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European Journal of Plant Pathology
Published in cooperation with the European Foundation for Plant Pathology

ISSN 0929-1873
Volume 142
Number 3

Eur J Plant Pathol (2015) 142:591-601
DOI 10.1007/s10658-015-0637-4

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A case study of FD and BN phytoplasma variability in Croatia: multigene sequence analysis approach

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Accepted: 6 March 2015 / Published online: 14 March 2015
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Abstract Uncultivable bacteria from the genus '*Candidatus* Phytoplasma' are associated with grapevine yellows (GY) diseases worldwide. In Euro-Mediterranean viticultural areas, GY are most frequently caused by Bois Noir (BN) and Flavescence Dorée (FD) phytoplasmas. Surveys of GY in Croatia have been conducted regularly since 1997. BN phytoplasmas have been found to be widespread, while FD phytoplasmas were recently discovered in restricted areas of the country. The aim of this study was to assess the variability of genotypes involved in GY pathosystems by a multilocus sequence typing (MLST) approach. Grapevine, weed and insect vector samples were collected from three locations. Species-specific *stamp* and *vmp*1 genes, together with house-keeping genes *tuf* and *sec*Y, were amplified from BN strains. In FD strains, the genes *sec*Y, *map* and *uvr*B-*deg*V were analyzed. MLST revealed a diversity of BN genotypes, one of which was prevalent and identified in samples of grapevine and the insect vector *Hyalesthes obsoletus*, corroborating their affiliation to the same pathosystem. Distinct BN strains found in bindweed and two grapevine samples indicated the presence of different BN pathosystems involving a yet unidentified vector, possibly from the genus *Reptalus*. Moreover, a co-occurence of BN and FD phytoplasma in the same vineyard was identified. The genotyping of FD strains from both grapevine and *Scaphoideus titanus* showed the presence of at least two distinct FD genotypes at two different locations, suggesting separate introductions of the disease into the country. In this study, MLST proved to be a useful and informative tool in advancing the understanding of GY epidemiology in Croatia.

Keywords Bindweed · '*Candidatus* Phytoplasma solani' · FD phytoplasma · Molecular epidemiology · MLST · Stolbur

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Introduction

Phytoplasmas are cell wall-less, plant pathogenic bacteria belonging to the class *Mollicutes*. They are phloem-limited and as such are transmitted by specific phloem-feeding insect vectors of the order Hemiptera. The lifecycle involves two ecological niches: plant phloem and insects. This contributes to the complexity of phytoplasma ecology and facilitates the evolution of unique phytoplasma strains (Bertaccini and Duduk 2009; Lee et al. 2010).

Grapevine yellows (GY) are diseases associated with phytoplasmas that are detected in all grapevine growing areas of the world and as such have a great economic impact on viticulture. Although GY symptoms are nearly identical among outbreaks, their etiology and epidemiology may differ (Pelletier et al. 2009). The two

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principal GY agents in Europe are Flavescence Dorée (FD) and Bois Noir (BN). FD phytoplasma belongs to the elm yellows 16SrV taxonomic group, subgroups C and D, and is persistently transmitted by the Cicadellidae leafhopper of American origin *Scaphoideus titanus* Ball. (Schvester et al. 1969; Pelletier et al. 2009). BN is associated with the stolbur phytoplasma (STOL) which is a member of the 16SrXII-A subgroup and is primarily transmitted by the occasional grapevine feeder, the planthopper *Hyalesthes obsoletus* Signoret (Sforza et al. 1998). However, another polyphagous cixiid species, *Reptalus panzeri* Low 1883, has recently been shown to be a successful vector of BN phytoplasma to the grapevine (Cvrković et al. 2014), while the field-trapped insects *Anaceratagallia ribauti* were able to transmit BN phytoplasma to *Vicia faba* seedlings (Riedle-Bauer et al. 2008) and *Catharanthus roseus* plants (Aryan et al. 2014). In addition to the 16Sr group classification system, there is a *Candidatus* species classification system with over 30 *Candidatus* species identified and a number of proposed 'species' within the genus. Stolbur and Bois Noir associated strains were recently assigned to a novel species taxon, '*Ca*. P. solani' (Quaglino et al. 2013). A name according to the *Candidatus* classification system has not yet been formally approved for FD phytoplasma. While FD phytoplasma is listed as a quarantine organism in the European Union due to its epidemic potential, BN phytoplasma is not disseminated as quickly but is widely distributed and more difficult to control (Pelletier et al. 2009; Maixner, 2011). FD is widespread in many viticultural regions of France and Italy. Currently, despite mandatory regulations for its control and eradication, FD is still spreading. It has been recorded in Spain, Switzerland and Austria while outbreaks have been detected in Portugal, Serbia and Slovenia (Kuzmanović et al. 2008; Mehle et al. 2011; EPPO 2014.). BN is a disease mainly reported from Europe, Asia Minor and the Mediterranean area. It is still an emerging disease with new outbreaks confirmed from various viticultural regions and in most countries is considered a predominant GY agent (Maixner 2011).

GY diseases have been continuously monitored and studied in Croatia since the late 1990s. Along with BN etiological agents that are present in most Croatian grape growing regions (Šeruga et al. 2000), FD phytoplasmas have also been detected in restricted continental areas bordering on Slovenia and Hungary since 2009 (Šeruga Musić et al. 2009; Šeruga Musić et al. 2011). The natural life-cycle of BN and FD phytoplasmas infecting grapevine have a variable epidemiology that creates dissimilar pathosystems with different phytoplasma natural reservoir species (Maixner, 2011). While *S. titanus* lives and feeds exclusively on the grapevine, *H. obsoletus* can feed on various herbaceous plants, though several species, such as *Convolvulus arvensis* L., *Calystegia sepium* L., and *Urtica dioica* L., have been reported as the most common hosts (Langer and Maixner, 2004). However, in Mediterranean BN pathosystems, other *H. obsoletus* plant hosts have been reported, including lavender (Sforza et al. 1999) and *Vitex agnus-castus* L. (Sharon et al. 2005), which may serve as potential BN phytoplasma reservoirs. In FD phytoplasma epidemiology, potential wild plant reservoirs have also been studied and identified. FD-related phytoplasmas similar to the Italian FD-C strain have been detected in *Clematis vitalba* L. plants collected from the vicinity of infected vineyards (Angelini et al. 2004) as well as in *Ailanthus altissima* Mill., an invasive tree species (Filippin et al. 2011). Furthermore, preliminary results have demonstrated that the polyphagous planthopper *Dictyophara europea*, commonly occurring on clematis, was able to transmit phytoplasma in experimental conditions from clematis to the grapevine (Filippin et al. 2009). Phytoplasmas associated with alder yellows (AldY), infecting *Alnus glutinosa* (L.) Gaertn., are phylogenetically related to FD phytoplasmas and belong to the same phylogenetic subclade (Arnaud et al. 2007). Nonetheless, they are transmitted by a different vector, the alder leafhopper *Oncopsis alni* Schrank (Maixner and Reinert 1999).

As the molecular analysis of 16S rDNA sequence alone may be insufficient for finer discrimination of phytoplasma strains, additional housekeeping genes together with specific variable genes encoding surface proteins are used for the genotyping of phytoplasma strains (Foissac et al. 2013a). The method Multi-locus sequence typing (MLST) is recognized as a reproducible and scalable system used globally in population, evolutionary and epidemiological studies of bacterial pathogens (Urwin and Maiden, 2003). This approach has already been successfully employed in studies on the genetic diversity of the fruit tree phytoplasmas '*Ca*. P. prunorum', '*Ca*. P. mali' and '*Ca*. P. pyri', where it also proved the existence of inter-species recombination between phytoplasmas (Danet et al. 2011). It has also been used for the detailed molecular characterization of

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'*Ca*. P. asteris' strain from oilseed rape (Šeruga Musić et al. 2014).

In studies of FD phytoplasmas and FD-related strains, the MLST approach proved to be crucial in the clarification of their relatedness to other members of the ribosomal group 16SrV. Phylogenetic analyses of *sec*Y, and particularly *map* and *uvr*B-*deg*V genetic loci have demonstrated that FD, AldY and palatinate grapevine yellows (PGY) phytoplasmas have a monophyletic origin and represent a distinct lineage among other members of the 16SrV group: '*Ca*. P. ziziphi', '*Ca*. P. ulmi' and rubus stunt phytoplasma (RuS). The existence of three FD phytoplasma strain clusters (FD1, FD2 and FD3) was also identified (Arnaud et al. 2007).

Along with the less variable *tuf* and *sec*Y genes, species specific *vmp*1 and *stamp* genes are used to assess the genetic diversity of BN phytoplasma strains (Cimerman et al. 2009; Pacifico et al. 2009; Fabre et al. 2011). The gene *stamp*, which encodes an antigenic membrane protein, is submitted to positive diversifying selection and may play a role in the interaction with the insect vector (Fabre et al. 2009), as shown for the protein AMP of the '*Ca*. P. asteris' (Suzuki et al. 2006; Galetto et al. 2011). Another gene unique to 'Ca. P. solani', *vmp*1, has been recognized as a relevant candidate gene for molecular typing. It encodes a putative transmembrane protein with an N-terminal hydrophilic part exposed to the phytoplasma cell surface. It is variable in size due to the variation in its repeated domains (Cimerman et al. 2009).

The aim of this research was to carry out a 1-year case study of FD and BN phytoplasma genotype variability by using multilocus sequence typing (MLST) on a limited number of samples at selected vineyard ecosystems in Croatia to demonstrate the advantages and the informative power of the multigene analyses approach. Moreover, these typing tools could enable tracing the route of introduction of phytoplasma strains and further clarification of the epidemiology of the two pathosystems in Croatia.

Materials and methods

Plant and insect samples

Throughout the 2011 growing season, three locations were chosen for a case study of grapevine phytoplasma pathosystems: Vukanovec as the BN focus, and Brckovština and Jagnjić Dol as the FD phytoplasma foci. All sampled locations are situated in the continental part of Croatia (central and north western part). In total, 15 grapevine samples exhibiting typical grapevine yellows symptoms were collected (two from Vukanovec, nine from Brckovština and four from Jagnjić Dol) together with 24 samples of the insect vector *H. obsoletus* (location Vukanovec) and 28 samples of *S. titanus* (14 from Brckovština and 14 from Jagnjić Dol). At the locations Vukanovec and Brckovština, the most abundant weed species were sampled: *Convolvulus arvensis* L. (12 samples from Vukanovec and 12 samples from Brckovština), *Urtica dioica* L. (nine samples from Vukanovec) and *Glechoma hederacea* L. (one sample from Vukanovec). In weeds, no typical phytoplasma symptoms were observed, though some of the sampled plants exhibited atypical reddening or yellowing. In order to compare the results, additional samples collected and analyzed in previous years were included in the MLST analyses: BN-positive samples 4–9 and 7–9 (location Vukanovec) from 2009, and FD-positive samples 3 and 4 (location Jagnjić Dol), and 18 and 19 (location Brckovština) from 2010. Reference phytoplasma strains, FD70 for the group 16SrV, and STOL, 19–25, PO, GGY and Charente-1 for the group 16SrXII have been maintained in periwinkle (*Catharanthus roseus* L.) by successive graft inoculation (kindly provided by Dr. X. Foissac, INRA, Bordeaux, France).

DNA extraction

Total nucleic acids (TNA) were extracted from 0.5 g of plant material using a modification of the cethyl-trimethyl-ammonium bromide (CTAB) procedure described by Šeruga et al. (2003). For nucleic acid extraction from individual insects, the commercial kit *OmniPrep*™ (G-Biosciences, USA) for the isolation of high quality genomic DNA was used according to the manufacturer's instructions. Nucleic acids were diluted in sterile deionized water to a final concentration of 20 ng/μl.

Phytoplasma detection

Insect and weed samples were tested by conventional PCR assays with phytoplasma universal primers P1/P7 amplifying 16S rDNA (Deng and Hiruki, 1991; Smart et al. 1996), followed by R16F2n/R2 in nested PCR and subsequent RFLP analysis (Lee et al. 1998). All

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grapevine samples were subjected to TaqMan triplex *real-time*-PCR assay amplifying the house-keeping gene *map* (Pelletier et al. 2009), which enables simultaneous detection of FD and BN phytoplasmas in the infected grapevine. *Real-time* PCR experiments were performed on the 7300 Real Time PCR System (Applied Biosystems, USA) in 96 well plates. Only positive samples were included for further analysis (Table 1).

Multilocus sequence typing (MLST) of phytoplasma strains

For further analyses of phytoplasma strains found in grapevine, insect and weed samples, the multilocus sequence typing (MLST) approach was used. The following gene regions were amplified from BN phytoplasma positive samples in direct and nested PCR: the *tuf* gene was amplified by the ftuf1/rtuf1 primer pair

Table 1 Detection of BN and FD phytoplasma from grapevine, insects and weed samples

Strain	Year	Host	16Sr group	Location
3	2010	Grapevine	16SrV (FD)	Jagnjić dol
4	2010	Grapevine	16SrV (FD)	Jagnjić dol
18	2010	Grapevine	16SrV (FD)	Brckovština
19	2010	Grapevine	16SrV (FD)	Brckovština
308	2011	Grapevine	16SrV (FD)	Jagnjić dol
309	2011	Grapevine	16SrV (FD)	Jagnjić dol
310	2011	Grapevine	16SrV (FD)	Jagnjić dol
311	2011	Grapevine	16SrV (FD)	Jagnjić dol
GBr1	2011	Grapevine	16SrV (FD)	Brckovština
GBr3	2011	Grapevine	16SrV (FD)	Brckovština
G8	2011	Grapevine	16SrV (FD)	Brckovština
G9	2011	Grapevine	16SrV (FD)	Brckovština
503	2011	*Scaphoideus titanus*	16SrV (FD)	Jagnjić dol
505	2011	*Scaphoideus titanus*	16SrV (FD)	Jagnjić dol
4K	2011	*Scaphoideus titanus*	16SrV (FD)	Jagnjić dol
9K	2011	*Scaphoideus titanus*	16SrV (FD)	Jagnjić dol
10K	2011	*Scaphoideus titanus*	16SrV (FD)	Jagnjić dol
11K	2011	*Scaphoideus titanus*	16SrV (FD)	Jagnjić dol
13K	2011	*Scaphoideus titanus*	16SrV (FD)	Jagnjić dol
14K	2011	*Scaphoideus titanus*	16SrV (FD)	Jagnjić dol
15K	2011	*Scaphoideus titanus*	16SrV (FD)	Jagnjić dol
16K	2011	*Scaphoideus titanus*	16SrV (FD)	Jagnjić dol
4S	2011	*Scaphoideus titanus*	16SrV (FD)	Brckovština
4-9	2009	Grapevine	16SrXII-A (BN)	Vukanovec
7-9	2009	Grapevine	16SrXII-A (BN)	Vukanovec
GVu1	2011	Grapevine	16SrXII-A (BN)	Vukanovec
GVu2	2011	Grapevine	16SrXII-A (BN)	Vukanovec
GBr2	2011	Grapevine	16SrXII-A (BN)	Brckovština
GBr4	2011	Grapevine	16SrXII-A (BN)	Brckovština
H17	2011	*Hyalesthes obsoletus*	16SrXII-A (BN)	Vukanovec
H18	2011	*Hyalesthes obsoletus*	16SrXII-A (BN)	Vukanovec
H21	2011	*Hyalesthes obsoletus*	16SrXII-A (BN)	Vukanovec
H24	2011	*Hyalesthes obsoletus*	16SrXII-A (BN)	Vukanovec
Br8	2011	Bindweed	16SrXII-A (BN)	Brckovština

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(Schneider et al. 1997) followed by ftufAY (Schneider et al. 1997) / rtufStol (modified from Schneider et al. 1997; 5′- CGTTGTCACCTGGCATAACC-3′); *sec*Y gene fragments were amplified using the primer pair PosecF1/PosecR1 followed by PosecF3/PosecR3 (Fialová et al. 2009); the *vmp*1 gene was amplified with STOLH10F1/STOLH10R1 (Cimerman et al. 2009) followed by the primer pair TYPH10F/TYPH10R (Fialová et al. 2009) and the *stamp* gene was amplified as described by Fabre et al. (2011).

MLST of FD strains was performed on the *sec*Y, *uvr*B-*deg*V and *map* genes. All PCR and nested PCR amplifications of the *sec*Y, *uvr*B-*deg*V and *map* genes were performed according to Arnaud et al. (2007).

PCR products were separated in 1 % agarose gel, stained with Olerup GelRed solution (Olerup SSP, Sweden) and visualized under UV light using a gel documentation system.

For routine BN strain typing, amplified products of the *tuf* and *vmp*1 genes were digested with *Hpa*II (Promega, USA) and *Rsa*I (*New England Biolabs*, UK) respectively, according to the manufacturer's instructions. To characterize FD phytoplasma strains, the amplified product of the *map* gene was double digested with *Alu*I and *Eco72*I restriction enzymes (*New England Biolabs*, UK), according to Arnaud et al. (2007) and following the manufacturer's instructions.

The restriction products were subsequently separated in 5 or 8 % polyacrylamide gel electrophoresis in 1× TBE buffer (90 mM Tris-borate, 1 mM EDTA), stained with ethidium bromide and visualized on an UV trans-illuminator. Only *tuf* gene amplicons were analyzed by 2.5 % agarose gel electrophoresis, stained with Olerup solution and visualized under UV light using a gel documentation system.

Sequencing and phylogenetic analysis

Sequencing of all obtained amplicons for the *sec*Y and *stamp* genes of BN strains, and the *sec*Y, *uvr*B-*deg*V and *map* genes of FD strains was performed by the commercial service Macrogen Inc. (Seoul, Republic of Korea). PCR products were directly sequenced on both strands and subsequent phylogenetic analyses were performed. Raw nucleotide sequences were assembled and edited with the Sequencher™ 4.7 software (http://www.genecodes.com/) and then aligned with ClustalX 2.0 (Thompson et al. 1997). Representative sequences were deposited in GenBank. Phylogenetic analyses were performed with MEGA 5 software (Tamura et al. 2011) by using the neighbour-joining method with the number of differences model and 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.

Results

Genetic variability of FD phytoplasma strains

The presence of FD phytoplasma was confirmed by *real-time* PCR and by conventional 16S rDNA PCR/RFLP in all four grapevine samples from Jagnjić Dol, in four of nine grapevine samples collected at Brckovština in 2011, and in four grapevine samples from 2010 (Table 1). The analyses of *S. titanus* samples from the corresponding locations resulted in 10 of 14 positive insects from Jagnjić Dol and only one of 14 from Brckovština (Table 1). Amplicons of all four analyzed gene regions were obtained for 13 of 19 FD-positive grapevine and insect samples from the 2011 study, and for all four grapevine samples from 2010. No FD phytoplasmas were detected in the grapevines from Vukanovec or in any of the tested weed samples.

RFLP analysis of doubly digested *map* gene amplicons revealed different restriction patterns. Both grapevines and insects originating from Brckovština showed a profile characteristic of the Map-FD2, while those from Jagnjić Dol had a profile of the Map-FD3 cluster (not shown). Results were corroborated by phylogenetic analyses of the amplified *map* gene. It was shown that sequences of samples originating from two different locations had clearly distinct genotypes regardless of the year of sampling, with *map* sequences of strains from Jagnjić Dol sharing 100 % identity (ID) with the sequence originating from Tuscany grapevine (Fig. 1a). Phylogenetic analyses of the *uvr*B-*deg*V and *sec*Y gene (Fig. 1b and c) were in agreement with the existence of two separate FD strain clusters that always corresponded to the strain clusters described for the *map* gene sequences. Moreover, the two gene regions were shown to be more variable, with single nucleotide polymorphisms (SNPs) found in both the *sec*Y and *uvr*B-*deg*V sequences. Phylogenetic trees always displayed the same topology regardless of the method used (neighbour-joining method or maximum parsimony).

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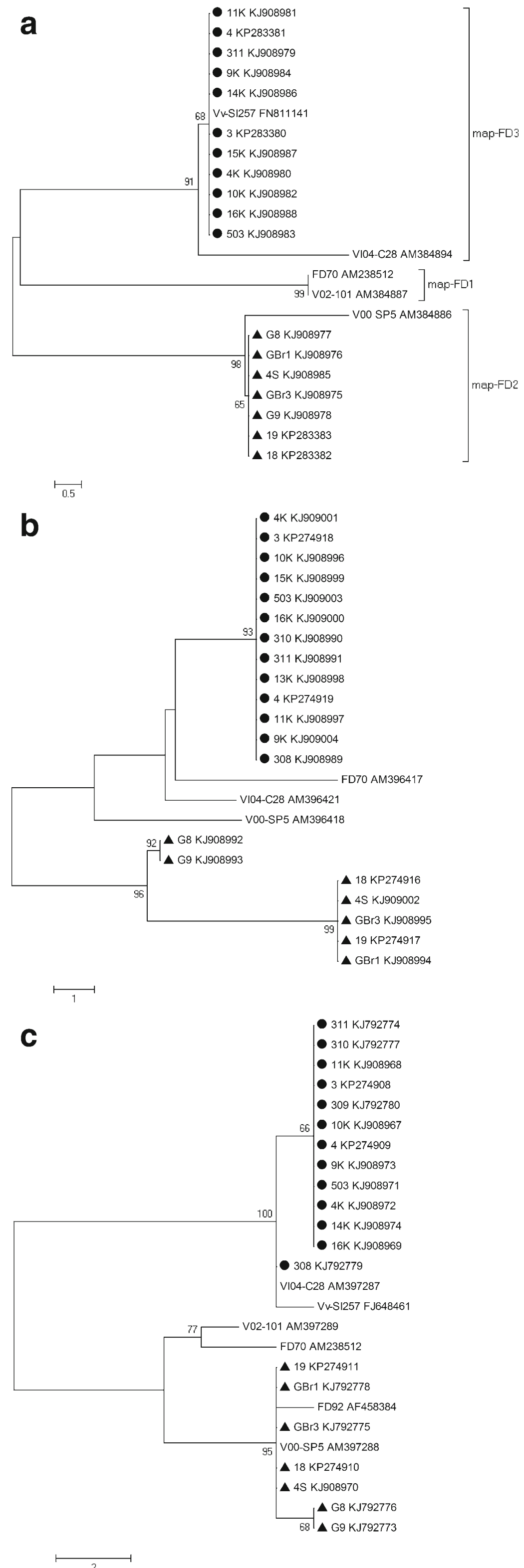

Fig. 1 Phylogenetic trees (NJ method, number of differences) ▶ constructed on the basis of FD phytoplasma (**a**) *map*, (**b**) *uvr*B-*deg*V and (**c**) *sec*Y gene. GenBank accession numbers are given next to the name of each strain. Grapevine and *Scaphoideus titanus* Ball samples from Jagnjić Dol are marked with black dots, while the black triangles denote grapevine and *S. titanus* samples from Brckovština

Genetic variability of BN phytoplasma strains

The presence of BN phytoplasma was confirmed in both symptomatic grapevines sampled at Vukanovec, in four of 24 *H. obsoletus* samples from 2011, and in samples 4–9 and 7–9 from 2009 (Table 1). No phytoplasmas were detected in any of the 12 weed samples collected from the same location. In Brckovština, BN phytoplasma was detected in two of nine tested grapevines and in one of nine tested bindweed samples, while no insect vectors were captured. Amplicons of all four genes for further analysis were successfully obtained from all BN-positive samples.

The restriction analysis with *Hpa*II revealed that all *tuf* gene amplicons from grapevine, insects and *C. arvensis* shared the same tuf-b restriction profile, except for one grapevine sample 7–9 from Vukanovec that had the pattern characteristic of tuf-a (Fig. 2). An identical *vmp*1 profile (V18) was obtained after all grapevine and insect amplicon RFLP analyses from the 2011 samples, whereas the amplicons obtained from the weed *C. arvensis* had a different restriction profile (V4). Samples 4–9 and 7–9 from 2009 showed RFLP profiles corresponding to V2 and V3, respectively (Fig. 3; Murolo et al. 2010, 2014; Cvrković et al. 2014). Phylogenetic analyses of the *sec*Y gene showed that sequences from grapevine and insects from 2011, and from the sample 7–9 belong to the same S6 group. The *sec*Y sequences of the grapevine sample 4–9 from Vukanovec and *C. arvensis* from Brckoviština showed an affiliation with the S4 phylogenetic group (Fig. 4a) (Foissac et al. 2013b). Phylogenetic analyses of the *stamp* gene also revealed greater variability and the presence of more than two *stamp* genotypes among the analyzed samples. All *stamp* sequences of grapevine and insects sampled in 2011 clustered together, while the one from bindweed grouped with the *stamp* gene sequence originating from the insect vector species of the genus *Reptalus* from Serbia (Fig. 4b; Cvrković et al. 2014). The sequence from the 4–9 grapevine sampled in 2009 shared 100 % identity (ID) with the sequences originating

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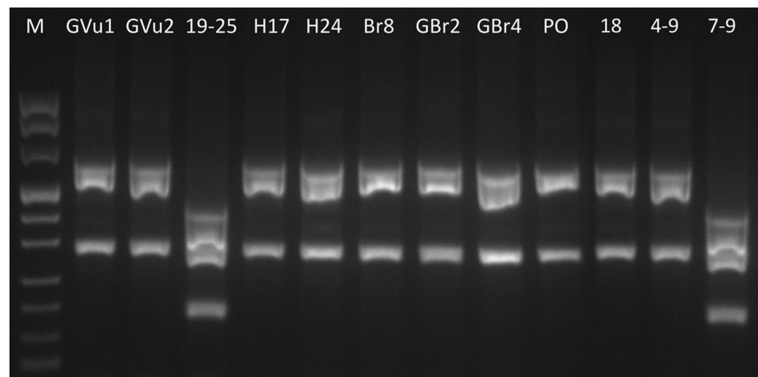

Fig. 2 Agarose gel (2.5 %) showing representative *Hpa*II RFLP profiles of BN phytoplasma *tuf* gene amplicons from grapevine (GVu1, GVu2, GBr2, GBr4, 18, 4–9, 7–9), *Hyalesthes obsoletus* Signoret (H17, H24) and bindweed (Br8) samples, as well as from reference strains 19–25 and PO. M - 1 kb DNA ladder

from Serbian grapevine and *R. quinquecostatus* (Fig. 4b; Cvrković et al. 2014), whereas the 7–9 *stamp* sequence was identical to that originating from *Urtica dioica* (Fig. 4b; Johannesen et al. 2012). The results of the phylogenetic analyses were consistent regardless of the method used (neighbour-joining method or maximum parsimony), with obtained trees always displaying the same topology.

Discussion

Several genes or gene regions have been employed as additional molecular markers in phytoplasma analyses for finer differentiation among BN and FD phytoplasma strains associated with GY diseases (Lee et al. 2010). This multiple-gene based system uses evolutionary differently conserved molecular markers – non-ribosomal housekeeping genes: *tuf*, *map*, *uvr*B-*deg*V, *sec*Y and variable genes encoding surface proteins: *vmp*A, *vmp*1 and *stamp* (Foissac et al. 2013a). The study of these genes using MLST gives insight into the diversity and distribution of different types of strains, and clarifies the interaction between members of the pathosystems (Danet et al. 2011).

In the scope of this study, FD phytoplasmas were detected at two of three tested locations: Jagnjić Dol and Brckovština. As FD phytoplasmas have only recently been reported in Croatia (Šeruga Musić et al. 2009; Šeruga Musić et al. 2011), this is also the first detailed genotyping of this pathogen. MLST analyses on the basis of the *map*, *sec*Y and *uvr*B-*deg*V genes clearly distinguished the genotypes found at each location. FD strains of grapevine and insects from the location Jagnjić Dol clustered with sequences belonging to the Map-FD3 group, corresponding to the reference strain FD-

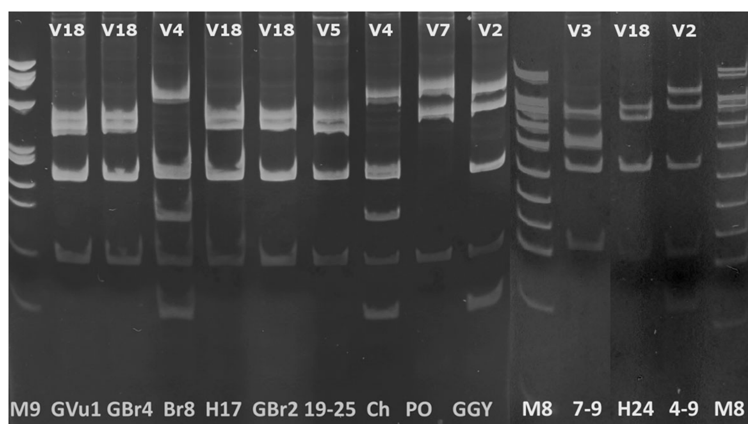

Fig. 3 Polyacrylamide gel (8 %) showing representative *Rsa*I RFLP profiles of *vmp*1 BN phytoplasma gene amplified from grapevine (GVu1, GBr2, GBr4, 4–9, 7–9), *Hyalesthes obsoletus* Signoret (H17, H24) and bindweed (Br8) samples, as well as from reference strains 19–25, Charente-1, PO and GGY. M8 – pUC mix molecular marker (Marker 8) with fragment sizes from 1118 to 19 bp; M9 - ΦX174 DNA digested with *Hae*III molecular marker (Marker 9) with fragment sizes from 1353 to 72 bp

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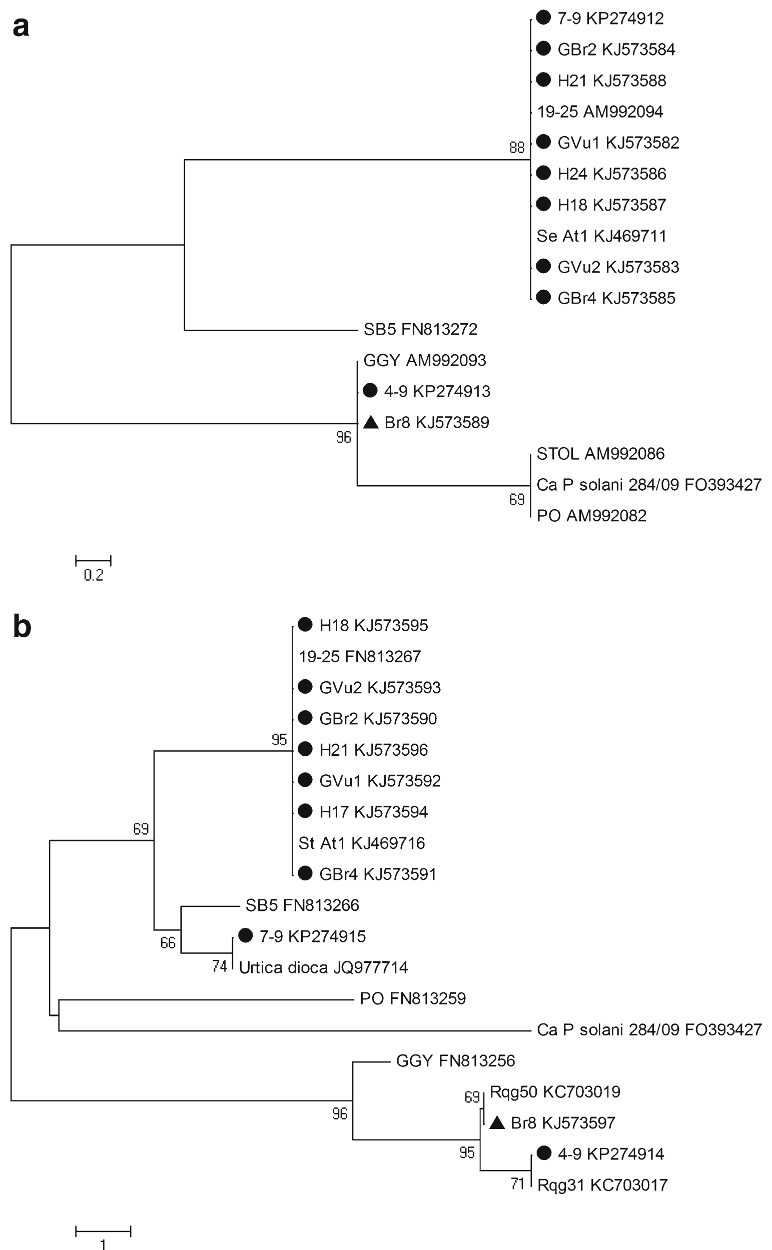

Fig. 4 Phylogenetic trees (NJ method, number of differences) constructed on the basis of BN phytoplasma (**a**) *sec*Y and (**b**) *stamp* gene. GenBank accession numbers are given next to the name of each strain. Grapevine and *Hyalesthes obsoletus* Signoret samples from Brckovština and Vukanovec are marked with black dots, while the black triangle denotes bindweed sample from Brckovština

C, while those from the location Brckovština grouped with the sequence from the cluster Map-FD2 corresponding to the reference strain FD92, according to Arnaud et al. (2007) (Fig. 1a). Two other analyzed loci corroborated this result, as the strains from the same location always formed a separate branch. However, greater variability was found for the *uvr*B-*deg*V and *sec*Y loci, allowing finer differentiation of the strains G8 and G9 belonging to the cluster Map-FD2 (Fig. 1b and c). Each of the previously described strain clusters differs in its geographic range; Map-FD2 is widespread and commonly detected in France and Italy whereas Map-FD3 is more restricted. It has been found in Italy (Arnaud et al. 2007), and in two countries neighbouring Croatia: Slovenia (Mehle et al. 2011) and Serbia (Kuzmanović et al. 2008). The presence of distinct strains and different genotypes at each of these two locations could indicate separate origins of the disease, i.e., separate introductions of FD phytoplasma into the country. Although FD-related phytoplasmas similar to

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the Italian FD-C strain were previously detected in *Clematis vitalba* L. from Istria (coastal Croatia) (Filippin et al. 2009), no clematis plants were found in the vicinity of sampled vineyards in this study. A survey on wild plant reservoirs potentially involved in FD epidemiology, such as alder or clematis, would be a useful direction for future study.

Multilocus sequence typing of the BN strains showed that all samples from grapevine and insects from Vukanovec collected in 2011 and grapevine samples from Brckovština shared the same genotype for all four analyzed genes: *sec*Y, *stamp*, *tuf* and *vmp*1. Moreover, it was revealed that this prevalent genotype shared 100 % sequence ID in the *stamp* and *secY* genes with the genotype named CPsM4_At1, which is also known to be prevalent in *U. dioica*, grapevine and *H. obsoletus* in Austria (Fig. 4; Aryan et al. 2014). This result is in accordance with the geographical positions of the investigated grapevines in continental Croatia and the open natural routes for vector migrations. As demonstrated by Aryan et al. (2014), this distinct BN genotype showed a clustering of the *stamp*, *sec*Y and *vmp*1 sequences with the nettle derived genotypes. However, in the RFLP analysis of the *tuf* gene, this strain displayed the tuf b pattern characteristic of those associated with bindweed. Nonetheless, the sequencing of the *tuf* gene revealed a single nucleotide change from C to T at position 666 responsible for the different restriction pattern when digested with *Hpa*II. Hence, this distinct *tuf* genotype is referred to as tuf b2. Both grapevine samples from Vukanovec collected in 2009 had specific genotypes that differed from those prevalent in 2011. In addition, the genotype characterized from sample 4–9 appeared to be similar to the comprehensive genotype Rqg31g found in grapevine and in *R. quinquecostatus* insects from Serbia (Fig. 4; Cvrković et al. 2014). The BN phytoplasma strain found in *C. arvensis* from Brckovština also showed considerable genetic variability in all of the analyzed genes except *tuf*. The phytoplasma strain from *C. arvensis* had a V4 restriction *vmp*1/*Rsa*I profile and the phylogenetic analysis showed that the *stamp* sequence of this strain also shared 100 % identity to the Rqg50 strain sequence found in *R. quinquecostatus* and *R. panzeri* insect vectors from Serbia. However, it differed in the *sec*Y genotype (Fig. 4; Cvrković et al. 2014). Overall, the results of MLST analyses of all BN strains from this study reflect the variability and dynamics of genotypes present at the studied locations. This suggests the co-existence of different BN phytoplasma genotypes and pathosystems at the same location, one involving *H. obsoletus* as the most common insect vector of BN phytoplasma in Europe, and the other involving a yet unidentified vector, possibly *R. panzeri* or *R. quinquecostatus*. Although *R. panz*eri is recognized as a vector of '*Ca*. P. solani' strains associated with maize redness disease (Jović et al. 2009), it also showed the ability to experimentally transmit BN phytoplasma to grapevine seedlings, while in the same study, *R. quinquecostatus* insects did not perform successful transmission (Cvrković et al. 2014). As *R. quinquecostatus* was found to harbour the BN genotype that is also present in the grapevine, it was speculated that *R. quinquecostatus* could be involved in an intermediate epidemiological cycle, increasing the phytoplasma incidence in reservoir-plants of vineyards for subsequent transmission to the grapevine by another successful vector. Moreover, Pinzauti et al. (2008) indicated that *R. quinquecostatus* had the ability to inoculate BN phytoplasma in an artificial feeding medium, thus strengthening the hypothesis that this species is capable of phytoplasma vectoring. Though the presence of *R. panzeri* and *R. quinquecostatus* has previously been recorded in the vineyards of continental and coastal Croatia (Mikec et al. 2006; Budinšćak 2008), in the present study, no insects of these two species were found at the sampling locations. Despite this, the involvement of different insect vector populations in the BN epidemiological cycles should be considered in the future with the use of genotyping tools such as MLST. The emphasis could be placed on the variability of the *vmp*1 gene, which is possibly involved in phytoplasma adaptation to different insect vector populations and plant hosts.

Overall, these results have shown the presence of considerable genetic variability amongst a relatively small number of analyzed FD and BN strains from Croatian grapevines, including the presence of different phytoplasma species and distinct genotypes of the same phytoplasma at a single location. Moreover, at the location Brckovština, the BN and FD phytoplasma pathosystems were found to co-occur.

In the present study, MLST proved to be a useful and informative tool for gaining insight into the diversity and prevalence of genotypes belonging to the same phytoplasma species. Furthermore, the results present a positive contribution to the better understanding and clarification of GY disease epidemiology in Croatia,

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aimed at enabling the tracing of the spread of phytoplasma strains in the future.

Acknowledgments The authors would like to acknowledge Dr. Xavier Foissac for kindly providing reference strains.

Compliance with Ethical Standards This research fully complies with Ethical Standards applicable for this journal and the relevant national and international ethics related rules and professional codes of conduct.

Funding This study was funded by the Croatian Ministry of Science Education and Sports (grant number 119-1191192-1222) and by the Ministry of Agriculture (National Survey of Quarantine Organisms Programme).

Conflict of interest The authors declare that they have no conflict of interest.

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