

## Molecular identification and seasonal monitoring of phytoplasmas infecting Croatian grapevines

by

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**S u m m a r y :** Phytoplasmas of the 16S rRNA RFLP group XII-A (stolbur) have been detected with tests carried out 4 times from September 1997 to April 1998 in Croatian Pinot gris grapevines showing yellows symptoms. This is the first report on the presence of stolbur phytoplasma in Croatian grapevines. Two asymptomatic vineyard weeds, *Taraxacum officinale* Web. and *Polygonum lapathifolium* L. showed the presence of the same prokaryotes.

**K e y w o r d s :** phytoplasmas, grapevine yellows, PCR, RFLP.

### Introduction

Grapevine yellows (GY) diseases encompass etiologically different grapevine diseases associated with the presence of phytoplasmas, formerly called mycoplasma-like organisms (GUNDERSEN *et al.* 1994; SEARS and KIRKPATRICK 1994). During the last 10 years advances in molecular biology-based techniques have enabled fast, reliable and highly sensitive diagnostics of GY-associated phytoplasmas. The PCR amplification of 16S rRNA gene portions followed by the restriction fragment length polymorphism analysis (RFLP) is the most widely used diagnostic tool, although hybridization and serology techniques can also be successfully employed (DAVIS *et al.* 1993; KUSZALA *et al.* 1993; DAIRE *et al.* 1993, 1994; BIANCO *et al.* 1996).

The molecular differences in PCR-amplified 16S rDNA showed that diverse and group-specific restriction patterns are the basis for the phytoplasma classifications proposed (NAMBA *et al.* 1993; SCHNEIDER *et al.* 1993; GUNDERSEN *et al.* 1994; LEE *et al.* 1993, 1998). Recently, some other phytoplasma sequences were used as alternatives for the differentiation and classification if the 16S rRNA gene is not variable enough to allow phytoplasma distinction (VIBIO *et al.* 1996; BOUDON-PADIEU *et al.* 1997; SCHNEIDER *et al.* 1997).

GY diseases are affecting various grapevine cultivars throughout the world (PRINCE *et al.* 1993; MAIXNER *et al.* 1995; ALMA *et al.* 1996; DAIRE *et al.* 1997). 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) groups. A number of authors have demonstrated mixed infections in the GY-affected vines with phytoplasmas from different groups or subgroups (BERTACCINI *et al.* 1995; ALMA *et al.* 1996; DAIRE *et al.* 1997).

Since 1994, symptoms typical for the GY diseases have been observed on Pinot gris and Chardonnay in the Zagreb area (northwestern Croatia). So far, no molecular evidence has proved the relationship between typical GY symptoms

and a phytoplasma agent (ŠARIC *et al.* 1997). In this study we present, for the first time, molecular evidence for the occurrence of the phytoplasma from the 16SrXII-A rRNA RFLP subgroup in the Pinot gris vines and vineyard weeds.

### Material and methods

**Plant samples and phytoplasma strains:** Grapevine samples showing yellows symptoms were collected in vineyards in the Zagreb area (Bozjakovina). Sampling was performed 4 times in 1997 and 1998 on 6 symptomatic plants, cv. Pinot gris (Table). This cultivar is prevalent in the area and showed the most severe symptoms. In order to investigate the phytoplasma distribution in plant midribs, cutting samples of wood below the graft and roots were taken in September 1997. At the same time 6 most common weeds were also collected randomly in the vineyards: *Taraxacum officinale* Web. (Asteraceae), *Polygonum aviculare* L., *Polygonum lapathifolium* L. (Polygonaceae), *Vicia cracca* L. (Fabaceae), *Equisetum silvaticum* L. (Equisetaceae), and *Amaranthus retroflexus* L. (Amarantaceae). In October 1997 grapevine leaves and canes were taken while in February and April 1998 only cuttings were available for testing.

The reference phytoplasma strains used in the experiments were: Maryland aster yellows (AY-1; I.-M. LEE, USDA, Beltsville, MD, USA); clover phyllody (CPh; L. N. CHYKOWSKI, Agriculture Canada, Ottawa, Ontario, Canada via I.-M. LEE) for the subgroups 16SrI-B and 16SrI-C, respectively (LEE *et al.* 1993) and Italian periwinkle virescence (IPVR) for the group 16SrXII-A (formerly subgroup 16SrI-G), (DAVIS *et al.* 1997). For the 16SrIII and 16SrV groups reference strains were apple proliferation (AP; L. CARRARO, University of Udine, Italy) and elm yellows (EY-1; H. GRIFFITH and W.A. SINCLAIR, Cornell University, USA), respectively.

**Nucleic acid extraction and PCR amplification:** Total nucleic acids were extracted from ca. 2 g of the plant tissue (leaf midribs, secondary roots, phloem scrapings of canes or wood) freshly ground in liquid nitro-

Table

Results of stolbur-related phytoplasma PCR detection tests performed in different periods and in different tissues of 6 Pinot gris vines

Sample	September 1997				October 1997		February 1998	April 1998
	leaf midribs	canes	wood below graft union	roots	leaf midribs	canes	canes	canes
PG1	+	-	+	-	-	-	+	-
PG2	+	-	-	-	-	-	+	+
PG3	+	-	+	-	-	-	-	-
PG4	+	-	-	-	-	-	-	+
PG5	-	-	+	-	+	-	-	-
PG6	+	-	+	-	-	-	+	-

gen. The extraction procedure has been described by PRINCE *et al.* (1993). Total nucleic acids, diluted with sterile deionized water to a final concentration of  $20 \text{ ng} \cdot \mu\text{l}^{-1}$ , were used for the direct PCR amplification with the universal phytoplasma primer pair R16F1/R0 followed by nested PCR with another general primer pair R16F2/R2 (LEE *et al.* 1994, 1995). Nested PCR experiments for the detection of phytoplasma 16S rRNA groups I and XII were carried out with the already published group I and XII specific primer pairs designated R16(I)F1/R1 (LEE *et al.* 1994). Occasionally other universal primers R16Fm1/Rm2 followed by R16F2n/R2 (GUNDERSEN and LEE 1996) were also employed to compare the sensitivity of the two systems. For all samples a universal primer pair M1/M2 (16R<sub>738f</sub>/16R<sub>1232r</sub>; GIBB *et al.* 1995) amplifying a 0.6 kb 16S rDNA sequence was also used in nested PCR. Additional direct PCR assays were performed using G35p/m (DAVIS *et al.* 1992) and "stolbur" primers (MAIXNER *et al.* 1995) specific for phytoplasmas in the groups 16SrI and 16SrXII. Each reaction was performed in a total volume of 25  $\mu\text{l}$  containing 2.5  $\mu\text{l}$  of the PCR buffer (SCHAFF *et al.* 1992), 200  $\mu\text{M}$  of deoxynucleotide triphosphates, 0.625 U of Taq polymerase (Perkin-Elmer, CA, USA) and 0.2  $\mu\text{M}$  of primer pair. Tubes with water instead of the template DNA were included in each reaction series as negative PCR controls. The conditions for 35 PCR cycles performed in an automated thermal cycler (Perkin-Elmer 480) have already been described (SCHAFF *et al.* 1992). Amplified DNAs were subjected to the electrophoresis in 1% agarose gels followed by ethidium-bromide staining and photographed under UV-light.

**Restriction fragment length polymorphism (RFLP) analysis:** PCR products amplified from different portions of phytoplasma 16S rRNA gene have been subjected to the digestion with restriction enzymes *Mse*I (New England Biolabs, Beverly, MA, USA), *Kpn*I and *Alu*I (Fermentas, MBI, Vilnius, Lithuania) at 37 °C for at least 16 h. Depending on the intensity of the ethidium-bromide stained DNA amplified bands in agarose gels, 6-10  $\mu\text{l}$  of the PCR product were used for the digestion with 4 U of enzyme.

The RFLP patterns, obtained after the electrophoresis through a 5% polyacrylamide gel and ethidium-bromide staining, were compared with those of the reference phytoplasma strains.

## Results

**Symptomatology:** The 6 symptomatic Pinot gris vines sampled have been designated PG1 to PG6 (Table). The vines have been exhibiting grapevine yellows symptoms since 1994 as already described (ŠARIC *et al.* 1997). Some of the plants (PG5, PG6) were severely symptomatic and almost dying. Randomly collected weeds in the September sampling demonstrated no apparent yellows symptoms on leaves.

**PCR amplification:** The first visible DNA products, 1.2 kb long, were yielded after the nested PCR with the R16F2/R2 general phytoplasma primer pair on the templates obtained by the direct amplification with the R16F1/R0 primers. Only one leaf sample from September (PG3) out of 6 was positive (Fig. 1 A). After the subsequent amplification step with 16SrI phytoplasma group-specific primer pair R16(I)F1/R1 the number of positive leaf samples raised to 3 (Fig. 1 B) while the use of M1/M2 primers showed 5 positive samples, in other words, all but PG5 (Fig. 2).

Two weed species, *Taraxacum officinale* Web. and *Polygonum lapathifolium* L., were also tested phytoplasma positive using the last primer pair (Fig. 2).

No positive results were obtained with phloem tissue from canes or roots (Table) or with water controls, the phytoplasma control strains were always positive according to the specificity of primers employed. The only vine (PG5) that tested negative with M1/M2 primers from leaves in September demonstrated phytoplasma 16S rDNA presence in the wood below the graft union together with samples PG1, PG3 and PG6 but no phytoplasma was detected in root samples (Table).

The use of primers M1/M2 in the October sampling, when only leaves and canes of grapevines were collected, show that the only sample that tested positive was the leaf sample of PG5 (Table).

In February tests on canes showed the presence of phytoplasmas in PG1, PG2 and PG6; in April positive canes were PG2 and PG4 (Table) always by using as a final nested the one with M1/M2 primers. In the direct PCR assays with the use of G35p/m and specific "stolbur" primers (see Materials and methods) no or only faint bands were obtained (data not shown).

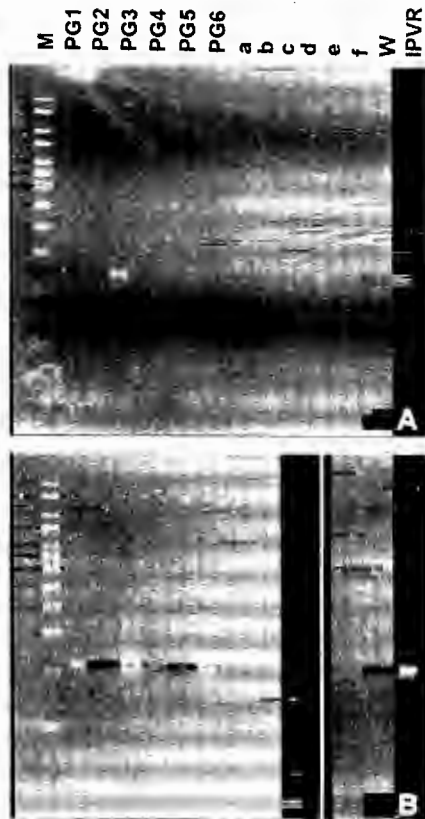


Fig. 1: Agarose gels of nested polymerase chain reaction (PCR) amplification products of phytoplasma 16S rDNA from grapevine leaves PG1/6 (as in the Table), phytoplasma control strain IPVR and vineyard weeds: *Taraxacum officinale* (a), *Polygonum lapathifolium* (b), *Vicia cracca* (c), *Equisetum silvaticum* (d), *Polygonum aviculare* (e), and *Amaranthus retroflexus* (f). Water (negative) control (W). M: 1 kb DNA ladder marker with fragment sizes, in kb, from top to bottom: 10.0, 8.0, 6.0, 5.0, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, 1.0, 0.5. The primers used are R16F2/R2 (in A) and R16(I)F1/R1 (in B).

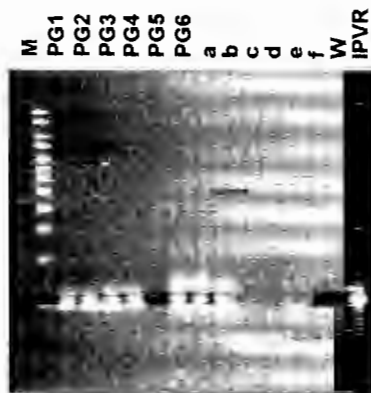


Fig. 2: Agarose gel of nested polymerase chain reaction (PCR) amplification products with primers R16738F/1232R (M1/M2) of phytoplasma 16S rDNA sequences from grapevine leaves PG1-6. For abbreviations see Fig. 1.

**RFLP analysis of the PCR products:** All products obtained by amplification of the phytoplasma 16S rDNA sequences from grapevines or weeds were subjected to the restriction analyses by the *MseI* (Figs. 3, 4 and 5), *Alu I* and *Kpn I* (data not shown). Amplified phytoplasma 16S rDNA from the reference strains were included in the RFLP experiments and the comparison of

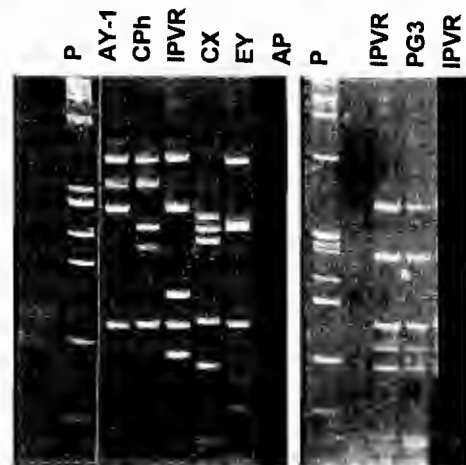


Fig. 3: Polyacrylamide gels (5 %) showing the *MseI* RFLP patterns of a phytoplasma 16S rDNA fragment of 1.2 kb obtained with primers R16F2/R2 from leaf sample PG3 and from phytoplasma reference strains from *Catharanthus roseus*: Aster yellows, AY-1; clover phyllody, CPh; Italian periwinkle virescence, IPVR; X-disease, CX; elm yellows, EY-1; apple proliferation, AP. P, marker  $\Phi$ X174 *HaeIII* digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

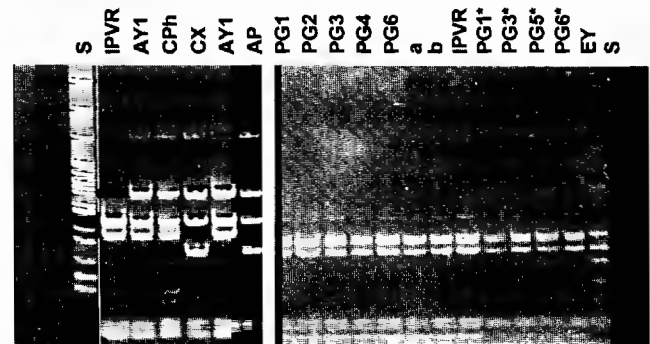


Fig. 4: Polyacrylamide gels (5 %) showing the RFLP patterns of phytoplasma 16S rDNA fragments of 0.6 kb obtained with primers R16738F/1232R (M1/M2). \*, Template DNA from wood below the grafting, other grapevine samples are from leaves. For abbreviations see Table and Figs. 1 and 3. S, marker pBR322 *MspI* digested; fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, 9.

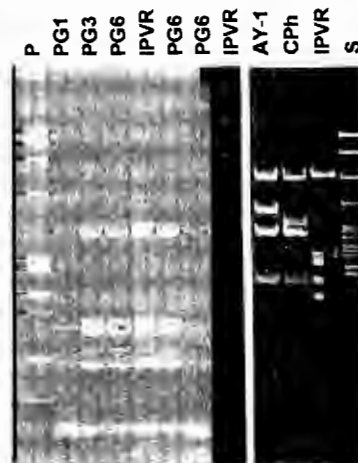


Fig. 5: Polyacrylamide gels (5 %) showing the RFLP patterns of phytoplasma 16S rDNA fragments of 1.1 kb obtained with primers R16(I)F1/R1. For abbreviations see Table and Figs. 1 and 3.

the RFLP patterns of all amplified products were those characteristic of 16SrXII-A phytoplasma subgroup and identical to the subgroup representative strain IPVR (Figs. 3, 4 and 5).

### Discussion

Positive results obtained in PCR experiments by amplifying portions of phytoplasma 16S rDNA confirmed the presence of phytoplasma in the Croatian grapevines showing GY symptoms. The amplification using the primer pair R16F1/R0 in direct PCR and R16F2/R2 in the subsequent nested PCR assays yielded only one positive sample (PG3). The use of the 16SrI group-specific primer pair R16(I)F1/R1 as well as M1/M2 primers in further nested PCRs enabled us to detect the phytoplasma in 5 out of 6 grapevine leaf samples from September. The only negative leaf sample (PG5) tested phytoplasma positive when the wood below the graft union was used.

The results of the 6 tested plants were different. They depended on the kind of tissue and the period of the year in which the tests had been performed. In addition, the system used for the amplification affected, in some cases, the results.

The most sensitive system proved to be the one that employed general primers R16F1/R0 followed by R16F2/R2 and specific primers R16(I)F1/R1 and/or M1/M2. The need for highly sensitive detection procedures is of outmost importance. The difficulties observed in PCR amplification can probably be attributed to the usual problems with phytoplasma detection in woody hosts, to the uneven pathogen distribution in grapevine and to seasonal variations of pathogen concentration. These and other problems also emerged in the October sample analyses when only one leaf sample (PG5) demonstrated the presence of phytoplasma. The samples had been collected at the very end of the vegetation season, i.e. close to leaf fall. For all these reasons it can not necessarily be assumed that the negative results from October are associated with the disappearance of phytoplasmas from the plants. The one positive October sample could thus be regarded as a confirmation of the September results especially with regard to the fact that it had identical RFLP patterns with the September samples.

All the phytoplasmas detected were identified as belonging to the 16SrXII-A rRNA RFLP group (DAVIS *et al.* 1997; LEE *et al.* 1998) in agreement to the reference strain (IPVR) pattern. IPVR has already been reported to be identical to stolbur, Bois Noir and phytoplasmas infecting grapevines in Italy (CAUDWELL 1993; BERTACCINI *et al.* 1995).

The Bois noir phytoplasmas have been reported in grapevine alone or in mixed infections with phytoplasmas from other groups in many countries west or north-west of Croatia. Among them are Italy (BIANCO *et al.* 1993; BERTACCINI *et al.* 1995; ALMA *et al.* 1996) and Slovenia (SELJAK and OSLER 1997) as well as the northeastern part of Croatia and Hungary (KÖLBER *et al.* 1997).

In Croatia, a disease with stolbur-like symptoms was reported in 1957 (PANJAN) in tomato, pepper, eggplant, po-

tato, *Cirsium* sp. and *Convolvulus arvensis*. Evidence by electron microscopy for the presence of phytoplasma in diseased Croatian potatoes (PANJAN *et al.* 1970) as well as in apples and pears (ŠARIC and CVJETKOVIC 1985) have already been reported. Molecular evidences for the presence of phytoplasma in Croatia were lacking until recently when phytoplasmas from the 16SrI group were identified in grapevines from Croatia and Slovenia tested in early spring (ŠARIC *et al.* 1997). In this paper we report for the first time the 16SrXII-A subgroup (stolbur) phytoplasmas in Croatian grapevines with GY symptoms.

Two weed species, *Taraxacum officinale* Web. and *Polygonum lapathifolium* L., were also found to host phytoplasmas of the same group. *Taraxacum* has already been known to be a phytoplasma host (ARZONE *et al.* 1995) but according to our knowledge no reports, on its phytoplasma identity have been published. Unlike other Eastern European countries where stolbur infection exists for some time as an economically important disease of solanaceous crops where 16SrXII-A subgroup phytoplasmas were identified (VIBIO *et al.* 1996), it has never been presented as an economically important disease in the Croatian agriculture.

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