP reactions were: PG3 = Croatian (16SrXII-A) stolbur strain from grapevine (ŠKORIĆ *et al.* 1998), Hyd-B = Belgian (16SrI-B) aster yellows strain causing Hydrangea phyllody and KVI = Italian (16SrIII-B) clover yellows strain causing Clover phyllody (BERTACCINI *et al.* 2000).

**Nucleic acid extraction and PCR amplification:** Two procedures were used for nucleic acid extraction. In the first one, total nucleic acid was extracted from approximately 1 g of leaf midribs and phloem scrapings according to the procedure described by PRINCE *et al.* (1993). This procedure was always used for the isolation of nucleic acid from standard phytoplasma strains. The second, less time consuming procedure in which 0.5 g of leaf midribs and phloem scrapings were used, followed the protocol of DAIRE *et al.* (1997 a) with some modifications introduced by ŠERUGA *et al.* (2003). In both procedures plant nucleic acid was di-

luted in sterile deionized water to 20 ng· $\mu\text{l}^{-1}$  and 1  $\mu\text{l}$  of this solution was used in PCR assays.

Amplification of phytoplasma 16S rRNA gene was performed in a direct PCR by using R16F1/R0 (LEE *et al.* 1995) universal phytoplasma primer pair. The nested PCRs were performed by using R16F2/R2 (LEE *et al.* 1993), 16R<sub>738F</sub>/R<sub>1232r</sub> (GIBB *et al.* 1995) and R16(I)F1/R1 primers (LEE *et al.* 1994), as described by ŠERUGA *et al.* (2000). Each reaction was performed in a total volume of 25  $\mu\text{l}$  containing 2.5  $\mu\text{l}$  of 10x PCR buffer, 1.5 mM MgCl<sub>2</sub>, 200  $\mu\text{M}$  of each dNTP (Eppendorf, Hamburg, Germany), 0.625 U of Taq polymerase (Eppendorf) and 0.2  $\mu\text{M}$  of each primer. Tubes containing water instead of a template DNA were included in each PCR experiment as negative controls.

Amplification of a major portion of the *tuf* gene coding for the elongation factor Ef-Tu was performed using the fTufu/rTufu primer pair (SCHNEIDER *et al.* 1997). PCR was performed as previously described by SCHAFF *et al.* (1992) with the exception of annealing temperature (55 °C in our experiments). Reaction mixture was prepared as for the amplification of 16S rDNA, with a difference in primer concentration which was 0.4  $\mu\text{M}$  instead of 0.2  $\mu\text{M}$  for each primer.

All PCRs were performed in an Applied Biosystems-GeneAmp PCR Sys 2700 cyclor. Amplicons were subjected to electrophoresis through 1 % agarose gels, stained with ethidium-bromide and examined on an UV-transilluminator at 312 nm.

**Restriction fragment length polymorphism (RFLP) analyses:** Amplified portions of 16S rDNAs (~200 ng) from all nested PCR assays were subjected to digestion with restriction enzymes *MseI* (=Tru9I), *AluI* and *KpnI* (Roche Diagnostics, Mannheim, Germany), while the amplified fragments of the *tuf* gene were digested with *Tru9I*, *Sau3AI*, *MaeI* (=BfaI) (Roche Diagnostics) and *TspEI* (=Tsp509I, *TasI*) (Fermentas, Vilnius, Lithuania). Each digestion was carried out with 5 U of enzyme according to the instructions given by the manufacturer. After digestion, DNA fragments were separated in 5 % polyacrylamide gels in 1X TBE buffer (0.09 M Tris-borate, 0.002 M EDTA) and stained with ethidium bromide.

## Results

All sampled vines exhibited severe grapevine yellows symptoms. On cv. Vranac the first symptoms usually appeared at the end of June as a light reddening along the main veins of the leaves that gradually became more intense. Dessication of inflorescences or young clusters also occurred as well as necrosis along the veins later in the season. The shoots matured irregularly or remained greenish until the end of the vegetative period and usually were killed by frost in winter. For Chardonnay vines, symptoms were clearly recognizable and typical for GY.

Amplification of phytoplasma 16S rDNA was not influenced by the nucleic acid extraction method. No matter which procedure was used, all grapevine samples gave strong 1.25 kbp amplification signals in the first nested PCR experiment, primed by a generic phytoplasma primer pair R16F2/R2, probably indicating relatively high concentration of

phytoplasmas in the phloem. Positive results obtained with a primer pair R16(I)F1/R1, specific for the groups 16SrI and 16SrXII (results not shown), as well as the restriction profiles which were identical to the stolbur (bois noir) reference strain PG3 (Fig. 1) enabled us to place phytoplasmas from all samples in the same ribosomal subgroup 16SrXII-A. RFLP patterns obtained after digestion with *AluI* and *KpnI* (not shown) supported this result. As for PCR amplicons, RFLP results were also not influenced by the nucleic acid extraction method.

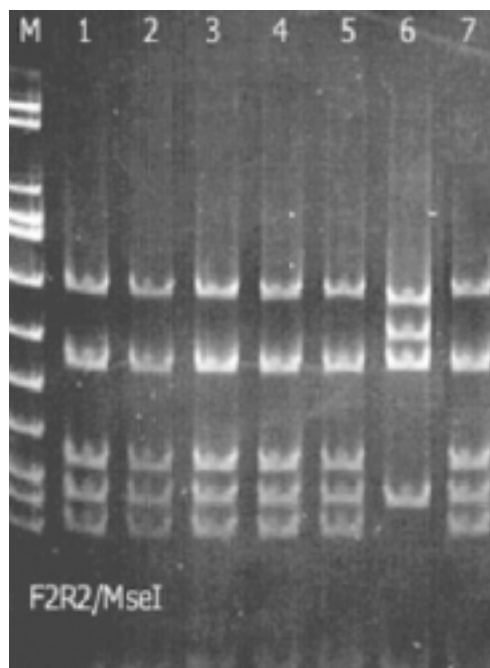


Fig. 1: Polyacrylamide gel (5 %) showing representative RFLP patterns of phytoplasma 16S rDNA amplified using R16F2/R2 primer pair from: cv. Chardonnay-Skopje (lane 1), cv. Vranac-Veles (lanes 2-5), aster yellows group (16SrI-B) control strain Hyd-B (lane 6) and stolbur (16SrXII-A) phytoplasma control strain PG3 (lane 7). The restriction enzyme used is *MseI*. Marker (M) is DNA molecular weight marker VIII (Roche Diagnostics) with fragment sizes (from the top): 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124 and 110 bp.

The region of the *tuf* gene (850 bp) primed with fTufu/rTufu primer pair was also amplified in all samples (Fig. 2). Restriction profiles of the PCR products digested with *Tru9I* (Fig. 3), *TspEI* (Fig. 4), *Sau3AI* and *MaeI* (results not shown), were also characteristic of the 16SrXII-A phytoplasma ribosomal subgroup. Amplification of *tuf* gene was not influenced by the nucleic acid extraction method, but when PCR products were digested, additional bands were revealed in all Vranac and Chardonnay samples taken in 2001 (not shown) and 2002 (Figs. 3, 4) if the nucleic acid was extracted as described in ŠERUGA *et al.* (2003). Those bands were not present in the RFLP if the nucleic acid from the same samples was extracted according to the procedure by PRINCE *et al.* (1993). The experiments were repeated twice for all samples, starting from nucleic acid extraction to RFLP analyses; the same results were obtained. This is the first molecular proof that indigenous and imported grapevines of this region are affected by phytoplasmoses.

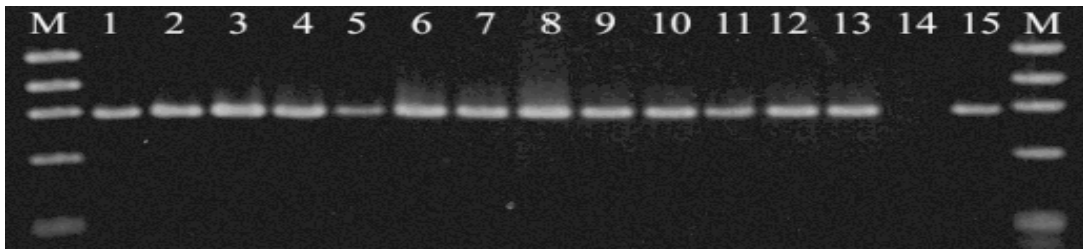


Fig. 2: Agarose gel (1 %) showing phytoplasma *tuf* gene DNA amplified using fTufu/rTufu primer pair from: cv. Vranac-samples collected in 2002 in Veles (lanes 2-4, 6-8), Vranac-samples collected in 2001 in Veles (lanes 10, 11), cv. Chardonnay-Skopje collected in 2002 (lanes 1, 5), cv. Chardonnay-Skopje collected in 2001 (lane 9), control strains Hyd-B (lane 12), KVI (lane 13) and PG3 (lane 15), negative control (lane 14), marker (M) is DNA molecular weight marker IX (Roche Diagnostics) with fragment sizes (from the top): 1353, 1078, 872, 603, 310, 281/271, 234, 194, 118 and 72 bp. In lanes 1-4, 9-11 nucleic acid was isolated according to ŠERUGA *et al.* (2003), and in lanes 5-8 according to PRINCE *et al.* (1993).

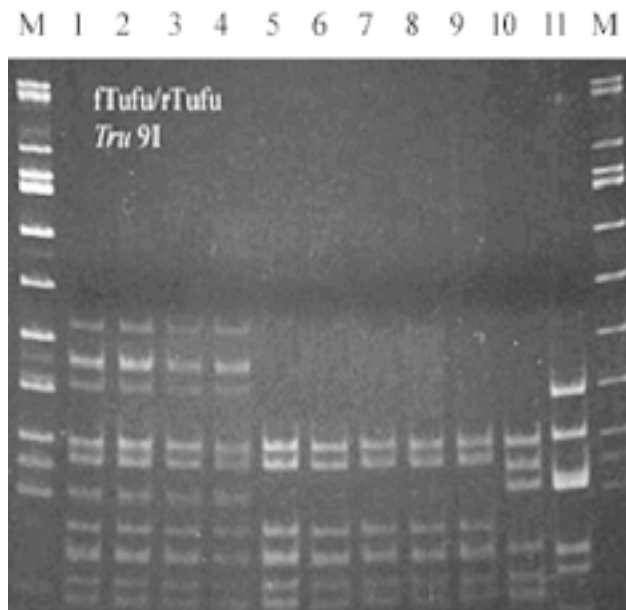


Fig. 3: Polyacrylamide gel (5 %) showing representative RFLP patterns of phytoplasma *tuf* gene amplified using fTufu/rTufu primer pair from: cv. Vranac-samples collected in 2002 in Veles (lanes 1-3; 5-7), cv. Chardonnay-Skopje (lanes 4, 8); control strains PG3 (lane 9), Hyd-B (lane 10) and KVI (lane 11), marker (M) (see Fig. 1). In lanes 1-4, nucleic acid was isolated according to ŠERUGA *et al.* (2003) and in lanes 5-8, according to PRINCE *et al.* (1993). The restriction enzyme is *Tru9I* (=MseI).

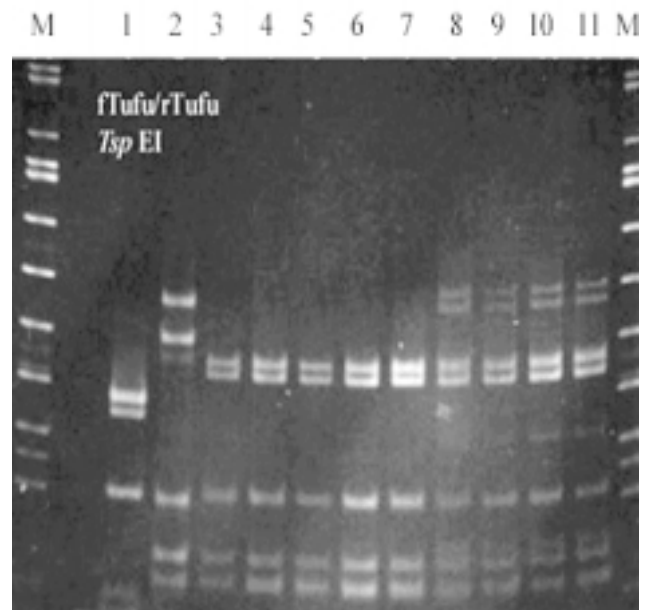


Fig. 4: Polyacrylamide gel (5 %) showing representative RFLP patterns of phytoplasma *tuf* gene amplified using fTufu/rTufu primer pair and cut with restriction enzyme *TspEI*: control strains KVI (lane 1), Hyd-B (lane 2) and PG3 (lane 3), cv. Vranac-samples collected in 2002 in Veles (lanes 4-6; 8-10), cv. Chardonnay-Skopje (lanes 7, 11), marker (M) (see Fig. 1). In lanes 8-11, nucleic acid was isolated according to ŠERUGA *et al.* (2003) and in lanes 4-7, according to PRINCE *et al.* (1993).

### Discussion

After grapevine yellows symptoms were observed in Macedonian vineyards, the presence of phytoplasmas was first confirmed by nested PCR experiments which amplified a 1.25 kbp long portion of 16S rRNA gene. RFLP patterns of those samples were comparable to the Croatian reference strain PG3 (bois noir). The results were positive for all 14 symptomatic samples collected in 2001 and 2002, and they were not dependent on the method of nucleic acid extraction.

In order to characterize Macedonian phytoplasma isolates in more detail, we also analyzed *tuf* gene. The *tuf* sequence is highly conserved, but not as much as 16S rDNA, and it is therefore considered suitable for finer differentiation and classification of closely related phytoplasmas

(MARCONE *et al.* 2000). However, restriction profiles of the *tuf* gene amplification products of the Macedonian samples were again comparable to those of the Croatian reference strain PG3 (16SrXII-A phytoplasma ribosomal subgroup). Additional bands that were revealed in all samples after digestion with *Tru9I* and *TspEI* did not appear if the nucleic acid extraction method of PRINCE *et al.* (1993) was used (Figs 3 and 4). The sum of the additional fragment sizes revealed that a gene portion of approximately the same length, but with a different restriction profile, was amplified when using the nucleic acid extraction procedure by ŠERUGA *et al.* (2003). A BLAST search in the NCBI database (<http://www.ncbi.nlm.nih.gov>) using *tuf* gene sequence as a criterion resulted in a number of very similar sequences belonging to a vast range of prokaryotic and eukaryotic organisms reflecting the ubiquitous nature of this gene. Primer pair

fTufu/rTufu had been designed for the universal amplification of the phytoplasma *tuf* gene but it was shown that at lower annealing temperatures (*e.g.* 45 °C) the *tuf* gene portion of other culturable mollicutes could also be amplified. The annealing temperature of 55 °C, also applied in our experiments, proved to be too high for the unspecific amplification (SCHNEIDER *et al.* 1997). There are no data whether the same primers amplify *tuf* gene of other microbial species that might be associated with grapevine. Further research is needed to reveal the exact nucleotide sequence and origin of these DNA fragments.

Although, the exact origin of additional RFLP bands remains unknown, our results unequivocally show that stolbur phytoplasmas were present in all tested samples. The phytoplasmas of grapevine have already been reported in neighboring countries, *e.g.* Serbia (DUDUK *et al.* 2003) and Greece (DAVIS *et al.* 1997), but this is the first report of the presence of phytoplasmas in Macedonian grapevines with GY symptoms. The scope and importance of the grapevine yellows diseases in the region remains to be determined.

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