Genetic Diversity of ‘Candidatus Phytoplasma solani’ Strains Associated with “Bois Noir” Disease in Croatian Vineyards

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Abstract—Variability of genotypes involved in “bois noir” (BN) pathosystem, disease induced by ‘Candidatus Phytoplasma solani’, was assessed by a multilocus sequence typing (MLST) approach. Genotyping was performed on almost ninety selected strains detected in the scope of an eight-year national survey encompassing grapevine samples, weeds and insects from all Croatian viticultural regions. The extensive -Variability of genotypes involved in “bois noir” (BN) pathosystem, disease induced by ‘Candidatus Phytoplasma solani’, was assessed by a multilocus sequence typing (MLST) approach. Genotyping was performed on almost ninety selected strains detected in the scope of an eight-year national survey encompassing grapevine samples, weeds and insects from all Croatian viticultural regions. The extensive

I. INTRODUCTION

‘Candidatus phytoplasma solani’ (ribosomal subgroup 16SrXII-B), also known as “stolbur” is associated with grapevine “bois noir” (BN) disease that is widespread in Euro-Mediterranean area (Quaglino et al. 2013; Langer and Maixner, 2004). The ‘Ca. P. solani’ life cycle involving two ecological niches – herbaceous plants hosting insect vectors - results in complex BN pathosystem comprising several, possibly intermixed epidemiological networks (Bertaccini and Duduk, 2009; Kosovac et al. 2016). While different cixiid planthoppers (Hemiptera: Cixiidae) are reported to transmit ‘Ca. P. solani’ in many herbaceous and woody plants, Hyalesthes obsoletus is considered to be the principal vector of ‘Ca. P. solani’ associated with BN in many countries (Maixner et al. 1995). Recently, a role in BN transmission has been proven also for Reptalus panzeri (Cvrković et al. 2014). H. obsoletus can feed on various herbaceous plants, but is most commonly associated with Convulvulus arvensis, Urtica dioica and Vítex agnus-castus as hosts for its nymphs and adults (Langer and Maixner et al. 2004; Sharon et al. 2005; Kosovac et al. 2016). Host plants/inoculum source for R. panzeri within BN pathosystem however, is still to be confirmed. BN involved ‘Ca. P. solani’ strains can be tied to different epidemiological cycle’s trough molecular characterization of several genes. Therefore, along with tuf (Schneider et al. 1997) and secY (Fialova et al. 2009) genes, the two variable surface protein genes - vmp1 (Cimerman et al. 2009) and stamp (Fabre et al. 2011) are frequently used to evaluate ‘Ca. P. solani’ genetic diversity. While surface proteins are especially important for understanding vector specificity to ‘Ca. P. solani’ phytoplasma of Cixiidae family (Suzuki et al. 2006), on the basis of the tuf gene ‘Ca. P. solani’ strains are linked to different natural epidemiologic cycles in the field, and two main genotypes: tuf-type a and tuf-type b were studied (Aryan et al. 2014).

The aim of the present study was: i) to assess the variability of ‘Ca. P. solani’ strains from infected grapevine, wild plants and insects by genotyping of tuf, secY, vmp1 and stamp genes and ii) to study the prevalence and distribution of ‘Ca. P. solani’ strains in Croatian viticultural regions.

II. MATERIAL AND METHODS

In the frame of a national survey programme, all Croatian grapevine growing regions were visually inspected for the presence of GY symptoms. In the eight year period (2009 – 2017) over 800 samples of grapevine and other wild/reservoir plants, along with insect samples of predominantly H. obsoletus together with C. wagneri and Reptalus sp. were collected countrywide. Plant total nucleic acids (TNA) were extracted following previously described CTAB extraction protocol (Maixner et al. 2014).
Grapevine DNA samples were then analysed by triplex real-time PCR assay according to Pelletier et al. (2009). Collected insects together with different weeds were tested by conventional PCR assays with phytoplasma universal primers P1/P7 (Deng and Hiruki, 1991; Smart et al. 1995), followed by R16F2n/R2 (Gundersen and Lee, 1996) in nested PCR. Selected ‘Ca. P. solani’ strains were characterised by MLST of tuf, secY, vmp1 and stamp genes (Schneider et al. 1997; Fialova et al. 2009; Cimerman et al. 2009; Fabre et al. 2011). Sequencing of obtained amplicons was performed by the commercial service Macrogen Inc. (Amsterdam, Netherlands). PCR products were directly sequenced on both strands. Raw nucleotide sequences were assembled and edited with Sequencher™4.7 software (http://www.geneious.com/) and Geneious (http://www.geneious.com/) and then aligned with ClustalX 2.0 (Thompson et al.1997). Phylogenetic analyses were performed with MEGA 7 software (Kumar et al. 2016) by using maximum parsimony with CNI on random trees method. Bootstrap analyses were performed (500 replicates) to estimate the stability of nodes and to support the inferred clades.

III. RESULTS

Out of 1212 grapevine samples analysed by triplex-real time PCR, 208 samples were BN positive belonging to subgroup 16SrXII-A. In addition to C. arvensis and V. agnus-castus, CPs was detected also in Ailanthus altissima, Robinia pseudoacacia and Polygonum aviculare plants. Although the sampling strategy was concentrated mostly on grapevine yellows distribution in number of different locations, ‘Ca. P. solani’ was also identified in 9 H. obsoletus out of 61 and 3 out of 11 sporadically collected samples from insects of the genus Ciixius.

At least 19 different ‘Ca. P. solani’ comprehensive genotypes were detected among the selected 84 isolates, for the most part analysed on the basis of tuf/secY/vmp1/stamp gene typing. The prevalent genotype was S6-ST6-V18-tuf-b2 infecting almost one third of the analysed samples and found both in H. obsoletus and grapevine samples. Two other comprehensive genotypes were also abundant while others were relatively scarce, sporadically distributed and represented only by a few samples. As expected stamp resulted to be most variable marker with at least 11 genotypes identified. Analysis of tuf gene revealed 3 genotypes (tuf-type a, tuf-type b1 and tuf-type b2) of ‘Ca. P. solani’ strains linked to both main natural epidemic cycles within BN pathosystem.

IV. DISCUSSION

On the basis of tuf/secY/vmp1/stamp gene typing, among the analysed samples at least 19 ‘Ca. P. solani’ comprehensive genotypes were detected thus revealing tremendous complexity of BN epidemiological network in Croatian winegrowing regions. The prevalent comprehensive genotype ST6-V18-tuf-b2 in Croatian vineyards is also the one considered as an emerging strain massively propagated by high H. obsoletus populations developing on U. dioica in Austria (Aryan et al. 2014). Moreover, several genotypes found in positive H. obsoletus captured on grapevine correlated with prevalent genotypes found in grapevine, thus suggesting a key role of this vector in spread of the BN disease in the country.

However, wide genetic diversity found in limited number of ‘Ca. P. solani’ strains and undocumented presence of H. obsoletus within BN foci in some regions suggests involvement of additional insect species as vectors in BN epidemiology. In light of recent BN epidemiological studies (Cvrković et al. 2014; Kosovac et al. 2016) it is possible to speculate about the active role of different, not host-specific vectors or vectors tied to new host/source plants with different ecological dynamics in diffusion of BN strains.

In addition to H. obsoletus, previous research identified the presence of other insects from Cixiidae family in Croatian vineyards (Budinšćak 2008). New findings of ‘Ca. P. solani’ infected insect from genus Ciixius opens additional questions and more importantly stresses the need to conduct new vector-searching oriented studies to address the role of possible other key players in BN epidemics in Croatia.

With as many as 11 stamp genotypes identified so far in Croatia, stamp revealed to be the highly discriminative marker. Formation of distinct stamp genotypes could be a result of ‘Ca. P. solani’ adaptations to a new vector since adaptation to new hosts have a significant impact on phytoplasma membrane proteins, resulting in number of different genotypes as previously reported by Fabre et al. (2011).

The data obtained in the present study reveals considerable genetic variability, epidemiological relevance and the geographical distribution of ‘Ca. P. solani’ BN involved genotypes in Croatia.

<table>
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ACKNOWLEDGMENTS

The authors thank the Croatian Phytosanitary Administration for their help and affability during the official survey of FD.

Funding. This study was partially supported Croatian Science Foundation grant no. UIP-2014-09-9744 and by the Ministry of Agriculture (National Survey of Quarantine Organisms Programme).

REFERENCES


