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Variability of stolbur phytoplasma strains infecting Croatian grapevine by multilocus sequence typing

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Abstract

In order to investigate the genetic variability among selected grapevine strains belonging to 16SrXII-A subgroup (stolbur) as a case study, multilocus sequence typing (MLST) approach was used. Three non-ribosomal house-keeping genes were analyzed: *tufB*, *secY* and *vmp1* gene, encoding a putative membrane protein of stolbur phytoplasma. Restriction analysis of *tufB* gene amplicons revealed the presence of both tuf-type a (VK-I) and tuf-type b (VK-II), with the latter being prevalent among the stolbur strains from Croatian grapevines tested. In genotyping of *vpm1* gene, five different RFLP profiles were obtained including a mixed pattern observed in one sample. Phylogenetic analyses of *secY* gene sequences were in accordance with the results of *tufB* and *vmp1* typing; however in some of the analyzed strains incongruences were observed. The presence of considerable genetic variability among relatively small number of 10 selected stolbur strains from grapevine was observed and the importance of MLST application in differentiation of closely related strains was verified.

Key words: MLST, *tufB*, *vmp1*, *secY*, phytoplasmas, grapevine.

Introduction

Main agents associated with grapevine yellows (GY) in Croatia are phytoplasmas belonging to the 16SrXII-A subgroup (stolbur) which are widespread and detected in most of the grapevine growing regions of the country (Šeruga Musić *et al*., 2009).

Natural life-cycle of stolbur phytoplasma infecting grapevine may be associated with different herbaceous plants and vector populations (Langer and Maixner, 2004). In order to clarify the epidemiology of the disease, variability of non-ribosomal house-keeping genes such as *tufB* and *secY* is usually studied. Recently, *vmp1* gene encoding a putative membrane protein and potentially involved in phytoplasma-host interactions was shown to be a valuable molecular marker in differentiation of stolbur strains and assessment of genetic variability (Cimerman *et al*., 2009).

The aim of this study was to examine the variability among selected stolbur phytoplasma strains infecting Croatian grapevine by using multilocus sequence typing (MLST).

Materials and methods

Ten phytoplasma strains from Croatian grapevine collected in different grapevine growing regions, previously characterized as members of 16SrXII-A subgroup (Šeruga Musić *et al*., 2009; unpublished) were chosen for MLST. Abbreviations of samples are listed in table 1.

The *tufB* gene fragments of approximately 940 bp were amplified in a nested PCR using fTufu/rTufu primer pair followed by ftufAY/rtufSTOL primers (Schneider *et al*., 1997; X. Foissac and A. Fabre, personal communication). Amplicons were digested with *Hpa*II (Langer and Maixner, 2004) and analyzed by electrophoresis in 2.5% agarose gel. The *vmp1* gene fragments were amplified in a nested PCR assay using StolH10F1/R1 primers, followed by TYPH10F/R primer pair, as described by Fialova *et al.* (2009). Fragments of approximately 1.1 to 1.5 kbp were digested with *Rsa*I and separated by electrophoresis in 2.5% agarose gel. Amplification of *secY* gene fragment of 998 bp was done in a nested PCR with PosecF1/R1 primers, followed by PosecF3/R3 (Fialova *et al.*, 2009). Sequencing of both strands of *secY* gene fragments was done by Macrogen Inc. (Seoul, Republic of Korea), using PosecF3/R3 primer pair. Obtained sequences were edited and assembled using Sequencher™ 4.10. demo version (http://www.genecodes.com/). Multiple alignments were done using ClustalX 2.0 (Thompson *et al*., 1997) and subsequent phylogenetic analyses performed using MEGA 4 (Tamura *et al*., 2007).

Results

Amplicons of *tufB* gene were successfully obtained from all samples. Restriction analysis with *Hpa*II enzyme indicated that samples SB1, SB5 and 21OS belong to tuf-type a (VKI), while all the other samples were typed as tuf-type b (VKII) according to Langer and Maixner (2004) (data not shown).

In *vpm1* gene typing, restriction analysis with *Rsa*I of fragments obtained in all samples showed the presence of 5 different profiles (figure 1) in the 10 analyzed samples. Restriction patterns of fragments from samples 8TO, 11VZ and 15PO were identical (V14 type). Another pattern (V3 type) was detected in samples SB1, SB5 and 21OS, while samples SI2 and VU6 showed a third type of *Rsa*I/vmp1-profile (V4 type). A unique profile was observed in sample 23DB (V5 type), while sample VU7 revealed a mixed restriction pattern of V2 and V18 types.

Phylogenetic analyses of *secY* gene sequences showed that the strains having the same tuf- and/or vmp1-profile grouped together in the same branch of the tree. Non-congruence was observed in samples 23DB and VU7 that were typed as tuf-type b, but grouped with the *secY* sequences of tuf-type a samples (data not shown).

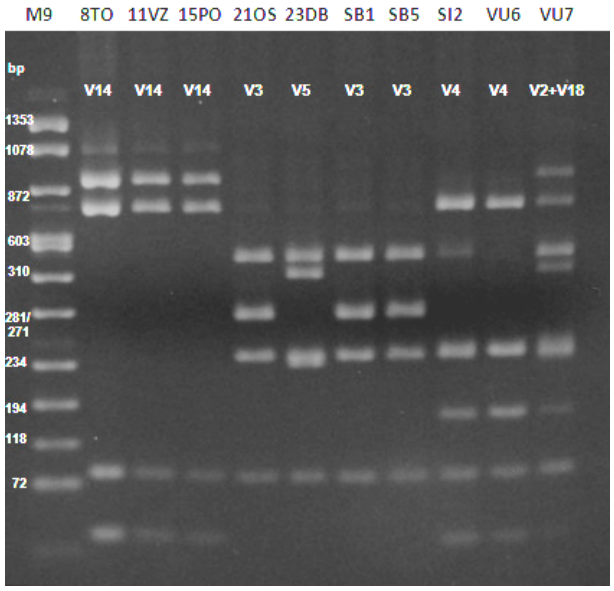

Figure 1. RFLP analysis of phytoplasma *vmp1* gene fragments amplified from Croatian grapevine extracts in a nested PCR assay using StolH10F1/R1, followed by TYPH10F/R primers and digested with *Rsa*I. Electrophoresis was performed in 2.5% agarose gel. Abbreviations of samples are same as in table 1. M9 - Marker 9; ΦX174 DNA/*Bsu*RI(*Hae*III) digested (Fermentas, Lithuania). Different vmp1-restriction profiles are marked as V-types.

Table 1. List of samples analyzed in this study.

Sample	Year	Cultivar	Location
SB1	2000	Chardonnay	Brodski Stupnik
SB5	2000	Chardonnay	Brodski Stupnik
SI2	2001	Debit	Drniš
VU6	2007	Chardonnay	Ilok
VU7	2007	Blaufränkisch	Ilok
8TO	2008	Chardonnay	Ilok
11VZ	2008	Chardonnay	Železna gora
15PO	2008	Chardonnay	Dajla
21OS	2008	Chardonnay	Erdut
23DB	2008	Chardonnay	Kneževi vinogradi

Discussion

Restriction analysis of *tufB* gene showed the presence of polymorphism with a prevalence of the tuf-type b, which is also the most diversified elsewhere in France and Italy (Pacifico *et al.*, 2009). Genotyping of *vpm1* revealed more variability with five different RFLP profiles observed in this study. Comparisons of phylogenetic analyses of *secY* sequences and RFLP analyses of *tufB* and *vmp1* genes proved that *vmp1* and *secY* possess greater variability than *tufB* hence are more informative markers for finer differentiation of closely related strains. Nevertheless, MLST has shown the presence of considerable genetic variability among the relatively small number of analyzed stolbur strains from Croatian grapevine. These results present a step forward in a better understanding and clarifying GY disease epidemiology in Croatia.

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References

CIMERMAN A., PACIFICO D., SALAR P., MARZACHÍ C., FOISSAC X., 2009.- Striking diversity of vmp1, a variable gene encoding a putative membrane protein of the stolbur phytoplasma.- *Applied and environmental Microbiology*, 75(9): 2951-2957.

FIALOVA R., VALOVA P., BALAKISHIYEVA G., DANET J-L., ŠAFAROVA D., FOISSAC X., NAVRATIL M., 2009.- Genetic variability of stolbur phytoplasma in annual crop and wild plant species in South Moravia.- *Journal of Plant Pathology,* 91: 411-416.

LANGER M., MAIXNER M., 2004.- Molecular characterisation of grapevine associated phytoplasmas of the stolbur-group based on RFLP-analysis of non ribosomal DNA.- *Vitis,* 43: 191-199.

PACIFICO D., ALMA A., BAGNOLI B., FOISSAC X., PASQUINI G., TESSITORI M., MARZACHÌ C., 2009.- Characterization of 'bois noir' isolates by restriction fragment length polymorphism of a stolbur-specific putative membrane protein gene.- *Phytopathology*, 99: 711-715.

SCHNEIDER B., GIBB K. S., SEEMÜLLER E., 1997.- Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas.- *Microbiology,* 143: 3381-3389.

ŠERUGA MUSIĆ M., ŠKORIĆ D., BUDINŠČAK Ž., KRIŽANAC I., MIKEC I., 2009.- Survey of phytoplasma diversity in heavily grapevine yellows-affected areas of Croatia.- *Le Progrès agricole et viticole HS, 16th Meeting ICVG*: 206-207.

TAMURA K., DUDLEY J., NEI M., KUMAR S., 2007.- MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.- *Molecular Biology and Evolution,* 24: 1596-1599.

THOMPSON J. D., GIBSON T. J., PLEWNIAK F., JEANMOUGIN F., HIGGINS D. G., 1997.- The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools.- *Nucleic Acids Research,* 24: 4876-4882.

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