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RESEARCH PAPER

## Multiple gene analyses identify distinct “bois noir” phytoplasma genotypes in the Republic of Macedonia

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**Summary.** “Bois noir” (BN) is a grapevine yellows disease, associated with phytoplasma strains related to ‘*Candidatus Phytoplasma solani*’, that causes severe losses to viticulture in the Euro-Mediterranean basin. Due to the complex ecological cycle of its etiological agent, BN epidemiology is only partially known, and no effective control strategies have been developed. Numerous studies have focused on molecular characterization of BN phytoplasma strains, to identify molecular markers useful to accurately describe their genetic diversity, geographic distribution and host range. In the present study, a multiple gene analyses were carried out on *16S rRNA*, *tuf*, *vmp1*, and *stamp* genes to study the genetic variability among 18 BN phytoplasma strains detected in diverse regions of the Republic of Macedonia. Restriction fragment length polymorphism (RFLP) assays showed the presence of one *16S rRNA* (16SrXII-A), two *tuf* (*tuf*-type a, *tuf*-type b), five *vmp1* (V2-TA, V3, V4, V14, V18), and three *stamp* (S1, S2, S3) gene patterns among the examined strains. Based on the collective RFLP patterns, seven genotypes (Mac1 to Mac7) were described as evidence for genetic heterogeneity, and highlighting their prevalence and distribution in the investigated regions. Phylogenetic analyses on *vmp1* and *stamp* genes underlined the affiliation of Macedonian BN phytoplasma strains to clusters associated with distinct ecologies.

**Key words:** grapevine yellows, stolbur, multiple gene analyses, membrane proteins.

### Introduction

“Bois noir” (BN) is a phytoplasma-associated grapevine yellows (GY) disease that induces severe crop losses in almost all grapevine varieties used for wine production in the Euro-Mediterranean area (Belli *et al.*, 2010; Foissac *et al.*, 2013) and in other continents (Botti and Bertaccini, 2006; Gajardo *et al.*, 2009; Karimi *et al.*, 2009; Duduk *et al.*, 2010). BN produces typical GY symptoms, including berry shrivel, desiccation of inflorescences, colour alterations and

curling of the leaves, reduction of growth, and irregular ripening of wood (Belli *et al.*, 2010).

Phytoplasmas are cell wall-less obligate intracellular parasites belonging to the *Mollicutes* class (Lee *et al.*, 2000). As preliminary data on phytoplasma cultivation in cell-free medium have been reported only recently (Contaldo *et al.*, 2013), their differentiation and classification is based on nucleotide sequence analysis of housekeeping genes (IRPCM, 2004; Martini *et al.*, 2007; Hodgetts *et al.*, 2008; Lee *et al.*, 2010; Valiunas *et al.*, 2013). On the basis of multiple gene (*16S rRNA*, *tuf*, *rplV-rpsC*, *secY*) sequence analysis, the etiological agent of BN has been attributed to phytoplasmas related to ‘*Candidatus Phytoplasma solani*’ (Quaglino *et al.*, 2013), subgroup 16SrXII-A (Lee *et al.*, 1998). BN phytoplasmas are transmitted

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from plant-to-plant by *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae), a polyphagous vector living preferentially on weeds inside and/or around vineyards (Sforza *et al.*, 1998; Alma *et al.*, 2002; Langer and Maixner, 2004; Berger *et al.*, 2009). The wide plant host range of *H. obsoletus* suggests that BN may exist in different ecosystems (Johannesen *et al.*, 2012). Furthermore, in vine-growing areas where *H. obsoletus* is absent, the presence of BN implies the existence of alternative vectors. Recently, *Reptalus panzeri* has been reported as natural vector of BN in Serbian vineyards (Cvrković *et al.*, 2014). Multiple gene analyses of BN phytoplasmas, based on RFLP-mapping and sequencing of *tuf* (Langer and Maixner, 2004), *secY*, *vmp1* (Fialová *et al.*, 2009) and *stamp* (Fabre *et al.*, 2011) genes have highlighted the presence of three *tuf*, 39 *secY*, 23 *vmp1*, and 56 *stamp* genotypes characterized by different distributions and prevalence in the Euro-Mediterranean basin (Foissac *et al.*, 2013).

Even though GY symptoms in vineyards of the Republic of Macedonia were observed from 1975 (Filip Pejčinovski, unpublished data), the first investigations of GY-associated phytoplasmas have been carried out only during the last few years. BN phytoplasmas (*tuf*-type b) were identified in all grape-growing areas of the Country (Seruga *et al.*, 2003; Mitrev *et al.*, 2007; Mitrev and Kostadinovska, 2013). A multiple gene typing analysis was carried therefore out on 16S rRNA, *tuf*, *vmp1*, and *stamp* genes to verify the presence of genetic variability among BN phytoplasmas in diverse regions of the Republic of Macedonia, in order to increase knowledge about BN epidemiology in this country.

## Materials and methods

### Sample collection and DNA extraction

During surveys carried out from mid-August to mid-September 2012, leaf samples were collected from 17 symptomatic plants of white grape variety Chardonnay and red varieties Vranec and Stanusina in vineyards of seven localities throughout the Republic of Macedonia. One *Cuscuta* spp. plant was also sampled in the investigated area (Table 1). Grapevine leaf veins, separated from laminae by a sterile razor, and all parts of the *Cuscuta* spp. plant, were stored at -80°C. Total nucleic acids were extracted from 1 g of frozen plant tissues by cetyltrimethylammonium bromide (CTAB) extraction procedure (Angelini *et al.*, 2001).

### Molecular identification of bois noir phytoplasmas

Phytoplasma detection was carried out by means of amplification of 16S rDNA in nested PCR assays primed by P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by primer pair R16F1/R16R0 (Lee *et al.*, 1995), and subsequent *AluI*-, *BfaI*-, *Bst*UI-, and *MseI*-RFLP assays on the amplicons obtained. PCR and RFLP reaction conditions were as previously described (Lee *et al.*, 1998; Quagliano *et al.*, 2009). PCRs were performed by using *Taq* polymerase (Promega) in an automated thermal cycler (MasterCycler Gradient, Eppendorf). PCR and enzymatic digestion products were electrophoresed through 1% and 3% agarose gel, respectively, in TBE buffer, stained with Midori Green Advance (Biosigma) and visualized under UV transilluminator. DNAs from periwinkle [*Catharanthus roseus* (L.) G. Don] plants infected by phytoplasma strains EY1 ('*Ca. P. ulmi*', subgroup 16SrV-A), STOL ('*Ca. P. solani*', subgroup 16SrXII-A), and AY1 ('*Ca. P. asteris*', subgroup 16SrI-B) were used as reference controls.

### Characterization of bois noir phytoplasmas through MLST

Molecular characterization of phytoplasma strains was performed by nested PCR/RFLP-based assays of three phytoplasma genomic portions, including *tuf*, *vmp1*, and *stamp* genes. Reaction mixtures and PCR-RFLP conditions used for amplifying and digesting the genomic segments of *tuf* (Schneider *et al.*, 1997; Langer and Maixner, 2004), *vmp1* (Pacifico *et al.*, 2009), and *stamp* (Fabre *et al.*, 2011) genes were as previously described. In detail, in the case of the *stamp* gene, full nucleotide sequences of '*Ca. P. solani*' phytoplasmas described by Cimerman *et al.* (2009) were retrieved from GenBank, compiled in FASTA format, and searched for single nucleotide polymorphisms (SNPs) in recognition sites for restriction enzymes by virtual RFLP analyses using the software pDRAW32 (www.acaclone.com). The enzyme *Hpy*188I, able to distinguish '*Ca. P. solani*' strains producing comprehensive restriction profiles, was selected for performing the digestion of *stamp* gene amplicons. Phytoplasma reference controls and visualization of PCR products and RFLP profiles were as described above for 16S rDNA.

PCR products of *vmp1* and *stamp* genes, amplified from BN phytoplasmas representative of the RFLP profiles obtained (Table 1), were sequenced in both

**Table 1.** Sample features and RFLP patterns of bois noir (BN) phytoplasma strains.

Sample/ strain	Locality	Region	Grapevine variety	RFLP profiles				Accession No.		BN phytoplasma genotype <sup>a</sup>
				16S rRNA	tuf	vmp1	stamp	vmp1	stamp	
MK19 <sup>b</sup>	Sveti Nikole	Pesirovo	Vranec	16SrXII-A	B	V14	S1	KF957602		Mac1
MK28 <sup>b</sup>	Veles	Sopot	Chardonnay	16SrXII-A	B	V4	S1	KF957603		Mac2
MK29 <sup>b</sup>	Veles	Sopot	Chardonnay	16SrXII-A	B	V2-TA	S1	KF957604		Mac3
MK44 <sup>b,c</sup>	Valandovo	Josifovo	Vranec	16SrXII-A	A	V3	S2	KF957605	KF957607	Mac4
MK47	Valandovo	Josifovo	Vranec	16SrXII-A	B	V2-TA	S1			Mac3
MK48	Valandovo	Josifovo	Vranec	16SrXII-A	B	V3	S2			Mac5
MK57	Valandovo	Josifovo	Chardonnay	16SrXII-A	B	V4	S1			Mac2
MK61	Gevgelija	Avlaki	Vranec	16SrXII-A	B	V14	S1			Mac1
MK62	Gevgelija	Avlaki	Vranec	16SrXII-A	B	V4	S1			Mac2
MK66 <sup>c</sup>	Amzibegov Pesirovo	Struga	Chardonnay	16SrXII-A	B	V14	S3		KF957608	Mac6
MK74	Amzibegov Pesirovo	Struga	Vranec	16SrXII-A	B	V14	S3			Mac6
MK86	Kavadarci	Krnjevo	Stanusina	16SrXII-A	B	V2-TA	S1			Mac3
MK94 <sup>b,c</sup>	Kavadarci	Krnjevo	Stanusina	16SrXII-A	A	V18	S1	KF957606	KF957609	Mac7
MK97	Kavadarci	Krnjevo	Vranec	16SrXII-A	B	V3	S2			Mac5
MK111	Kavadarci	Krnjevo	Vranec	16SrXII-A	B	V4	S1			Mac2
MK103	Sarcievo	Sarcievo	Vranec	16SrXII-A	B	V4	S1			Mac2
MK104	Sarcievo	Sarcievo	Vranec	16SrXII-A	B	V14	S1			Mac1
MK501	Argulica	Tupanec	<i>Cuscuta</i> spp.	16SrXII-A	B	V14	S1			Mac1

<sup>a</sup> Determined by collective RFLP profiles of 16S rRNA, tuf, vmp1, and stamp genes.

<sup>b</sup> Strain representative of vmp1 (V) RFLP profiles, defined by RsaI restriction profiles, selected for sequencing.

<sup>c</sup> Strain representative of stamp (S) RFLP profiles, defined by Hpy188I restriction profiles, selected for sequencing.

senses by a commercial service (Primm) to achieve at least 4× coverage per base position. Nucleotide sequence data were assembled by employing the Contig Assembling program of the software BioEdit version 7.0.5 ([www.mbio.ncsu.edu/bioedit/bioedit.html](http://www.mbio.ncsu.edu/bioedit/bioedit.html)). Assembled sequences were deposited in the GenBank database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) under the accession numbers listed in Table 1.

### Phylogenetic analysis

Nucleotide sequences of vmp1 and stamp genes obtained in the present study (Table 1), and of previously described ‘*Ca. P. solani*’ strains (Cimerman

*et al.*, 2009; Murolo *et al.*, 2010, 2013; Johannesen *et al.*, 2012; Cvrković *et al.*, 2014) retrieved from NCBI GenBank, were employed for phylogenetic analyses. Vmp1 and stamp nucleotide sequences were compiled in FASTA format. Vmp1 sequences were trimmed to approx. 1300-nt fragments (TYPH10F/TYPH10R fragments; Fialová *et al.*, 2009), and stamp sequences to 550-nt fragments (StampF1/StampR1 fragments; Fabre *et al.*, 2011), and aligned using the “ClustalW Multiple Alignment” application of the software BioEdit version 7.0.5. Minimum evolution analysis was carried out using the neighbour-joining method and bootstrap replicated 1000 times with the software MEGA5 (Tamura *et al.*, 2011).

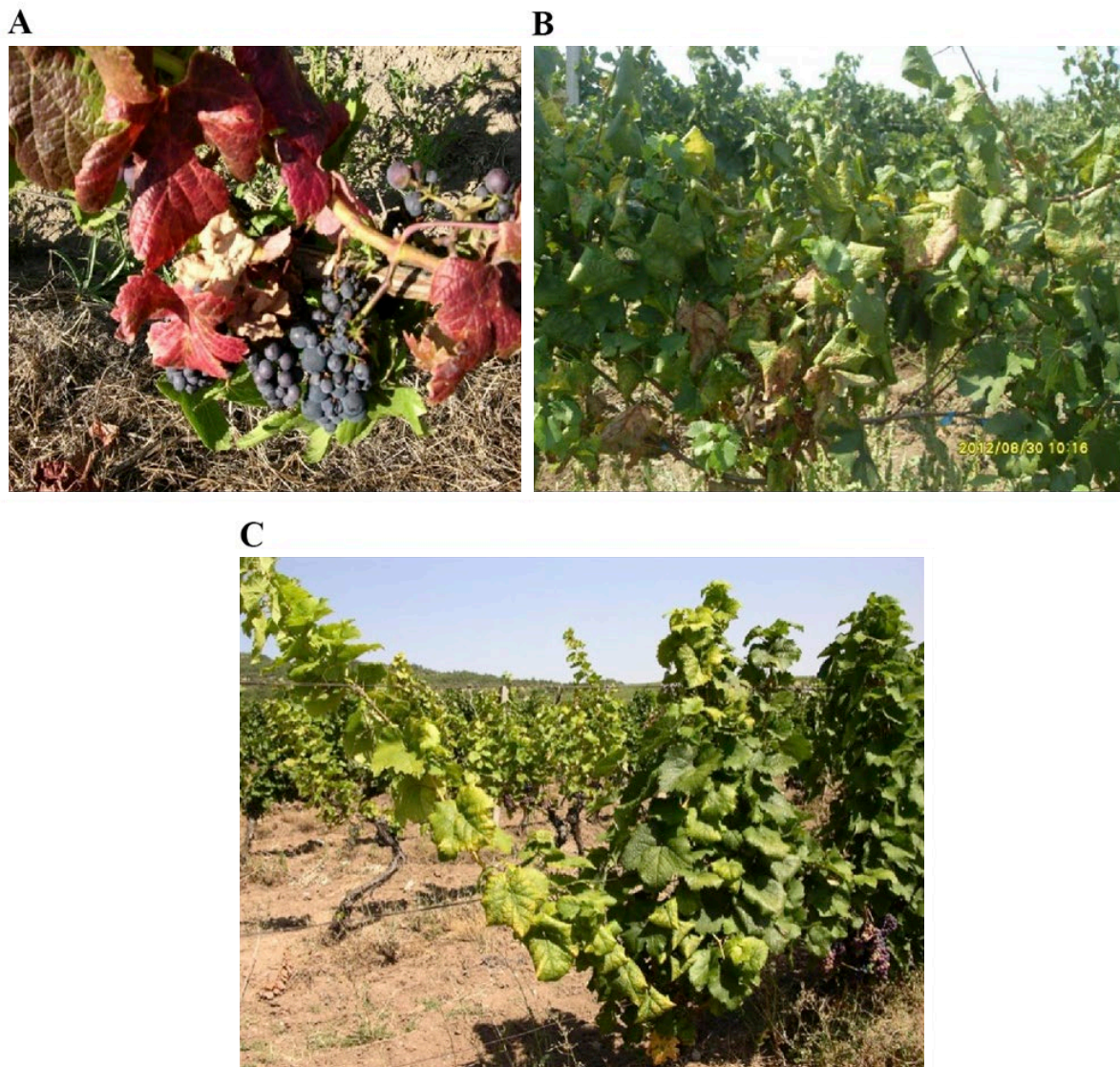
## Results

### Grapevine yellows symptoms

Typical GY symptoms were observed on Chardonnay and in Vranec grapevine varieties. Chardonnay showed leaf yellowing, while Vranec showed leaf reddening (Figure 1A and 1B). The autochthon red variety Stanusina, cultivated in the region Krnjevo, exhibited leaf yellowing (Figure 1C).

### Molecular identification of bois noir phytoplasmas

PCR-based amplification of *16S rRNA* gene showed that all the examined samples were infected by phytoplasmas. DNA amplification was obtained from periwinkles infected by phytoplasma reference strains, while no amplification was present in healthy periwinkle and negative controls (PCR mixture devoid of DNA). *AluI*, *BfaI*, *BstUI*, and *MseI*



**Figure 1.** Grapevine yellows symptoms observed on Vranec (A), Chardonnay (B) and Stanusina (C) grapevine varieties. Vranec and Chardonnay showed typical grapevine yellows symptoms, while Stanusina (red grape variety) exhibited white variety-like symptoms.

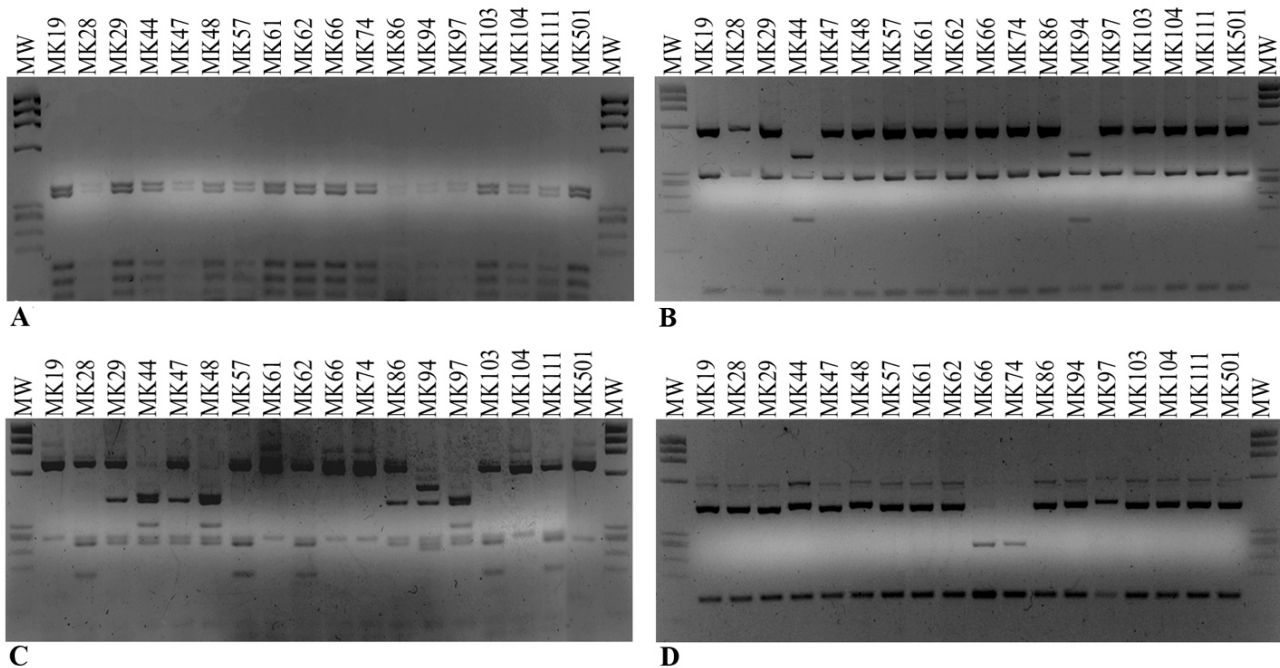
digestion analysis showed that all the identified phytoplasma strains belong to subgroup 16SrXII-A, since their restriction patterns were indistinguishable from one another and from the patterns characteristic of the STOL (16SrXII-A) reference strain (*MseI* RFLP patterns are shown in Figure 2A; *AluI*, *BfaI*, *BstUI* RFLP profiles are not presented). Moreover, PCR-positivity of all the grapevine samples analyzed confirmed the association between specific GY disease symptoms and phytoplasma presence in the examined vineyards.

### Characterization of bois noir phytoplasmas through MLST

Collective RFLP patterns, obtained by multiple gene analyses, revealed the presence of seven distinct BN phytoplasma genotypes, designated as Mac1 to Mac7 (Table 1). RFLP-based results confirmed that these genotypes are distinguishable by non-ribosomal gene sequence analyses (*tuf*, *vmp1*, and *stamp*). Two, three and five restriction patterns

were evidenced based on *tuf*, *stamp*, and *vmp1* genes, respectively (Table 1; Figure 2B, 2C, 2D). In detail, it was possible to identify: (i) *HpaII* RFLP profiles associated with *tuf*-type a (two strains) and *tuf*-type b (16 strains), formerly named VK-I and VK-II (Langer and Maixner, 2004) (Table 1; Figure 2B); (ii) *RsaI* RFLP profiles associated with *vmp1* patterns V2-TA (three strains), V3 (three strains), V4 (five strains), V18 (one strain), and V14 (six strains) described in previous studies (Murolo *et al.*, 2010, 2013; Cvrković *et al.*, 2014) (Table 1; Figure 2C); and (iii) *Hpy188I* RFLP profiles S1 (*stamp1*) (15 strains), S2 (three strains), and S3 (two strains) described for the first time in this study (Table 1; Figure 2D). Attribution of *vmp1* profiles was confirmed by pDRAW32 virtual restriction analyses of *vmp1* nucleotide sequences of BN phytoplasmas representative of *vmp1* (V) RFLP profiles obtained in the present study and comparison with restriction patterns from previous studies (Figure 3).

Based on *RsaI* RFLP digestions of *vmp1* gene amplicons, the profiles V4 (associated with *tuf* type-b/S1 genotype) and V14 (associated with *tuf*-type b



**Figure 2.** RFLP patterns discriminating bois noir phytoplasma genotypes. (A) Profiles from *MseI* digestions of R16F1/R16R0 PCR products (*16S rRNA* gene); (B) profiles from *HpaII* digestions of fTufAY/rTufAY PCR products (*tuf* gene); (C) profiles from *RsaI* digestions of H10F2/H10R2 PCR products (*vmp1* gene); (D) profiles from *Hpy188I* digestions of stampF1/stampR1 PCR products (*stamp* gene). Phytoplasma acronyms are listed in Table 1. [MW: marker Fx174 digested by *HaeIII* (Invitrogen)].

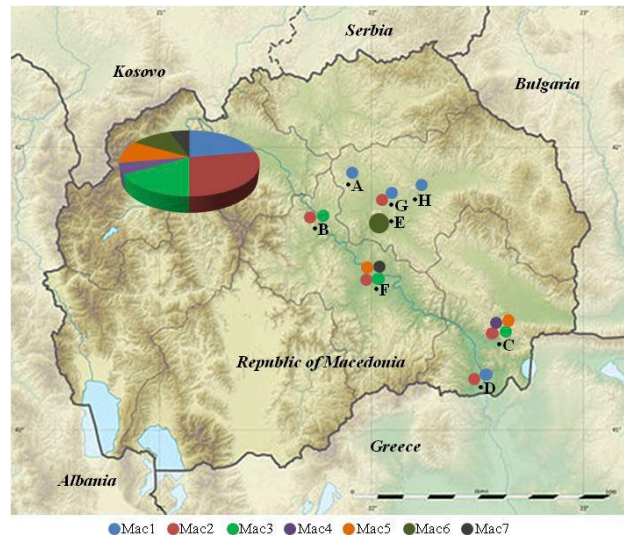




**Figure 3.** Virtual *RsaI* profiles of representative bois noir phytoplasmas. Nucleotide sequences of phytoplasmas MK19, MK28, MK29, MK44, and MK94 (Table 1) were compiled in FASTA format, trimmed to TYPH10F/TYPH10R fragments (approximately 1300-nt fragments), and *RsaI* virtually digested. Virtual RFLP profiles were visualized on 3% agarose gel. FX174 digested by *HaeIII* was selected as marker.

/S1 and *tuf*-type b/S3 genotypes) were prevalent among the analyzed BN phytoplasmas (Table 1). S2 (*stamp*) patterns were found exclusively associated with V3 (*vmp1*) profiles, and S3 exclusively with V14 profiles. Pattern S1 was identified in BN phytoplasmas characterized by patterns V2-TA, V4, V18, and V14 (Table 1).

Considering the BN phytoplasma genotypes determined by collective RFLP patterns, Mac1 (*tuf*-type b/V14/S1), Mac2 (*tuf*-type b/V4/S1), and Mac3 (*tuf*-type b/V2-TA/S1) were identified in the majority of the samples (12/18) throughout the examined viticultural regions (Table 1; Figure 4). On the other hand, Mac5 (*tuf*-type b/V3/S2) and Mac6 (*tuf*-type b/V14/S3) were found each in two plants. Mac4 (*tuf*-type a/V3/S2) was identified in only one BN-diseased Vranec plant, and Mac7 (*tuf*-type a/V18/



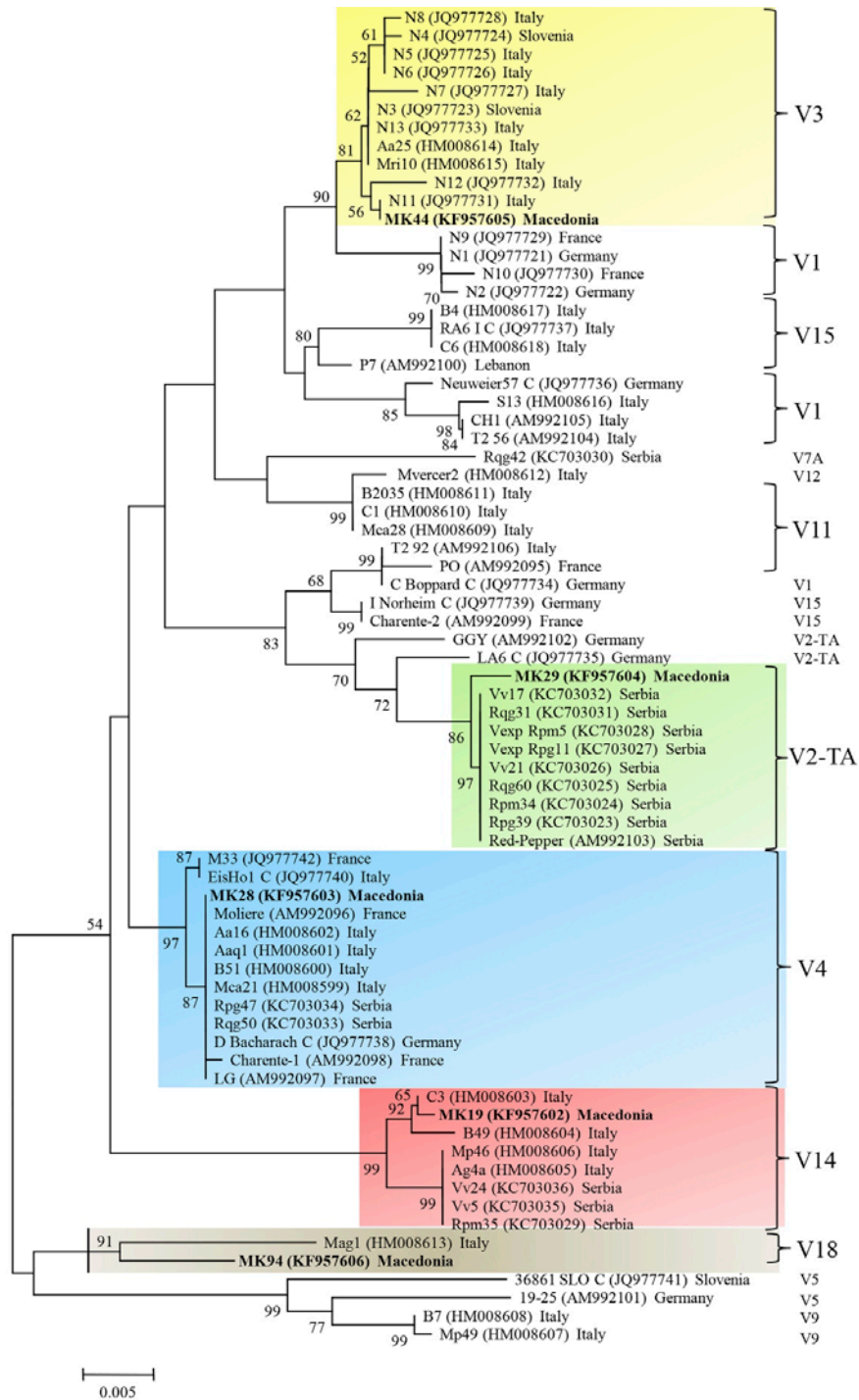
**Figure 4.** Distribution of bois noir (BN) phytoplasma genotypes in the Republic of Macedonia. Vineyard localities are represented as capital letters: A (Sveti Nikole), B (Veles), C (Valandovo), D (Gevgelija), E (Amzibegov Pesirovo), F (Kavadarci), G (Sarcievo), H (Argulica). BN phytoplasma genotypes (Mac1 to 7) are reported at marked localities as shown in the legend. Spot diameter is related to the number of BN phytoplasma strains. Pie graphic shows the prevalence of BN phytoplasma genotypes.

S1) was identified only in one diseased Stanusina plant (Table 1; Figure 4).

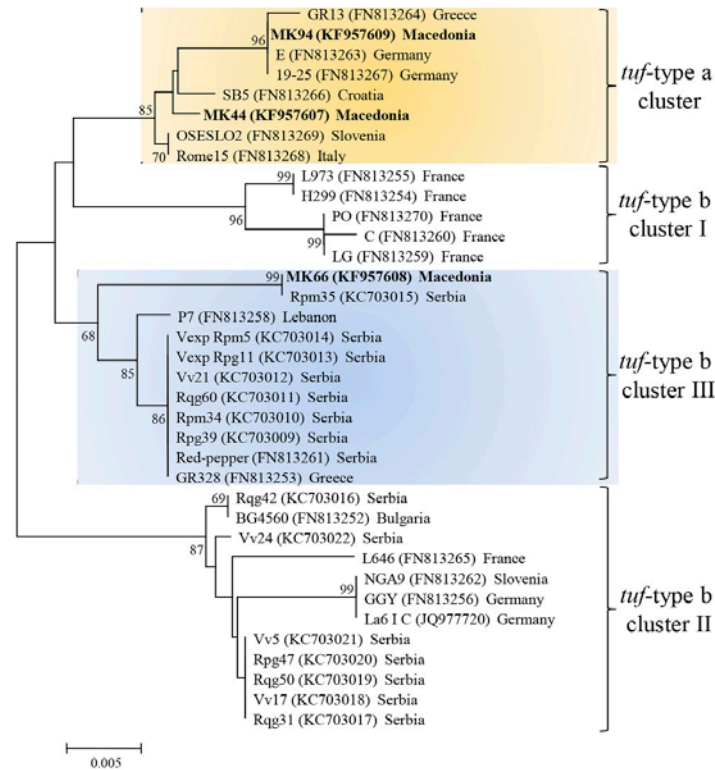
### Phylogenetic analyses

The *vmp1* phylogenetic tree confirmed that BN phytoplasmas identified in the present study and selected as representative of *RsaI* RFLP profiles V2-TA (strain MK29), V3 (MK44), V4 (MK28), V18 (MK94), and V14 (MK19), clustered with previously reported BN phytoplasmas having the same *RsaI* RFLP profiles (Figure 5).

The *stamp* phylogenetic tree highlighted that BN phytoplasmas (*tuf*-type a) carrying *Hpy188I* RFLP profiles S2 (strain MK44) and S1 (MK94) were positioned within the *tuf*-type a cluster, while the BN phytoplasma MK66 (*tuf*-type b) carrying *Hpy188I* RFLP profiles S3, grouped within the *tuf*-type b cluster, including BN phytoplasmas from Balkan and Eastern parts of Mediterranean basin (Greece, Republic of Serbia, Romania, Russia, Lebanon, and Azerbaijan) (Figure 6).



**Figure 5.** Unrooted phylogenetic tree inferred from 'Ca. P. solani' strain nucleotide sequences of gene *vmp1*. Minimum evolution analysis was carried out using the neighbor-joining method and bootstrap replicated 1,000 times. Names of phytoplasma strains included in phylogenetic analysis are written on the tree image. GenBank accession number of each sequence is given in parenthesis; gene sequences obtained in the present study are indicated in bold characters. Clusters encompassing bois noir phytoplasmas from the present study are shown as colored boxes.



**Figure 6.** Unrooted phylogenetic tree inferred from ‘*Ca. P. solani*’ strain nucleotide sequences of gene *stamp*. Minimum evolution analysis was carried out using the neighbor-joining method and bootstrap replicated 1,000 times. Names of phytoplasma strains included in phylogenetic analysis are written on the tree image. GenBank accession number of each sequence is given in parenthesis; gene sequences obtained in the present study are indicated in bold characters. Clusters encompassing bois noir phytoplasmas from the present study are shown as colored boxes.

## Discussion

In previous years, typical GY symptoms were observed in Chardonnay and in Vranec grapevine varieties in vineyards of the Republic of Macedonia (Mitrev *et al.*, 2007). Typically, white grape varieties exhibit leaf yellowing, while red varieties show leaf reddening (Belli *et al.*, 2010). In the present study, however, symptoms associated with white varieties (leaf yellowing) were observed in the red variety Stanusina. In the same geographic area, other red varieties (e.g. Plovdina) showed leaf yellowing when infected by phytoplasmas associated with “flavescence dorée” disease (Kuzmanovic *et al.*, 2006). To our knowledge, the behaviour of such phytoplasma-infected red varieties, showing leaf yellowing instead of reddening, is unique and seems to be present only in these geographic areas. The characteristic leaf col-

our alteration associated with phytoplasma infection is caused by the accumulation of flavonoids (mainly flavonols, anthocyanins, and proanthocyanidins) which are different in white and red varieties (Bogs *et al.*, 2005; Hren *et al.*, 2009). Comparative studies on physiology and transcriptome/proteome of healthy and phytoplasma-infected plants of these red grape varieties, carried out as in previous studies (Hren *et al.*, 2009; Margaria and Palmano, 2011), could provide useful data to assist understanding this phenomenon.

Multiple gene analysis has been proposed and employed to distinguish ecologically separated phytoplasma populations (Lee *et al.*, 2004; Seemüller and Schneider, 2004; Malembic-Maher *et al.*, 2011; Davis *et al.*, 2013; Quaglino *et al.*, 2013). This approach has also been applied for investigating genetic diversity among phytoplasmas associated with several dis-



eases in order to identify strain-specific molecular markers useful for improving knowledge of complex phytoplasma ecologies (Arnaud *et al.*, 2007; Casati *et al.*, 2010, 2011; Adkar-Purushothama *et al.*, 2011; Durante *et al.*, 2012). In order to gain an insight into the genetic diversity among BN phytoplasmas in the Republic of Macedonia, the PCR-RFLP based analysis was performed on a gene (*tuf*) coding for the translational elongation factor Tu (EF-Tu), whose sequence mutations were reported to be associated with different weed hosts of the BN vector *H. obsoletus* (Langer and Maixner, 2004), and on two genes (*vmp1* and *stamp*) coding for membrane proteins putatively involved in the interaction of BN phytoplasma with its hosts (Cimerman *et al.*, 2009; Fabre *et al.*, 2011), whose sequence mutations seem to be associated with the geographic distribution and host range of this phytoplasma (Murolo *et al.*, 2010, 2013; Johannesen *et al.*, 2012; Foissac *et al.*, 2013; Cvrković *et al.*, 2014).

Previous research showed that only the BN *tuf*-type b, prevalent in south-eastern and central Europe (Foissac *et al.*, 2013), was detected in the Republic of Macedonia (Seruga *et al.*, 2003), suggesting a key role of *H. obsoletus* haplotypes feeding preferentially on bindweed (*Convolvulus arvensis* L.) (Johannesen *et al.*, 2012) in the diffusion of BN phytoplasmas in this country. Here, identification of the *tuf*-type a could support the idea that nettle (*Urtica dioica* L.) is involved also in BN epidemiology in the investigated geographic regions.

Based on *RsaI* RFLP digestions of *vmp1* gene amplicons, the profiles V4 (associated with *tuf*-type b/S1 genotype) and V14 (associated with *tuf*-type b/S1 and *tuf*-type b/S3 genotypes) were prevalent among the analyzed BN phytoplasmas (Table 1). These data confirmed the widespread presence of pattern V4 throughout the Euro-Mediterranean basin and the specific association of pattern V14 with East Europe (Foissac *et al.*, 2013). Moreover, profile V3 was found in association with both *tuf*-types a and b, in disagreement with previous evidence indicating the exclusive association of V3 and *tuf*-type a (Foissac *et al.*, 2013). In addition, BN phytoplasmas from three grapevines exhibited the *RsaI* pattern V2-TA, previously reported only in grapevines and in the insects *Reptalus panzeri* and *R. quinquecostatus* in Serbia (Cvrković *et al.*, 2014). The identification of V2-TA profile in the vineyards examined in the present study, and the proven *R.*

*panzeri* vectorship of BN phytoplasmas to grapevine in Serbia suggest a possible role of *R. panzeri* as BN vector also in the Republic of Macedonia. Moreover, strain MK44 clustering along with phytoplasma strains from European nettles reinforced that this weed can also play a role in the BN diffusion in the Republic of Macedonia. Moreover, strains MK19 and MK29 were grouped in clusters including BN phytoplasmas identified in Serbian *R. panzeri* insects (Cvrković *et al.*, 2014), able to transmit BN phytoplasmas from grapevine and maize to grapevine, and in Italian grapevines (Murolo *et al.*, 2010, 2013). This suggests a new scenario about the possible existence of *R. panzeri*-related BN diffusion, specific for the strains showing V2-TA and V14 *RsaI* patterns.

The identification of two distinct BN genotypes (Mac3 and Mac7) in the red grape variety Stanusina suggests that no specific BN phytoplasmas could be related to the symptoms occurring in white varieties. Moreover, the identification of the genotype Mac1 (frequently reported in grapevine) in the *Cuscuta* spp. plant (Table 1) suggests that it may also be a reservoir of BN phytoplasmas.

Preliminary results showed that BN phytoplasmas from the Republic of Macedonia belonged only to cluster II of *tuf*-type b, including BN phytoplasmas from Central Europe and Balkan (Germany, Czech Republic, Hungary, Croatia, and Bulgaria) (Foissac *et al.*, 2013; Cvrković *et al.*, 2014). Based on information from the present and previous studies, we conclude that BN in the Republic of Macedonia is associated with phytoplasmas strongly related with those reported from Central Europe to the Eastern part of Mediterranean basin and from Balkan regions (Foissac *et al.*, 2013).

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