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The molecular epidemiology of bois noir grapevine yellows caused by '*Candidatus* Phytoplasma solani' in the Republic of Macedonia

Biljana Atanasova · Miljana Jakovljević · Dušan Spasov · Jelena Jović · Milana Mitrović · Ivo Toševski · Tatjana Cvrković

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Abstract Bois noir (BN), which is induced by '*Candidatus* Phytoplasma solani' ('*Ca*. P. solani'), is an important grapevine yellows disease that causes severe damage in viticultural regions throughout the Euro-Mediterranean basin. An epidemiological survey to determine potential insect vectors and the primary reservoir plants of BN phytoplasma in Macedonian vineyards was undertaken between 2012 and 2013 in the southeastern part of the country. A study on the species diversity from the suborder Auchenorrhycha revealed the prevalence of the principal vector of '*Ca*. P. solani', which is the planthopper *Hyalesthes obsoletus*. *Reptalus panzeri*, which is the second-most documented BN vector, was not recorded in Macedonian vineyards. Three leafhopper species, namely *Psammotettix alienus*, *Artianus manderstjernii* and *Euscelis incisus*, were also widespread in the BN-affected vineyards, but only *H. obsoletus* tested positive for '*Ca*. P. solani'. Molecular characterizations were performed by the sequencing and/or RFLP typing of *tuf*, *vmp1* and *stamp* genes, and the results were used to gain detailed insight into the molecular diversity of the '*Ca*. P. solani' strains associated with grapevines, tentative reservoir plants (*Urtica dioica* and *Convolvulus arvensis*) and the *H. obsoletus* associated with these plants. Among the 91 '*Ca*. P. solani' strains detected in diverse plant and insect hosts, three *tuf*, five *vmp1* and 11 distinct *stamp* genotypes were identified. Twelve comprehensive genotypes of '*Ca*. P. solani' were detected according to the *tuf*/*vmp1*/*stamp* genotyping. The highest diversity of genotypes was detected among the strains from *H. obsoletus* individuals associated with *U. dioica*, of which the most frequent genotype was tuf-ab/V18/M1 (43 %). The tuf-b/V2-TA/STOL comprehensive genotype was found in 33 % of naturally infected grapevines. Two '*Ca*. P. solani' genotypes were associated with *U. dioica*, namely (i) tuf-ab/V18/M1 (60 %) and tuf-a/V3/M4 (40 %), and only one genotype (tuf-b/V2-TA/Rqg50) was associated with *C. arvensis*.

Keywords Grapevine yellows · Molecular epidemiology · *Hyalesthes obsoletus* · *Stamp* variability · Stolbur

B. Atanasova · D. Spasov
Štip, Faculty of Agriculture, Goce Delčev University, Goce Delčev bb, 2400 Strumica, R. Macedonia

M. Jakovljević · J. Jović · M. Mitrović · I. Toševski · T. Cvrković (✉)
Department of Plant Pests, Institute for Plant Protection and Environment, Banatska 33, 11080 Zemun, Serbia
e-mail: tanjacvrkovic@yahoo.com

I. Toševski
CABI, 1 Rue des Grillons, 2800 Delémont, Switzerland

Introduction

Bois noir (BN) is an important grapevine yellows disease, and it is induced by the stolbur phytoplasma from the 16SrXII-A subgroup that was recently described as '*Candidatus* Phytoplasma solani' ('*Ca*. P. solani') (Quaglino et al. 2013). The disease is widespread all over Europe, the Mediterranean area and in the Middle

East and causes serious economic losses in grapevine production (Johannesen et al. 2012; Aryan et al. 2014; Cvrković et al. 2014).

Phytoplasmas are wall-less, non-helical prokaryotes, and they are members of the class *Mollicutes* that colonize plant phloem. These organisms are obligatorily transmitted by insects, grafting or parasitic plants (Weintraub and Beanland 2006). The insect vectors of phytoplasmas include leafhoppers, planthoppers and psyllids, and these insects belong to the suborders Auchenorrhyncha and Sternorrhyncha, order Hemiptera.

'*Ca*. P. solani' is an emerging plant pathogen that causes yellows diseases in grapevines (bois noir) and various cultivated plants of economic importance including potatoes, maize, sugar beets and others (Gatineau et al. 2002; Jović et al. 2009, 2011). The documented vectors that transmit '*Ca*. P. solani' to grapevines are polyphagous planthoppers of xerothermic habitats, namely *Hyalesthes obsoletus* and *Reptalus panzeri*, both of which belong to the Cixiidae family (Maixner 1994; Cvrković et al. 2014). Nevertheless, other species such as *R. quinquecostatus* and *Anaceratagallia ribauti* are also known to harbor '*Ca*. P. solani' infections and were demonstrably able to transmit the pathogen to experimental plants or artificial feeding medium; however, their ability to vector the pathogen to grapevines has not yet been demonstrated (Pinzauti et al. 2008; Riedle-Bauer et al. 2008; Aryan et al. 2014).

The epidemiology of phytoplasma-induced diseases is determined by the interaction between the vector and pathogen and their common natural hosts. In the case of BN, grapevines are an erroneous food substrate for *H. obsoletus* and, consequently, a dead-end host for '*Ca*. P. solani' (Johannesen et al. 2012). The epidemiology of BN is coupled to the infection of herbaceous host plants, which are the primary food sources for *H. obsoletus* nymphs and pathogen reservoirs. According to observations of the elongation factor Tu (*tuf*) gene, the '*Ca*. P. solani' consists of two genetically divergent strain types, *tuf-a* and *tuf-b*, which are involved in the two diverse epidemiological cycles of BN (Langer and Maixner 2004). The *tuf-b* type is primarily associated with field bindweed (*Convolvulus arvensis*), the major host plant of *H. obsoletus* in the eastern and southeastern occurrences of the disease (Langer and Maixner 2004; Johannesen et al. 2012), although it infects a number of diverse weedy plants (Riedle-Bauer et al. 2008; Johannesen et al. 2012; Cvrković et al. 2014). *Tuf-a* strains of '*Ca.* Phytoplasma solani' are spread via an epidemic cycle sourced in stinging nettle (*Urtica dioica*) which is the only documented herbaceous reservoir of this '*Ca*. P. solani' genotype (Langer and Maixner 2004; Johannesen et al. 2012). The nettle-sourced epidemiological cycle is the most common in the northwestern disease range (Germany, Switzerland and northern France), and it was recently registered as the cause of an epidemic BN outbreak in Austria (Aryan et al. 2014).

In addition to the epidemiological significance of the *tuf* housekeeping gene, two stolbur-specific genes encoding putative membrane proteins that are involved in host recognition and interaction, namely *vmp1* and *stamp*, are proposed for the characterization of genetic diversity in '*Ca*. Phytoplasma solani' through a multilocus sequencing approach (Cimerman et al. 2009; Fabre et al. 2011; Pacifico et al. 2009). These two genes have higher sequence variability than the *tuf* gene, and thus they are more widely used in epidemiological studies of BN phytoplasmas (Johannesen et al. 2012; Aryan et al. 2014; Cvrković et al. 2014; Kostadinovska et al. 2014).

The grapevine is one of the most important cultivated plants and has a long tradition of cultivation and economic significance in the Republic of Macedonia. The presence of BN has been reported in different viticultural regions of Macedonia (Šeruga et al. 2003), infecting diverse grapevine varieties with a constantly increasing incidence of infection throughout the vineyards (Kostadinovska et al. 2014). However, there is a lack of information about the diversity of planthoppers and leafhoppers in affected vineyards and their surroundings, potential insect vectors and the putative reservoir plants that are involved in the epidemiological cycle(s) of BN phytoplasma in Macedonian vineyards. Therefore, the aim of this study was as follows: (i) to identify insect species from the suborder Auchenorrhyncha that occur in BN-affected vineyards and that harbor '*Ca*. P. solani', and (ii) to examine the epidemiologically informative *tuf*, *vmp1* and *stamp* genes of '*Ca*. P. solani' strains in naturally infected grapevines, insects and reservoir plants to elucidate the epidemiological cycle(s) involved in BN transmission in Macedonia.

Material and methods

A survey of potential insect vectors

A survey of potential phytoplasma vectors in the suborder Auchenorrhyncha was performed in 2012 and 2013.

The survey sites included three vineyards with symptoms of phytoplasma infection in the southeastern Macedonian viticulture region. Insects were collected every 15 days from May 15th until the end of September. Potential hemipteran vectors including leafhoppers, planthoppers and cixiids were collected from grapevines, along the inter-rows, in the rows and around the vineyard on different herbaceous and woody plants, with a focus on the major documented hosts of '*Ca*. P. solani' - *H. obsoletus*, *C. arvensis* and *U. dioica*. Insects were collected with sweep nets and mouth aspirators. The insects collected for PCR analyses were placed in 2-ml plastic vials (Sarstedt) containing 96 % ethanol, and they were subsequently identified to the species level with taxonomic keys provided by Holzinger et al. (2003) and Biedermann and Niedringhaus (2004).

Plant sampling

Symptomatic grapevines and the predominant weeds inside and around the vineyards were collected for '*Ca*. P. solani' detection and multilocus sequence typing.

In the beginning of September 2012, leaves with symptoms of phytoplasma infection, such as the rolling of leaf margins and partial discoloration, were sampled from vineyards. Fresh leaf veins and petioles were dissected, distributed into 1 g aliquots and stored at −20 °C prior to DNA extraction.

During August and at the beginning of September 2013, 118 samples of the two most abundant weeds, *C. arvensis* and *U. dioica*, were collected from the study vineyards. Because they were asymptomatic, the weeds were sampled randomly, dug out with roots that were later sliced, distributed into 0.5-1.0 g aliquots and stored at −20 °C until DNA extraction.

DNA extraction

Total nucleic acids were extracted from fresh grapevine leaf midribs and petioles and from weed roots by using the CTAB protocol described by Angelini et al. (2001).

To detect '*Ca*. P. solani' in the insects, we analyzed species that were represented by more than a 100 individuals per collection year, along with all the specimens of cixiid species that were present in the vineyards. The insects were analyzed in pools of 3–5 adults, depending on the specimen size, or individually in the case of cixiids. DNA was isolated by applying a modified CTAB method according to Gatineau et al. (2001).

'*Ca*. P. solani' detection in plants and insects

The presence of '*Ca*. P. solani' in field-collected plant and insect material was detected by using a modification of the stolbur-specific Stol11 protocol with an F2/R1 primer pair for direct PCR, followed by F3/R2 for nested PCR (Clair et al. 2003). DNA amplification was performed in a 20 μl reaction volume by following amplification conditions according to Radonjić et al. (2009). DNA extracts of BN-infected grapevines from Serbia (Cvrković et al. 2014) were used as a positive control in all amplification reactions. Amplified products were separated on a 1 % agarose gel by electrophoresis in TBE buffer (Tris-Borate 90 mM, EDTA 1 mM), stained with ethidium bromide and visualized under a UV transilluminator.

Characterization of '*Ca*. P. solani' based on RFLP and sequence typing

The amplification of the following three phytoplasma genomic loci was performed for the molecular characterization of '*Ca.* P. solani' strains detected in grapevine plants, insects and weedy reservoir plants: (i) the *tuf* gene encoding the translation elongation factor Tu, (ii) the *vmp*1 gene encoding a putative '*Ca.* P. solani' membrane protein, and (iii) the *stamp* gene encoding the antigenic membrane protein in '*Ca.* P. solani'.

Tuf gene

Tuf gene amplification was performed by nested PCR with the primers Tuf1f/r, followed by TufAYf/r as described by Langer and Maixner (2004). The amplicons obtained by nested PCR were subjected to restriction analysis with *Hpa*II endonuclease to obtain information about the *tuf* type present in the collected material. Restriction analyses were performed according to the manufacturer's instructions (Fermentas, Lithuania). The restriction products were separated by automated capillary electrophoresis by using a QIAxcel advanced system (Qiagen) with a Screening Gel Cartridge (Qiagen) under the following parameters from the applied method: sample injection voltage 5 kV, sample injection time 8 s, separation voltage 6 kV and separation time 320 s. The QX alignment marker for 15 bp/5 kb (Qiagen) was

used to align the resulting restriction fragments and the QX DNA size marker FX174/HaeIII (Qiagen) was used for fragment size comparisons. The DNA of '*Ca*. P. solani' *tuf-a* and *tuf-b* types was isolated from naturally infected *H. obsoletus* from the Middle-Rhine and Mosel regions of Germany, respectively (as provided by M. Maixner, Bernkastel-Kues), and they were used as the reference controls to compare with the restriction profiles. Strains with the *tuf-b* restriction profile that were associated with *stamp* and *vmp1* types and generally considered to be associated with the nettle were additionally subjected to a sequencing analysis of the *tuf* nested products. Sequencing was performed by Macrogen Inc. (Seoul, South Korea) and the sequences are deposited in the NCBI GenBank under the accession numbers KP337324-7. The *tuf* sequences were edited by using FinchTV v.1.4.0 (http://www.geospiza.com) and aligned with the reference strains (Aryan et al. 2014) by using Clustal W as integrated into MEGA5 software (Tamura et al. 2011) to compare the SNPs associated with each of the tuf-a/-b strains.

Vmp1 gene

The *vmp*1 gene amplification was performed as a nested PCR with the primer pair StolH10F1/R1 (Cimerman et al. 2009), followed by primer pair TYPH10F/R (Fialová et al. 2009), by using reaction conditions specified by Fialová et al. (2009). The TYPH10F/R amplicons of all the characterized strains were digested with *Rsa*I restriction enzyme, and for some of the resulting profiles, an additional *Taq*I and *Alu*I digestion was performed to distinguish between the V2 and V2-TA *vmp1* profiles. The restriction fragments were separated by capillary electrophoresis as described above. The phytoplasma strains employed as references for *vmp1* restriction pattern comparison were taken from Cvrković et al. (2014) or provided by X. Foissac (Bordeaux-France) (Fig. 1).

Stamp gene

The *stamp* gene, which encodes the antigenic membrane protein in '*Ca*. P. solani', was amplified by nested PCR with StampF/R0 followed by StampF1/R1 primers, with PCR conditions according to Fabre et al. (2011). The resulting amplicons were sequenced by Macrogen Inc. by using the forward primer only and the sequences are deposited in the GenBank database under accession numbers KP337309-23.

The *stamp* sequences were edited with FinchTV v.1.4.0 and compared with reference *stamp* strains (Fabre et al. 2011; Johannesen et al. 2012; Aryan et al. 2014; Cvrković et al. 2014; Kostadinovska et al. 2014).

All phylogenetic analyses were conducted under the GTR+I+G nucleotide substitution model as chosen by jModeltest 2.1.7 (Darriba et al. 2012) according to the Akaike information criterion (AIC). Bayesian and maximum parsimony (MP) approaches were applied. The Bayesian analysis was performed in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) with the settings as follows: two simultaneous Markov Chain Monte Carlo (MCMC) runs for one million generations, with a sampling frequency of 100 generations and a relative burn-in of 25 %. The convergence of the MCMC chains and their stationarity were checked by using Tracer 1.5 (Rambaut and Drummond 2009). The MP analysis was conducted with PAUP* 4.0b10 (Swofford 2002). One hundred replicates of a heuristic search were performed with an initial random stepwise addition of sequences and tree bisection-reconnection (TBR) branch-swapping. The trees obtained from both analyses were visualized in FigTree 1.4 (Rambaut 2012).

Results

Auchenorrhyncha species in the vineyards of Macedonia

The surveys performed in and around vineyards in 2012 and 2013 led to the collection of 1180 Auchenorrhyncha specimens, which belonged to 29 species from the following six families: Cicadellidae (21), Aphrophoridae (3), Cixiidae (2), Delphacidae (1), Dictyopharidae (1) and Issidae (1) (Table 1). Despite the high diversity of Auchenorrhyncha, only three species were found in numbers greater than 200 over the 2 years survey. The predominant species was the primary BN vector *H. obsoletus*, followed by leafhoppers *Psammotettix alienus*, *Artianus manderstjernii* and *Euscelis incisus*; the species *Dictyophara europaea*, *Cicadella viridis*, *Doratura impudica* and *Anaceratagallia ribauti* were collected in numbers <50. The other cixiid vector of BN, *R. panzeri*, was not recorded in the inspected vineyards, and only four *R. quinquecostatus*

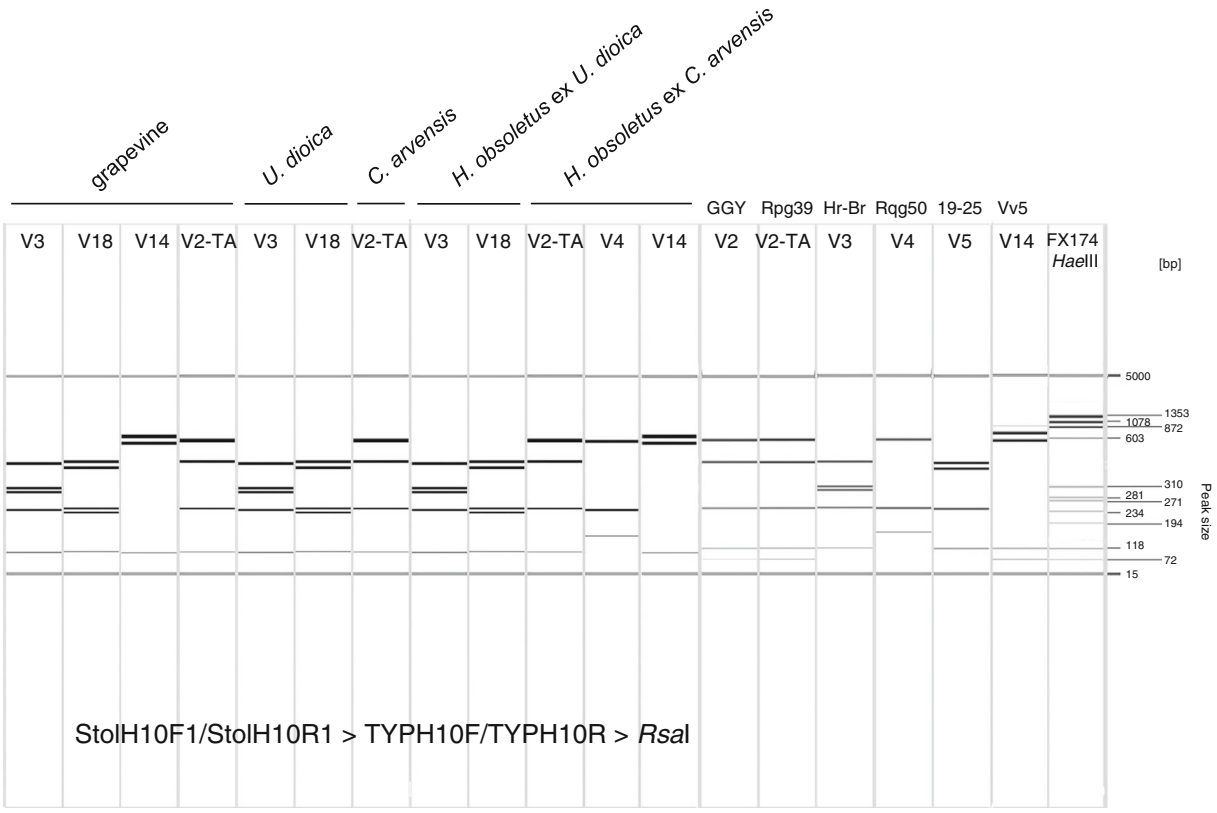

Fig. 1 *Rsa*I RFLP profiles of the *vmp1* marker for '*Candidatus* Phytoplasma solani' associated with different hosts in BN-diseased vineyards in Macedonia and reference strains. The restriction fragments were separated by automated capillary electrophoresis by using the QIAxcel advanced system (Qiagen). The following phytoplasma strains were employed as references for *vmp1* restriction pattern comparisons: GGY, Sp-infected grapevine from Germany, V2 profile; Rpg39, Sp-infected *R. panzeri* from Serbia, V2-TA profile; Hr-Br (HR-BR18-09), Sp-infected grapevine from Croatia, V3 profile; Rqg50, Sp-infected *R. quinquecostatus* from Serbia, V4 profile; 19–25, Sp-infected grapevine from Germany, V5 profile; and Vv5, Sp-infected grapevine from Serbia, V14 profile. Reference strains were taken from Cvrković et al. (2014) or provided by X. Foissac (Bordeaux-France). FX174/*Hae*III: QX DNA size marker (Qiagen). The fragment sizes (bp) of the marker (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72) and alignment marker QX 15 bp/5 kb (15 and 5000) are designated

individuals were collected as suspect vectors along the weed-covered borders adjacent to the vineyards. For 18 species, <10 total specimens were identified in all the inspected vineyards during the 2-year survey.

The majority of all *H. obsoletus* specimens (75 %) were collected from *Urtica dioica*, and approximately 25 % of individuals were associated with *Convolvulus arvensis*.

'*Ca*. P. solani' detection in plants and insects

The presence of '*Ca*. P. solani' was detected in all 12 symptomatic grapevine samples that were subjected to analyses, and in 22 % (15 of 68) and 14 % (7 of 50) of asymptomatic *U. dioica* and *C. arvensis* plants, respectively (Table 2).

Because the amount of symptomatic grapevine plants that occurred in the studied vineyards was high (ca. 50 %), we analyzed the specimens of the most abundant leafhoppers and planthoppers species as tentative vectors, as well as *R. quinquecostatus*, which was the only cixiid species present in the vineyards in addition to *H. obsoletus*. PCR amplifications with stolbur-specific Stol11 primers indicated that out of the five analyzed insect species in BN-affected vineyards, only *H. obsoletus* harbored '*Ca*. P. solani' (Table 1). '*Ca*. P. solani' were detected in *H. obsoletus* individuals at a rate of approximately 18 % (Table 2). None of the four analyzed *R. quinquecostatus* specimens was positive for the '*Ca*. P. solani' presence.

Table 1 Auchenorrhyncha species present in and around BN-infected vineyards in southeastern Macedonia during the 2 years survey

Family	Subfamily	Species	Individuals collected	Stolbur-positive
Cixiidae	Cixiinae	*Hyalesthes obsoletus*	304	57
		Reptalus quinquecostatus	4	0
Delphacidae	Delphacinae	*Kelisia sp.*	3	–
Dictyopharidae	Dictyopharinae	*Dictyophara europaea*	36	–
Issidae	Issinae	*Issus coleoptratus*	21	–
Aphrophoridae	Aphrophorinae	*Neophilaenus campestris*	4	–
		Philaenus spumarius	13	–
		Aphrophora alni	9	–
Cicadellidae	Macropsinae	*Macropsis fuscula*	6	–
	Agalliinae	*Anaceratagallia ribauti*	17	–
		Dryodurgades reticulatus	1	–
	Aphrodinae	*Aphrodes diminuta*	7	–
		Aphrodes makarovi	12	–
		Aphrodes sp.	5	–
	Cicadellinae	*Cicadella viridis*	23	–
	Typhlocybinae	*Typhlocyba sp.*	10	–
	Deltocephalinae	*Fieberiella septentrionalis*	7	–
		Neoaliturus fenestratus	2	–
		Macrosteles sp.	1	–
		Doratura impudica	19	–
		Platymetopius guttatus	1	–
		Allygus cf. mixtus	7	–
		Allygus communis	1	–
		Allygidius commutatus	1	–
		Euscelis incisus	113	0
		Artianus manderstjernii	264	0
		Psammotettix alienus	287	0
		Jassargus obtusivalvis	1	–
		Enantiocephalus cornutus	1	–

The molecular typing of the '*Ca*. P. solani' strains

A molecular differentiation of the'*Ca*. P. solani' strains that were infecting grapevines and tentative reservoir plants and were harbored by the insects, was performed by PCR-RFLP and sequence typing for three presumably epidemiologically informative genes, that is, *tuf*, *vmp1* and *stamp*.

All 91 positive plant and insect samples yielded successful amplifications of the *tuf* gene with Tuf1f/r and TufAYf/r primers. The *Hpa*II restriction profiles showed the presence of the so-called nettle-associated *tuf-a* type as defined by Langer and Maixner (2004) in 25 % of infected grapevines (3 of 12), 40 % of '*Ca*. P. solani'-infected nettle plants (6 of 15) and 49 % of '*Ca*. P. solani'-infected *H. obsoletus* that were collected from nettles (21 of 43). PCR-RFLP analysis revealed the presence of the so-called bindweed-associated *tuf-b* type in 75 % of '*Ca*. P. solani'-infected grapevines, all the infected bindweeds and *H. obsoletus* collected on bindweeds. However, 9 out of 15 (60 %) stinging nettles and 45 % of *H. obsoletus* collected on *U. dioica* surprisingly exhibited RFLP profiles corresponding to the *tuf-b* type. In order to confirm these results, the tuf fragments were sequenced for these samples, in addition to the grapevine samples. A sequence comparison determined the presence of the following three *tuf* types: *tuf-a*, *tuf-b* and a third type that was genealogically intermediate

Table 2 '*Candidatus* Phytoplasma solani' genotypes hosted by grapevines, *Urtica dioica*, *Convolvulus arvensis* and *Hyalesthes obsoletus* ex *U. dioica* and *C. arvensis* in Macedonian vineyards

Host	No. of analyzed/no. of stolbur-positive (percent) samples	No. (percentage) of *tuf*/*vmp1*/*stamp* comprehensive genotypes	*tuf*/*vmp1*/*stamp* genotype[a]	Known genotypes[b]
Vitis vinifera	12/12 (100 %)	3 (25 %)	tuf-a/V3/M3	
		2 (17 %)	tuf-ab/V18/19-25	CPsM4_At1
		3 (25 %)	tuf-b/V14/Vv24	Vv24g
		4 (33 %)	tuf-b/V2-TA/STOL	STOLg
Hyalesthes obsoletus ex *U. dioica*	43/227 (19 %)	3 (7 %)	tuf-a/V3/SB5	
		10 (23 %)	tuf-a/V3/M2	
		8 (19 %)	tuf-a/V3/M3	
		3 (7 %)	tuf-ab/V18/19-25	CPsM4_At1
		19 (44 %)	tuf-ab/V18/M1	
Urtica dioica	15/68 (22 %)	6 (40 %)	tuf-a/V3/M4	
		9 (60 %)	tuf-ab/V18/M1	
Hyalesthes obsoletus ex *C. arvensis*	14/ 77 (18 %)	5 (35 %)	tuf-b/V2-TA /Rqg50	
		4 (29 %)	tuf-b/V14 /Rqg50	Rqg50g = CPsM4_At12
		4 (29 %)	tuf-b/V4/GGY	CPsM4_At9
		1 (7 %)	tuf-b/V4/M5	
Convolvulus arvensis	7/50 (14 %)	7 (100 %)	tuf-b/V2-TA /Rqg50	

[a] The *tuf*/*vmp1*/*stamp* genotypes of '*Ca.* P. solani' detected in this study

[b] The comprehensive genotypes of '*Ca.* P. solani' according to the reference strains (Aryan et al. 2014; Cvrković et al. 2014)

between *tuf-a* and *tuf-b* and was designated in this study as *tuf-ab* (Table 2) that clusters within the *tuf-a* type, which is the nettle-associated lineage *tuf-b2* as defined by Aryan et al. (2014). The presence of an intermediate *tuf* type was revealed in all nettles and the nettle-associated *H. obsoletus* designated as the *tuf-b* type according to RFLP typing, in addition to 17 % of grapevine samples (2 of 12; Table 2).

Vmp1 gene amplicons of approximately 1450 bp in length were obtained from all 91 '*Ca*. P. solani' strains. A restriction digestion with *Rsa*I, *Taq*I and *Alu*I enzymes allowed us to identify five diverse *vmp1* profiles among the phytoplasmas infecting the grapevines, *H. obsoletus*, nettles and bindweeds called V2-TA, V3, V4, V14 and V18 (Fig. 1). All the detected *vmp1* profiles were previously published and designated with these names (Murolo et al. 2010, 2013; Cvrković et al. 2014).

The V18 and V3 profiles were the most common with 36 and 33 % of all the strains assigned to these types, respectively. All V18 *vmp1* profiles were associated with an intermediate *tuf-ab* type, and they were detected in grapevines, *U. dioica* and its corresponding *H. obsoletus* populations. The V3 profile was found to be uniquely associated with the *tuf-a* type in grapevines, stinging nettles and *H. obsoletus* specimens collected on nettles. The less frequently identified V2-TA, V4 and V14 profiles were detected in grapevines and bindweed-associated *H. obsoletus*. The V14 type was found only in grapevines, V4 was detected only in *H. obsoletus* associated with bindweed, and V2-TA was shared by grapevines, bindweeds and bindweed-associated *H. obsoletus*.

The *stamp* gene-based phylogeny that employed 91 sequences revealed the highest diversity of '*Ca*. P. solani' strains and allowed us to identify 11 distinct genotypes with a maximum variability of 4.9 %. Among the identified *stamp* genotypes, six were identical to previously published reference strains: 19–25, SB5, Rqg50, Vv24, GGY and STOL (Fabre et al. 2011; Cvrković et al. 2014), and five were unique and designated as M1, M2, M3, M4 and M5 (Fig. 2, Table 2). Both Bayesian and MP phylogeny revealed two major phylogenetic groups, each of which was associated with bindweed and nettle (Fig. 2). The first one consisted of the three clusters *b-I*, *b-II* and *b-III*,

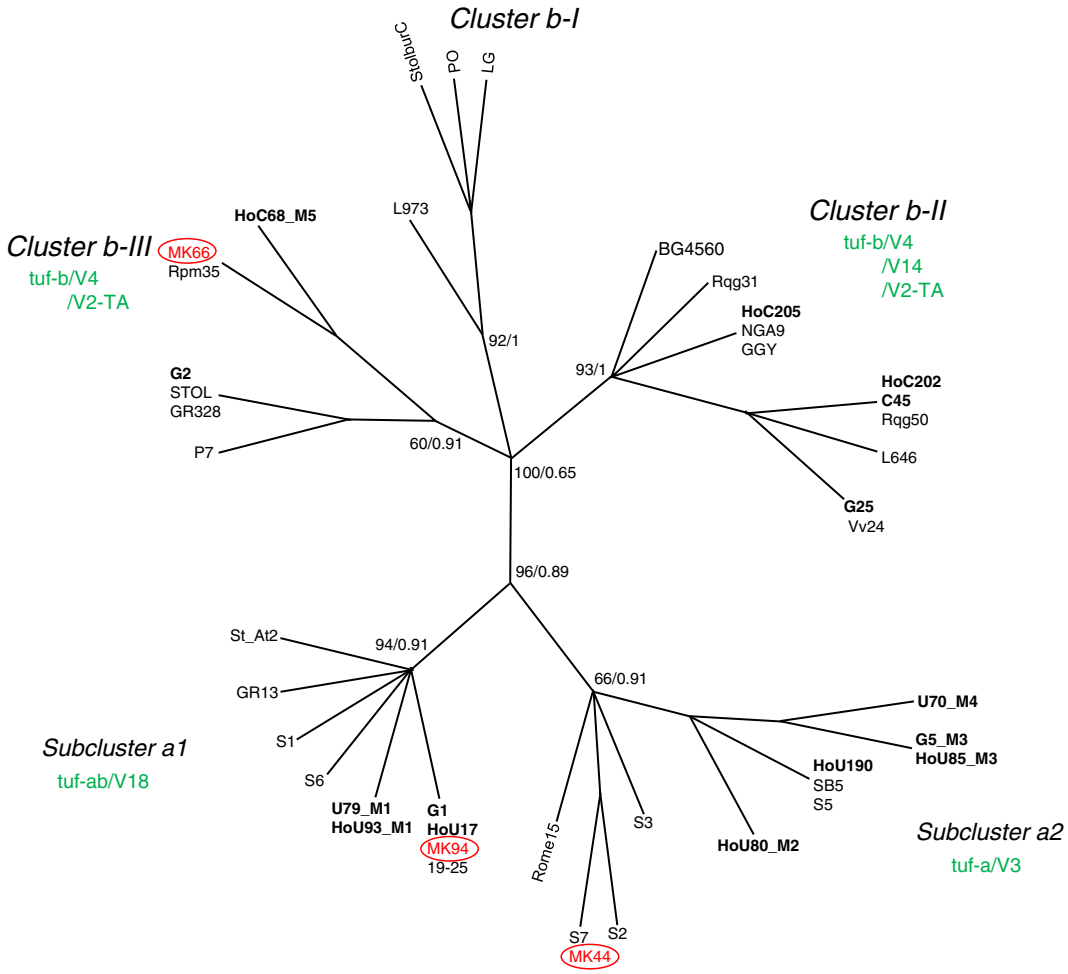

Fig. 2 The MP cladogram obtained from *stamp* sequences of '*Candidatus* Phytoplasma solani' strains as detected in grapevines, weeds and *H. obsoletus* in the Republic of Macedonia and in reference strains (Fabre et al. 2011; Johannesen et al. 2012; Aryan et al. 2014; Cvrković et al. 2014; Kostadinovska et al. 2014). The strains of each *stamp* genotype detected in this study are designated in bold letters. Strains from the *stamp* genotype as previously detected in grapevines from Macedonia according to Kostadinovska et al. (2014) are designated in red. Maximum parsimony bootstrap values/Bayesian posterior probabilities are indicated for each cluster node

among which the '*Ca*. P. solani' strains from Macedonian vineyards clustered within the latter two. The second cluster associated with nettle consisted of two subclusters, which were designated *a1* and *a2*, both of which encompassed the strains detected in this study. Four of the *stamp* genotypes that were detected for the first time in this study (M1-M4) were clustered within nettle-associated cluster *a* (three in *a2* subcluster and one in *a1*) and the M5 genotype belonged to cluster *b-III* (Fig. 2). The majority of the characterized *stamp* sequences belonged to the newly identified M1 genotype.

Four diverse *stamp* genotypes were identified in grapevines. A sequence comparison and a maximum parsimony phylogenetic analysis revealed that two of them clustered within the *b* group and had 100 % sequence similarity with reference strains STOL (cluster *b-III*) and Vv24 (cluster *b-II*) from Serbia. The third genotype clustered within the *a1* subcluster, and it had sequence characteristics identical to those of reference strains 19–25. The fourth genotype was clustered within the *a2* subcluster and has a unique sequence designated here as M3, which is present only in grapevines and *H. obsoletus* collected on nettles.

Five *stamp* genotypes were identified among the *H. obsoletus* specimens collected on nettles. Three of them, namely M1, M2 and M3, have unique sequences

in comparison with the reference strains. The other two genotypes are identical to reference strains 19–25 from Germany and SB5 from Croatia, respectively. However, in *U. dioica*, only two different *stamp* genotypes were identified, that is, M1 and M4, and they belong to the *a1* and *a2* subclusters, respectively, and have unique sequences in comparison with the reference strains. The first one was also detected in *H. obsoletus* associated with nettle, and the second is characteristic only for the nettles that share a 99.6 % sequence similarity with the M3 genotype detected in grapevines and nettles (with a 2 nt difference).

All '*Ca*. P. solani' strains from bindweed and bindweed-associated *H. obsoletus* belonged to cluster *b-II*. The majority of *H. obsoletus* and all the bindweed strains had sequences that were identical to one that was previously found in tentative insect vectors, *H. obsoletus* and grapevines in Serbia and Austria (Rqg50; Aryan et al. 2014; Cvrković et al. 2014). Four *H. obsoletus* strains have an identical sequence to that of *H. obsoletus* individuals collected from bindweed in Germany and Slovenia (GGY and NGA9; Fabre et al. 2011).

The comprehensive *tuf*/*vmp1*/*stamp* genotypes of '*Ca*. P. solani' hosted by grapevines, reservoir plants and *H. obsoletus*

Overall, twelve *tuf*/*vmp1*/*stamp* comprehensive genotypes of '*Ca*. P. solani' were detected in grapevines, nettles, bindweeds and associated populations of *H. obsoletus* in the vineyards of southeastern Macedonia.

Four '*Ca*. P. solani' genotypes were detected among naturally infected grapevines, six among nettle and nettle-associated population of *H. obsoletus* and four in bindweed and its associated insects. According to the *tuf*/*vmp1*/*stamp* genotyping, the most frequent genotypes were those associated with nettle, i.e., tuf-ab/V18/M1 detected in 60 % of naturally infected nettles and in 44 % of its corresponding *H. obsoletus* populations, and tuf-a/V3/M3 detected in 25 % of analyzed grapevine and in 19 % of nettle-associated *H. obsoletus*. The majority of '*Ca*. P. solani' genotypes were detected among the strains from *H. obsoletus* that were associated with *U. dioica*: (i) tuf-ab/V18/M1, (ii) tuf-a/V3/M2, infecting 23 % of analyzed samples, (iii) tuf-a/V3/M3, infecting 18 % of analyzed samples, (iv) tuf-ab/V18/19-25, and (v) tuf-a/V3/SB5, both of which infected approximately 7 % of the analyzed samples (Table 2). All '*Ca*. P. solani' strains associated with *C. arvensis* and 35 % of strains associated with its corresponding *H. obsoletus* populations belonged to the tuf-b/V2-TA/Rqg50 genotype. However, *tuf-b* strains found in naturally infected grapevine were associated with V14/Vv24 and V2-TA/STOL *vmp1*/*stamp* types.

Discussion

Bois noir infections of diverse grapevine varieties have been recorded in the vineyards of the Republic of Macedonia (Šeruga et al. 2003), and a molecular characterization of '*Ca*. P. solani' infecting BN-affected grapevines have recently been documented (Kostadinovska et al. 2014).

An increased incidence of BN throughout Macedonian vineyards initiated a study on genetic diversity of '*Ca*. P. solani' in symptomatic grapevine samples, potential insect vectors and herbaceous host plants as the primary reservoirs of infection, in order to clarify the epidemiology of the BN. During the two-year survey, we detected the presence of '*Ca*. P. solani' in all analyzed grapevine samples, the major '*Ca*. P. solani' vector *H. obsoletus* and the two principal '*Ca*. P. solani' reservoirs, bindweed and nettle. A qualitative analysis of the planthoppers and leafhoppers captured in the vineyards of southeastern Macedonia determined a high diversity with 29 species recorded. Of all the analyzed species, only *H. obsoletus* tested positive for the presence of '*Ca*. P. solani' despite the high diversity of Auchenorrhyncha species.

The incidence and dispersal of vector-borne plant pathogens depends upon the abundance of the vector(s), their interplant movement and a high infection rate (Power 1992; Orenstein et al. 2003; Trivellone et al. 2005). The infection rate of *H. obsoletus* specimens collected from *U. dioica* and *C. arvensis* was approximately 20 %, and the abundance of their populations was high in southeastern Macedonia. Additionally, the other cixiid species *R. panzeri*, a documented vector of BN, was not present in the studied vineyards, and the potential vector *R. quinquecostatus* was present in a negligible number. This finding clearly indicates that *H. obsoletus* plays a major role in the BN epidemiology of the vineyards we studied, and diminishes the hypothesis made by Kostadinovska et al. (2014) that *R. panzeri* could be a vector of '*Ca*. P. solani' in Macedonian

vineyards. However, the G2 genotype (tuf-b/V2-TA/STOL) was detected in 33 % of the diseased grapevines but in none of the *H. obsoletus* found infected. A similar incidence (42 %) was observed in northeastern Serbia where this strain proved to be the only strain transmitted by *R. panzeri* (Cvrković et al. 2014).

A molecular characterization of the '*Ca*. P. solani' strains indicates the presence of two epidemiological BN cycles in Macedonian vineyards, with one sourced by *C. arvensis* and the other by *U. dioica* as weedy phytoplasma reservoirs. Overall, 12 genotypes were detected according to the *tuf*/*vmp1*/*stamp* typing. Four genotypes were found to be associated with grapevines, with equally distributed *tuf-a* and *tuf-b* types. According to the comprehensive *tuf*/*vmp1*/*stamp* typing, one of the grapevine-associated BN genotypes that occurred in Macedonian vineyards corresponded to the most prevalent genotype in Serbian grapevines and planthoppers (STOLg), and another was identical to the Vv24g associated with BN-affected grapevines in Serbia (Cvrković et al. 2014). Regarding the *tuf-a* and intermediate *tuf-ab* '*Ca*. P. solani' types, they were found in grapevines, nettles and nettle-associated *H. obsoletus*. One of them corresponded to the tuf-ab/V18/19-25 that was previously found in grapevines from Macedonia (Kostadinovska et al. 2014) and in grapevines, *H. obsoletus* and nettles from Austrian vineyards (genotype CPsM4_At1) where it was found to be a major genotype that induced the current BN epidemics (Aryan et al. 2014). The second is a new *stamp* genotype, known as comprehensive tuf-a/V3/M3, which was found to be restricted to grapevines and *H. obsoletus* associated with nettles. Two new genotypes were associated with nettles, called tuf-ab/V18/M1 and tuf-a/V3/M4; the former was also associated with the corresponding population of *H. obsoletus*, and the latter restricted solely to nettle. The high genotype diversity of '*Ca*. P. solani' was detected in *H. obsoletus* collected from both bindweeds and stinging nettles. In addition to the above mentioned genotypes, five genotypes were associated exclusively with *H. obsoletus*. Two genotypes were found in nettle-associated *H. obsoletus*, with tuf-a/V3/SB5 as previously reported in Croatia (Fabre et al. 2011) and a new genotype called tuf-a/V3/M2. The following three genotypes were detected in bindweed-associated *H. obsoletus*: tuf-b/V4/GGY, tuf-b/V4/M5 and tuf-b/V14/Rqg50. The third genotype corresponds to the Rqg50g identified in Serbian vineyards and to the CPsM4_At12 genotype that occurs in Austrian vineyards. Only one genotype was associated with bindweed, called tuf-b/V2-TA/Rqg50, which was previously reported in grapevines from Montenegro (A. Kosovac, personal communication). Profile V3 was only found in association with type *tuf-a*, which is consistent with previous evidence (Foissac et al. 2013), and profile V18 was always found in association with the intermediate type *tuf-ab*.

H. obsoletus adults were found to feed on diverse plant species, but for nymphal development, during which the insects acquire phytoplasmas, only a few are preferred (Langer and Maixner 2004). *Convolvulus arvensis* is generally considered as both the primary host plant and '*Ca*. P. solani' reservoir, but within the last decade, *U. dioica* has become an equally preferred host plant (Johannesen et al. 2012). Until recently, stinging nettle was considered a primary host only in Italy (Lessio et al. 2007). However, the latest studies emphasize that *tuf-a* type associated with stinging nettles is the most common '*Ca*. P. solani' strain and *U. dioica* is the dominant host plant in Germany, northeastern France, Switzerland and Austria, where it is predominantly responsible for the mass occurrence of *H. obsoletus* and severe BN outbreaks (reviewed in Johannesen and Riedle-Bauer 2014).

Our research indicates that BN epidemiological cycles in the Republic of Macedonia are correlated with both bindweed and stinging nettle as reservoir plants, with the prevalence of tuf type *a* and *H. obsoletus* associated with *Urtica dioica*, which is the preferred host plant for *H. obsoletus* in this country. The high incidence of nettle-associated *tuf-a* and *tuf-ab* types might be related to the agricultural praxis in southeastern Macedonia, which includes intensive irrigation during hot and dry summers and provides suitable habitats and environmental conditions for the growth of stinging nettles in vineyard surroundings. Because stinging nettles do not express the symptoms of phytoplasma infection and the plants were randomly selected for '*Ca*. P. solani' identification, only two different genotypes were identified in infected reservoir plants. However, a high diversity of *stamp* genotypes was detected in '*Ca*. P. solani' strains from *H. obsoletus* collected on *U. dioica*, in addition to strains from *H. obsoletus* associated with *C. arvensis*.

The increased BN incidence since the first report in 2003 in the Republic of Macedonia (Šeruga et al. 2003) and the high abundance of *H. obsoletus* on stinging nettles suggest the possible occurrence of sudden outbreaks and the existence of two host races for the vector,

with one specialized in stinging nettle and one in bindweed. Further research should be focused on the host-race diversification of *H. obsoletus* among Macedonian host-plant populations and the design of adequate management strategies.

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