

**DETECTION OF XIII PHYTOPLASMA GROUP ON CULTIVAR  
ZUPLJANKA IN ZUPA VINEYARD REGION BY RFLP ANALYSIS  
OF 16S rDNA SEQUENCES**

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“Bois noir” (BN) is an important grapevine disease associated with  
phytoplasmas belonging to ribosomal subgroup 16SrXII-A. Phytoplasmas  
cause diseases in several hundred plant species. The number of infected  
cultivars is growing each year and it is important to follow the spreading of  
the phytoplasma in the different regions and identify which strains are  
present in specific regions on specific cultivars. Phytoplasmas are identified

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and classified based on direct sequencing of phytoplasma 16S rDNA or the 16S to 23S intergenic spacer region, but this approach is not always practical when a large number of unknown phytoplasmas is to be analyzed. Classification by RFLP analysis has provided a simple and rapid method that can be used to differentiate and identify a large number of unclarified phytoplasmas. Our objective was to investigate presence of phytoplasmas of 16SrXII-A group (Stolbur) in Zupa vineyard region. Detection was based on RFLP analysis of 16s rDNA sequences using four restriction enzymes: *TruI*, *AluI*, *KpnI* and *TaqI*. We identified phytoplasmas of XIIA group on two of three investigated cultivars (Zupljanka and Frankovka, but not on Plovdivina) in the Zupa vineyard regions (Gornje Rataje and Tules locality). This is the first report of Stolbur phytoplasma on cv. Zupljanka in Zupa region.

*Key words* cv. Zupljanka,; nested-PCR, phytoplasmas XIIA group, RFLP analyses

## INTRODUCTION

Phytoplasmas are wall-less prokaryotes of the class *Mollicutes* that cause diseases in several hundred plant species. They occur in phloem cells of the host plant and are transmitted by phloem-feeding insects, especially leafhoppers. Characterization of these pathogens has been difficult because they cannot be cultured *in vitro*. In the past two decade, huge efforts have been focused on the characterization and identification of new uncultured plant pathogens and, in 1994., simple name “phytoplasma” officially replaced “mycoplasma-like organism” (GUNDERSEN *et al.*, 1994). Substantial progress has been made towards improved differentiation and classification of phytoplasmas by the application molecular methods including nucleic acid hybridization and restriction fragment length polymorphism (RFLP) analysis of chromosomal DNA. Currently, phytoplasma detection and characterization are based predominantly on PCR amplification of rRNA genes (rDNAs), especially the 16S rDNA (JOMANTIENE *et al.*, 1998). This gene is present in all the prokaryotes and its conserved and variable regions make it ideal for phylogenetic classification (AHRENS and SEEMÜLLER, 1992). The preferred methods for phytoplasma classification have become restriction fragment length polymorphism (RFLP) analysis and sequence analysis of rDNA. Several major groups and subgroups (RFLP groups) of related phytoplasmas have been identified by restriction analysis of the 16S rDNA gene. Phytoplasmas constitute a large, genetically diverse group. Currently, 17 groups and more than 40 subgroups have been classified based on RFLP analysis of 16S rDNA gene (LEE *et al.*, 1993).

Grapevine is world-wide affected by yellows diseases associated with phytoplasmas. The main phytoplasmas responsible of this disease belong to group 16SrV and 16SrXII. We observed the incidence of 16SrXII-A phytoplasma in different vineyards of Serbian Zupa region, especially on cv. Zupljanka, using PCR assays with the universal primer pair P1/P7 for the amplification of phytoplasma 16S

rRNA gene, R16F2n/R16R2 primer pair for nested PCR and RFLP patterns of nested products obtained by restriction endonucleases (specific for the 16SrXII-A group).

## MATERIALS AND METHODS

Samples of grapevine (*Vitis vinifera*) were collected during September 2009 from plants showing leaf roll, leaf redness, vein chlorosis, necrosis and absence of lignification. Total nucleic acid was extracted separately from leaf midveins and stem bark collected from 40 symptomatic and 6 asymptomatic plants from Zupa region (Gornje Rataje and Tules). We tested 30 plants of *cv.* Zupljanka, 5 plants *cv.* Frankovka and 5 plants *cv.* Plovdina from this region and earlier detected Stolbur infected plants of *cv.* Chardonnay from Vrsac vineyard region (JOSIĆ *et al.*, 2006). FD-C, AY and 16SrXII-A strains were used as control strains as described by KUZMANOVIC (2007). DNA for use in PCR was extracted by an intact phytoplasma enrichment procedure (DAIRE *et al.* 1997). Direct PCR, using universal ribosomal primers P1/P7, followed by a nested amplification with the group-specific primer pairs R16F2n/R2 (LEE *et al.*, 1993), was used to identify samples infected by phytoplasmas. Amplification reactions and cycling conditions were as previously described (PASQUINI *et al.*, 2001). The primers were purchased from Metabion, *Taq* native polymerase, restriction endonucleases and dNTPs from Fermentas, Lithuania. PCR amplifications were performed by using an Eppendorf Master Cycler Personal. Electrophoresis was carried out on a horizontal 1.5% agarose gel in TBE electrophoresis buffer as described by KUZMANOVIC (2007). RFLP analysis of 16s rDNA sequences was done using four restriction enzymes: *TruI*, *AluI*, *KpnI* and *TaqI*. Restriction fragments were separated on 2,5% agarose gel in TBE electrophoresis buffer. The gels were stained with ethidium bromide and photographed under UV illumination.

## RESULTS AND DISCUSSION

Phylogenetic analyses based on 16s rDNA of the uncultured phytoplasmas provided a basis for establishing a phylogenetically valid classification (SEEMÜLLER *et al.*, 1998). RFLP analysis of PCR-amplified 16S rDNA sequences with a number of restriction enzymes was used to differentiate various phytoplasmas on the basis of distinct RFLP patterns. The phytoplasma groups identified based on comprehensive RFLP analyses with sufficient number of restriction enzymes have been consistent with phylogenetic groups delineated based on sequence data (LEE *et al.* 1993).

We investigated grapevine (*Vitis vinifera*) plants from Zupa vineyards regions: Tules and Gornje Rataje. Symptomatic plants belonged to three cultivars: Zupljanka, Frankovka and Plovdina. Phytoplasmas were detected using universal primer pair P1/P7 with amplified fragment of 1800bp, followed by R16F2n/R16R2 in nested PCR with amplified fragment of 1200bp shown in Figure 1. The results from healthy plants tissues were negative. Also, 1200 bp amplicons were obtained in direct amplification in some plants with very intensive leaf redness.

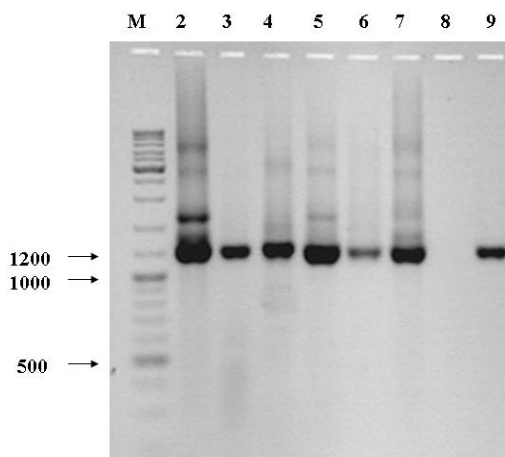


Figure 1. R16F2n/R16R2 in nested PCR with amplified fragment of 1200bp.

Lane 1: M- GeneRuler DNA Ladder mix SM0331 (Fermentas); Lane 2: Zupljanka; 3: Frankovka; 4: Plovdiva 5: Chardonnay; 6: control 16SrXII group; 7: control FD-C phytoplasma; 8: blank control; 9: control aster yellow (AY) phytoplasma

The 1200bp products were digested with *TruII*, *AluI*, *KpnI* and *TaqI* restriction endonucleases and analysis of RLFP pattern (Figure 2-5) showed presence of 16SrXII-A group (Stolbur). The molecular characterization of grapevines collected in different vineyards, showed that Stolbur phytoplasma were present on two investigated cultivars: Zupljanka and Frankovka. Seventeen out of 30 symptomatic plants of cv. Zupljanka and 4 out of 5 symptomatic plants of cv. Frankovka were infected by Stolbur phytoplasma. Cultivar Plovdiva was already shown to be sensitive to FD phytoplasma (KUZMANOVIC *et al.*, 2007; 2009) and all 5 symptomatic plants were infected by this strain.

The host range of subgroup 16SrXII-A (Bois Noir or Stolbur) strains is known to include plants such as nectarine, pear and vineyard weeds (LEE *et al.*, 1995). The presence of Stolbur phytoplasma was reported in many regions in Europe and several neighboring countries. PASQUINI *et al.* (2007) detected Stolbur isolates in grapevines, weeds and insects collected in central and southern Italy. Bois Noir phytoplasmas were detected in vineyards of the eastern and north-western Croatia and the most frequently affected cultivar was 'Chardonnay' (CURKOVIC-PERICA *et al.*, 2001). Phytoplasmas from the ribosomal subgroup 16SrXII-A were also detected by PCR and RFLP analyses of 16S rDNA gene in symptomatic grapevines (cvs Chardonnay and Vranac) from the Veles and Skopje areas in Macedonia, reported by SEGURA *et al.* (2003).

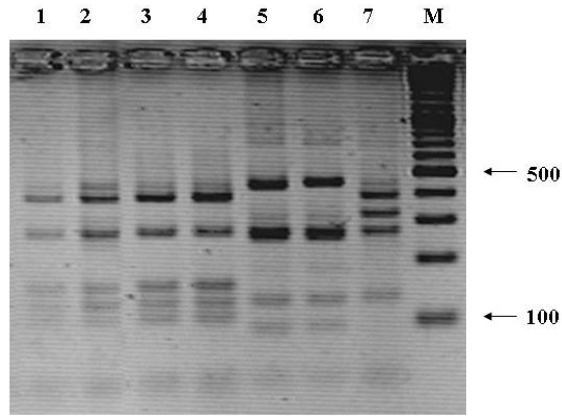


Figure 2. The nested R16F2n/R16R2 amplified fragment digested with *TruI*.

Lane 1: Zupljanka; 2: Frankovka; 4: Chardonnay; 5: Control 16SrXII group; 6: Plovdina; 7: control FD-C phytoplasma; 8: control AY phytoplasma; 9: M- GeneRuler DNA Ladder mix SM0331

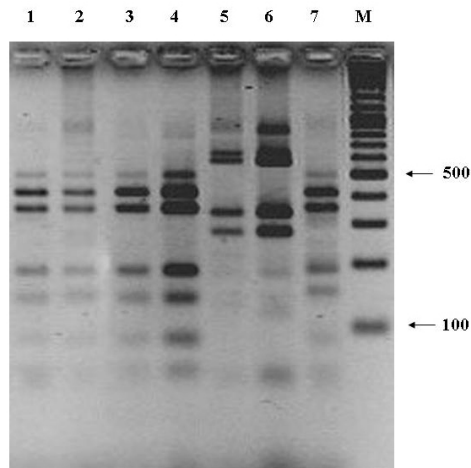


Figure 3. The nested R16F2n/R16R2 amplified fragment digested with *AluI*.

Lane 1: Zupljanka; 2: Frankovka; 3: Chardonnay; 4: Control 16SrXII group; 5: Plovdina; 6: control FD-C phytoplasma; 7: control AY phytoplasma; 8: M- GeneRuler DNA Ladder mix SM0331

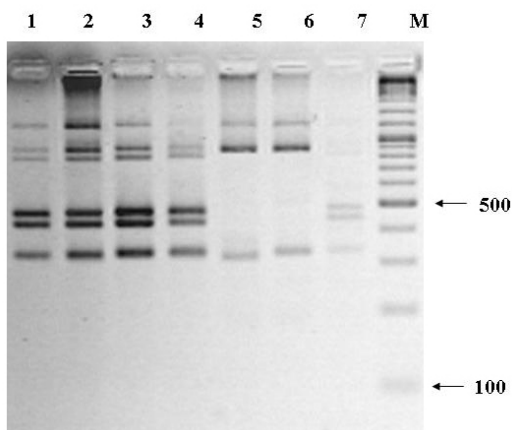


Figure 4. The nested R16F2n/R16R2 amplified fragment digested with *KpnI*.

Lane 1: Zupljanka; 2: Frankovka; 4: Chardonnay; 5: Control 16SrXII group; 6 and 7: Plovdivina 8: control AY phytoplasma; 9: GeneRuler DNA Ladder mix SM0331 (Fermentas)

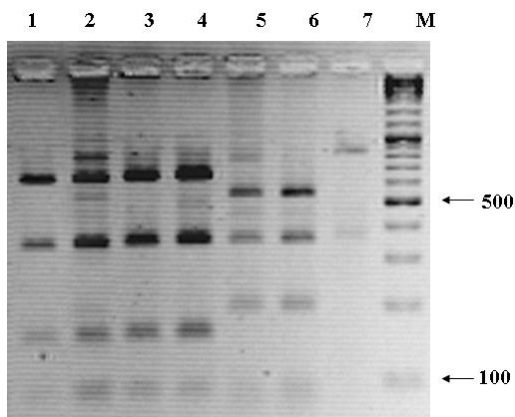


Figure 5. The nested R16F2n/R16R2 amplified fragment digested with *TaqI*.

Lane 1: Zupljanka; 3: Frankovka; 5: Chardonnay; 6 Control 16SrXII group; 7 and 8: Plovdivina; 9: control AY phytoplasma; Lane 10: GeneRuler DNA Ladder mix SM0331

Recent investigations of Stolbur phytoplasma presence on different grapevine cultivars in different regions in Serbia showed that *cv.* Zupljanka was infected only in Vojvodina regions (Vrsac and Deliblatska pescara) and with very low percent of infected plants (KUZMANOVIC *et al.*, 2008). So far, Stolbur phytoplasma was never detected on Zupljanka cultivar in Zupa region, but this investigation showed that Stolbur phytoplasma have spread in this region too.

Phytoplasmas belonging to ribosomal subgroup 16SrXII-A cause important disease “Bois noir” (BN) associated not only with grapevine; it is commonly spread in a wide range of host plants including wild and cultivated herbaceous hosts (PASQUINI *et al.* 2007). Better understanding of phytoplasma ecology is pertinent for the development of efficient control measures to suppress disease. Therefore, it is of great importance to monitor presence, incidence and progression of BN disease of grapevine.

### CONCLUSIONS

Using nested PCR with universal primer pair P1/P7 followed by R16F2n/R16R2 on three cultivars of *Vitis vinifera* plants from Zupa region (Gornje Rataje and Tules), we confirmed presence of phytoplasma on these cultivars. Based on RFLP analyses of phytoplasma 16S rDNA sequences, using *TruI*, *AluI*, *KpnI* and *TaqI* restriction endonucleases, we identified 16Sr XII-A phytoplasma group on *Vitis vinifera* plants (cultivars Zupljanka and Frankovka) and FD on *cv.* Plovdina. No infected plants of Zupljanka cultivar were detected in Zupa region until now. This investigation showed that Stolbur phytoplasma has spread on *cv.* Zupljanka in the Zupa region.

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**DETEKCIJA XIIA GRUPE FITOPLAZMI SORTE ŽUPLJANKA U ŽUPSKOM VINOGRJU RFLP ANALIZOM 16S rDNA SEKVENCI**

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**I z v o d**

"Bois noir" (BN) je značajna bolest vinove loze izazvana fitoplazmama 16SrXII-A grupe. Fitoplazme izazivaju bolesti kod nekoliko stotina biljnih vrsta. Ovaj broj se povećava svake godine, pa je veoma značajno pratiti širenje fitoplazmi u različitim regionima i utvrditi koji izazivač je prisutan u nekom regionu i na određenoj sorti. Identifikacija i klasifikacija fitoplazmi bazirana je na sekvenciranju 16S rDNA ili intergenskog regiona 16S - 23S, ali ovaj metod se retko primenjuje na analizu velikog broja uzoraka. Primena RFLP analize omogućava brzo i jednostavno detektovanje, diferenciranje i klasifikaciju velikog broja fitoplazmi. Cilj ovog rada je detektovanje fitoplazmi 16SrXII-A grupe (Stolbur) u Župskom vinogorju. Detekcija je izvršena RFLP analizom 16s rDNA sekvenci korišćenjem restrikcionih enzima: *TruI*, *AluI*, *KpnI* and *TaqI*. Identifikovane su fitoplazme XIIA grupe na dve od tri ispitivane sorte (na Župljanki i Frankovki, ali ne na Plov dini) u Župskom vinogorju (lokaliteti Gornje Rataje i Tuleš). U ovim istraživanjima je prvi put dokazano prisustvo Stolbur fitoplazme na sorti Župljanka u Župskom regionu.

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