Identification and Characterization of New '*Candidatus* Phytoplasma solani' Strains Associated with Bois Noir Disease in *Vitis vinifera* L. Cultivars Showing a Range of Symptom Severity in Georgia, the Caucasus Region

Fabio Quaglino, Department of Agricultural and Environmental Sciences–Production, Landscape, Agroenergy (DISAA), University of Milan, Milano, Italy; **David Maghradze,** Institute of Horticulture, Viticulture and Oenology, Agricultural University of Georgia, Tbilisi, Georgia; **Paola Casati,** DISAA, University of Milan; **Nona Chkhaidze,** Laboratory of Plant Anatomy and Physiology, Agricultural University of Georgia, Tbilisi; **Mzagho Lobjanidze,** Institute of Entomology, Agricultural University of Georgia, Tbilisi; and **Adriano Ravasio, Alessandro Passera, Giovanni Venturini, Osvaldo Failla,** and **Piero Attilio Bianco,** DISAA, University of Milan

Abstract

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Evidence from a preliminary survey highlighted that '*Candidatus* Phytoplasma solani', the etiological agent of bois noir (BN) disease of grapevine, infects grapevine varieties in Georgia, a country of the South Caucasus. In this study, field surveys were carried out to investigate the BN symptom severity in international and Georgian native varieties. '*Ca.* P. solani' was detected and identified by polymerase chain reaction-based amplification and restriction fragment length polymorphism analysis of 16S ribosomal DNA, and further characterized by multiple gene typing analysis (*vmp1* and *stamp* genes). Obtained data highlighted that the majority of Georgian grapevine varieties showed moderate and mild symptoms, whereas international cultivars exhibited severe symptoms. Molecular characterization of '*Ca.* P. solani' from grapevine revealed the presence of 11 distinct phytoplasma types. Only one type (VmGe12/StGe7) was identical to a strain previously reported in periwinkle from Lebanon; the other '*Ca.* P. solani' types are described here for the first time. Phylogenetic analyses of *vmp1* and *stamp* gene concatenated nucleotide sequences showed that '*Ca.* P. solani' strains in Georgia are associated mainly with the bindweed-related BN host system. Moreover, the fact that '*Ca.* P. solani' strains are distributed in grapevine cultivars showing a range of symptom intensity suggests a different susceptibility of such local cultivars to BN.

Grapevine yellows (GY) are a phytoplasma-associated disease complex that induces severe crop losses in almost all varieties used for wine production. GY have been described in Europe, Australia, and several American countries, and more than seven genetically different phytoplasmas have been associated with such disease complex (Laimer et al. 2009). Typical GY symptoms include berry shrivel, desiccation of inflorescences, color alterations and curling of the leaves, reduction of growth, and irregular ripening of wood (Belli et al. 2010).

Among GY, bois noir (BN) is responsible for serious crop losses in the Euro-Mediterranean area and on other continents (Belli et al. 2010; Foissac et al. 2013). Phytoplasmas are cell-wall-less obligate intracellular parasites of the Mollicutes class, classified through the nucleotide sequence analysis of their housekeeping genes (Davis et al. 2013; IRPCM Phytoplasma/Spiroplasma Working Team–Phytoplasma Taxonomy Group 2004). On the basis of unique biological properties and exclusive molecular markers within multiple genes, the etiological agent of BN disease has been attributed to phytoplasma strains (BNp) belonging to the species '*Candidatus* Phytoplasma solani', subgroup 16SrXII-A (Quaglino et al. 2013). In Euro-Mediterranean regions, BNp strains are transmitted to grapevine by *Hyalesthes obsoletus* Signoret (Homoptera: Cixiide), a polyphagous vector living preferentially on nettle (*Urtica dioica* L.), bindweed (*Convolvulus arvensis* L.), and chaste tree (*Vitex agnus-castus* L.) inside or around vineyards (Kosovac et al. in press; Langer and Maixner 2004; Sharon et al. 2005). Moreover, *Reptalus panzeri* has been reported as a natural vector of BNp in Serbian vineyards (Cvrković et al. 2014). Additionally, *Anaceratagallia ribauti* and *R. quinquecostatus* were experimentally confirmed as vectors of '*Ca.* P. solani' but not to grapevine (Pinzauti et al. 2008; Riedle-Bauer et al. 2008); therefore, currently, such insects are not considered to be involved in BNp transmission to grapevine. Other studies showed that several weeds host BNp and can play a role in BN diffusion (Mori et al. 2015). The biological complexity of BN disease has stimulated research on molecular markers of BNp genetic diversity. Multilocus sequence typing, based on molecular characterization of *tuf* (Langer and Maixner 2004), *secY*, *vmp1* (Fialová et al. 2009), and *stamp* (Fabre et al. 2011) genes, highlighted the presence of numerous genetically distinct strains characterized by different distribution and prevalence in the Euro-Mediterranean basin (Atanasova et al. 2015; Foissac et al. 2013; Kosovac et al. in press; Kostadinovska et al. 2014; Murolo and Romanazzi 2015).

Due to this complexity, it is difficult to design efficient control strategies against BN. Because insecticides applied to the grapevine canopy influence neither the disease nor the presence of *H. obsoletus*, the management of *H. obsoletus* host plants in the vineyards and surrounding areas is considered crucial for BN control (Mori et al. 2012). Thus, preventive measures such as checking the sanitary status of propagation materials and treating diseased mother plants through thermotherapy are applied to limit long-distance dissemination and in-field spread of the disease. Other strategies for reducing BN spread or incidence are based on (i) preventive removal of the grape suckers on which *H. obsoletus* could feed after grass mowing, (ii) trunk cutting above the engagement point of the symptomatic grapevines, and (iii) treatments by resistance inducers (Belli et al. 2010).

An ambitious strategy for phytoplasma disease control is based on the selection of resistant, tolerant, or not susceptible plant varieties, cultivated or not-cultivated, as source of resistance genes for plant breeding programs. Previous studies identified the presence of plant species or varieties showing low susceptibility to phytoplasma

Corresponding author: F. Quaglino; E-mail: fabio.quaglino@unimi.it

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infection (Bianco et al. 2011). Unfortunately, up to now, none of the examined *Vitis* spp. and *Vitis vinifera* L. varieties have been found immune or resistant to the phytoplasma associated with GY (Laimer et al. 2009).

In the last decade, the Georgian grapevine cultivars and minor germplasm have been objects of intense research activities from both a scientific and a vinicultural perspective (Chkhartishvili and Maghradze 2012; Maghradze et al. 2009). The Georgian native germplasm consists of more than 500 cultivars, grown and vinified today only in part (Chkhartishvili and Maghradze 2012), but the best cultivars, such as 'Saperavi' (red) and 'Rkatsiteli' (white), are well known outside of the country and are cultivated in eastern Europe and central Asia. From the genetic point of view, the Georgian native germplasm demonstrated a very unique genetic pool, distinct from the European (Imazio et al. 2013) and central Asiatic (Bacilieri et al. 2013) ones. This specificity, and some relationships among the Georgian wild (representative in the native flora) and cultivated grapevines, are in agreement with archaeological evidence related to the antiquity and originality of Georgian viniculture and cultivar assortment. These genetic characteristics have a phenotypic correspondence in terms of vine morphology, phenology, and cultural and enological traits, which show a large variability inside the Georgian germplasm associated with a distinctness from neighboring as well as geographically distant grapevine germplasm (Maghradze et al. 2009). Moreover, recent studies reported that grapevine varieties selected in domestication centers of *V. vinifera* such as Georgia (the country of the South Caucasus) showed possible tolerance or resistance to plant pathogens such as *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni, associated with downy mildew (Bitsadze et al. 2014). For that reason, among others, Georgian native *V. vinifera* varieties were employed in breeding-based constitution of new varieties (Vakhtangadze et al. 2010). Evidence from a preliminary survey on GY in Georgia highlighted that '*Ca*. P. solani' infects international and local varieties (Giorgadze 2005; Quaglino et al. 2014).

In the present study, field surveys and multiple gene-typing analyses were carried out to study (i) the GY symptom severity in international and Georgian native varieties and (ii) the genetic diversity among '*Ca*. P. solani' strain populations in Georgia.

Materials and Methods

Symptom observation and plant sampling. Surveys on GY symptoms were carried out from 14 to 20 September 2013 in vineyards and *V. vinifera* germplasm collections in Khaketi and Shida (Inner) Kartli regions in eastern Georgia. Based on the severity of typical GY and GY-like symptoms observed, international and native Georgian grapevine varieties were classified into group I (mild symptoms: color alterations and curling of the leaves; normal production), group II (moderate symptoms: color alterations and curling of the leaves, partial irregular ripening of wood, and mild berry shrivel; partially reduced production), and group III (severe symptoms: berry shrivel, desiccation of inflorescences, color alterations and curling of the leaves, reduction of growth, and irregular ripening of wood; complete loss of production). Leaf samples were collected from 81 symptomatic grapevine plants of four western European or international ('Chardonnay', France; 'Freisa', Italy; 'Carignano' or 'Carignan', Spain; and 'Muscat a Petites Grains Blanc' or 'Moscato Bianco', international) and 37 native Georgian varieties. Moreover, in Mukhrani vineyards (Shida Kartli region), leaf samples were collected from six plants of the wild species *C. arvensis*, known as one of the main wild plants involved in BNp diffusion (Table 1).

Phytoplasma detection based on polymerase chain reaction and restriction fragment length polymorphism analysis of the 16S ribosomal RNA gene. Total DNA was extracted from examined plants as described by Angelini and colleagues (2001), with some modifications. Briefly, leaf veins and petioles (0.5 g) were separated from the lamina with sterile scalpels, immersed in liquid nitrogen, and ground using sterile pestles and mortars. Prewarmed cetyltrimethylammonium bromide (CTAB)-based buffer (2.5% [wt/vol] CTAB, 100 mM Tris [pH 8.0], 1.4 M NaCl, 50 mM EDTA [pH8], 1% PVP-40, and 0.5% ascorbic acid) was added to the crushed tissues, homogenized by mechanical pestle, and held at 60°C for 20 min. After incubation, DNA was extracted by adding chloroform/isoamyl alcohol (24:1, vol/vol) solution and precipitated by incubation with isopropanol at −20°C for 20 min. A nucleic acid pellet was washed with 70 and 80% ethanol, air dried, suspended in 50 µl of deionized autoclaved water, and maintained at −30°C until use.

Detection of phytoplasmas was carried out by means of amplification of 16S ribosomal DNA (rDNA) in nested polymerase chain reaction (PCR) assays primed by universal primer pairs P1/P7 and R16F2n/R16R2, and subsequent *Alu*I-, *Bfa*I-, *Bst*UI-, and *Mse*I-restriction fragment length polymorphism (RFLP) assays on the obtained amplicons. PCR and RFLP conditions were as previously described (Quaglino et al. 2009). PCR assays were performed by using *Taq* polymerase (Promega, Milan, Italy) in an automated thermal cycler (MasterCycler Gradient; Eppendorf, Milan, Italy). PCR and enzymatic digestion products were electrophoresed through 1 and 3% agarose gel, respectively, in Tris-borate-EDTA (TBE) buffer, stained with Midori Green Advance (Biosigma, Venice, Italy), and visualized under a UV transilluminator. Total nucleic acids from periwinkle (*Catharanthus roseus* (L.) G. Don) plants infected by phytoplasma strains elm yellows 1 (EY1, '*Ca.* P. ulmi', subgroup 16SrV-A), stolbur (STOL, '*Ca.* P. solani', subgroup 16SrXII-A), and aster yellows 1 (AY1, '*Ca.* P. asteris', subgroup 16SrI-B) were used as reference controls. Total nucleic acids extracted from healthy periwinkle and PCR mixture devoid of nucleic acids were used as negative controls.

Molecular characterization of '*Ca*. P. solani' strains through sequence analysis of *vmp1* and *stamp* genes. BNp strains, identified in the present study by PCR-RFLP-based assays on 16S rDNA, were typed by nested PCR amplification of the gene *vmp1* and subsequent molecular characterization by RFLP assays and sequence analyses. Reaction mixtures and PCR conditions were as previously described (Fialová et al. 2009). Phytoplasma reference controls and visualization of PCR products were as described above for 16S rDNA. Amplified PCR products were analyzed by RFLP technique using the enzyme *Rsa*I (Fialová et al. 2009). Enzymatic digestion products were electrophoresed through 3% agarose gel in TBE buffer, stained with Midori Green Advance (Biosigma), and visualized under a UV transilluminator.

The *vmp1* gene fragments amplified from 15 BNp strains, representative of the obtained *Rsa*I-RFLP profiles, were selected for nucleotide sequence analysis. The *vmp1* gene amplicons were sequenced in both senses by a commercial service (Primm, Milan, Italy) to achieve at least 5× coverage per base position. Nucleotide sequence data were assembled by employing the Contig Assembling program of the software BioEdit version 7.0.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/) under accession numbers shown in Table 2. The *vmp1* nucleotide sequences of '*Ca.* P. solani' strains previously published were retrieved from GenBank (Supplementary Table S1) and used for comparison with the sequences obtained in this study. In detail, nucleotide sequences were compiled in FASTA format and trimmed to the fragment delimited by annealing sites of the primers TYPH10F and TYPH10R (Fialová et al. 2009). To comprehensively compare *Rsa*I-RFLP profiles of BNp strains from Georgia with profiles previously described (Aryan et al. 2014; Cimerman et al. 2009; Cvrković et al. 2014; Kostadinovska et al. 2014; Murolo et al. 2013), trimmed nucleotide sequences were searched for single-nucleotide polymorphisms in recognition sites for the enzyme *Rsa*I by virtual RFLP analyses using the software pDRAW32 (http://www.acaclone.com/). The association between the *vmp1*-RFLP profiles and BN symptom severity was evaluated by χ^2 test using IBM SPSS Statistics V. 22.0 for Windows (IBM Corporation, Armonk, NY).

The *stamp* gene was amplified from the same 15 BNp strains selected for *vmp1* gene sequence characterization. Reaction mixtures and PCR conditions were as previously described (Fabre et al. 2011). Phytoplasma reference controls and visualization of PCR products were as described above for 16S rDNA. The *stamp* gene

amplicons were sequenced, assembled, and deposited in the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers shown in Table 2. Nucleotide sequences of the '*Ca*. P. solani' *stamp* gene previously published were retrieved from GenBank and utilized for comparison with the sequences obtained in this study. Nucleotide sequences were compiled in FASTA format and trimmed to the fragment delimited by annealing sites of the primers StampF1 and StampR1 (Fabre et al. 2011).

The *vmp1* and *stamp* gene nucleotide sequences were aligned using the ClustalW Multiple Alignment application and analyzed for sequence identity determination using the Sequence Identity Matrix application of the software BioEdit, version 7.0.5. Based on sequence identities, '*Ca*. P. solani' strains were grouped into *vmp1* and *stamp* genetic variants, and into collective *vmp*/*stamp* types for which nomenclature was designated in the present study (Table 3; Supplementary Tables S2 and S3). Strains of each variant or type shared 100% sequence identity.

Phylogenetic analysis and selective pressure of '*Ca*. P. solani' from Georgia and other geographical regions. Nucleotide sequences of *vmp1* and *stamp* genes of BNp strains identified in the present study (Table 2) and of one strain representative of each *vmp1* and *stamp* genetic variant identified among '*Ca*. P. solani' strains previously described in GenBank were employed for phylogenetic analyses. GenBank accession numbers are shown in Supplementary Table S1 and on the phylogenetic trees. Moreover, *vmp1* and *stamp* gene sequences of '*Ca*. P. solani' from this and previous studies (Tables 2 and 3) were concatenated with the BioEdit software and used for phylogenetic analyses. In detail, minimum evolution analysis was carried out using the neighbor-joining method and bootstrap was replicated 1,000 times with the software MEGA6 (Tamura et al. 2013).

Table 1. Symptom severity and phytoplasmas in Georgian vineyards

Location	Plant host	Severity	*N*[b]	rXII-A[c]	*vmp1*[a]					
					1 (V1)	2 (V14)	3 (V15)	4 (und1)	5 (und2)	6 (und3)
Tsinandali collection, Khaketi,	Carignano	+++	1	1	…	…	…	…	1	…
Winery "Shumi"	Freisa	+++	1	1	…	…	…	…	1	…
	Grdzelmtevana	+	1		…	…	…	…		…
	Saperavi Pachkha	++	1	…	…	…	…	…	…	…
Kondoli vineyards 1, Khaketi,	Chardonnay	+++	5	5	3	1	…	…	1	…
Winery "Telavi Wine Cellar"	Kisi	+++	2	2	1	…	…	…	1	…
	Mtsane Kakhuri	+	1	…	…	…	…	…	…	…
	Rkatsiteli	+	2	2	2	…	…	…	…	…
	Saperavi	++	3	2	2	…	…	…	…	…
Kondoli vineyards 2, Khaketi, Winery "Bessini"	Chardonnay	+++	4	4	1	1	…	…	…	…
Kindzmaraulis collection, Khaketi,	Adznizhi	+	1	…	…	…	…	…	…	…
Winery "Kindzmaraulis Marani"	Amlakhy	+	1	1	…	…	…	…	…	1
	Asuretuli Shavi	+	1	…	…	…	…	…	…	…
	Buera	++	1	1	1	…	…	…	…	…
	Chinuri	+	1	…	…	…	…	…	…	…
	Chkhaverii	+	1	…	…	…	…	…	…	…
	Chuberi	+	2	…	…	…	…	…	…	…
	Goruli Mtsvane	++	1	…	…	…	…	…	…	…
	Khikhvi	+	1	1	…	…	…	…	1	…
	Khikhvi variation	+	1	…	…	…	…	…	…	…
	Kikhvi Loladzis	+	1	1	…	…	1	…	…	…
	Kisi	+++	1	1	…	…	…	…	…	…
	Korkaula	+	3	2	…	…	1	…	…	…
	Mtredisphekha	+	1	…	…	…	…	…	…	…
	Mujuretuli	+	1	1	…	…	…	…	…	…
	Rkatsiteli	+	1	1	…	…	1	…	…	…
	Saperavi	++	1	…	…	…	…	…	…	…
	Saperavi Budeshuri	+	2	1	…	…	…	…	1	…
	Tavkveni Saperaviseburi	+	1	1	…	…	…	…	…	…
	Tavkveri	+	1	1	…	…	1	…	…	…
	Tshnoris Tetri	+	1	…	…	…	…	…	…	…
	Tsitska	+	1	1	…	…	…		1	…
	Tsolikouri	+	1	1	…	…	…	…	…	…
	Usakhelouri	+	1	1	…	…	1	…	…	…
Mukhrani vineyards, Shida Kartli,	Chardonnay	+++	11	11	4	3	…	2	2	…
Winery "Chateau Mukhrani"	*Convolvulus arvensis*	+++	6	6	…	4	…	2	…	…
	Goruli Mtsvane	++	3	1	…	1	…	…	…	…
	Moscato Bianco	+	3	3	…	1	…	…	…	…
	Rkatsiteli	+	2	1	…	…	…	…	…	…
	Saperavi	++	2	2	…	1	…	…	1	…
Shida Kartli vineyards	Rkatsiteli	+	4	…	…	…	…	…	…	…
	Saperavi	++	7	5	2	…	…	…	1	…
Overall	…	…	87	61	16	12	5	4	11	1

[a] Designation of V1, V14, and V15 *Rsa*I restriction fragment length polymorphism (RFLP) profiles according to the SEE-ERANET nomenclature (X. Foissac, INRA, Bordeaux, France); und1, und2, and und3 are new, previously undescribed restriction patterns.
[b] Number of samples.
[c] Subgroup defined by RFLP analysis of 16S ribosomal RNA F2n/R2 PCR fragments.

The ratio between the proportion of nonsynonymous and synonymous substitutions (*dN/dS* ratio) for each gene was determined for the nucleotide sequences of (i) the overall '*Ca*. P. solani' strain populations (both strains identified in Georgia in this study and previously identified strains from Euro-Mediterranean areas), (ii) the '*Ca*. P. solani' strain populations identified in Georgia, and (iii) the '*Ca*. P. solani' strain populations from Euro-Mediterranean areas. The *dN/dS* ratios and the null hypothesis of no selection (H0: $dN = dS$) versus the positive selection hypothesis (H1: $dN > dS$) were calculated using the Nei-Gojobori method in a codon-based *Z*-selection test (Nei and Gojobori 1986). The analysis was carried out in MEGA6, and the variance of the differences was computed using the bootstrap method (1,000 replicates; Tamura et al. 2013). The synonymous and nonsynonymous nucleotide substitution rates for the nucleotide sequences of the genes *vmp1* and *stamp* were calculated. Positive selection happens when *dN/dS* ratio >1.0 and *P* value for the *Z* test < 0.05; on the other hand, a ratio < 1.0 suggests a purifying selection process (Nei and Kumar 2000).

Results

Symptoms observed on grapevine in Georgia. Severe symptoms were observed in 3 international varieties (Chardonnay, Carignano, and Freisa) and 1 local Georgian variety ('Kisi') (Fig. 1A and B); moderate symptoms were observed in 4 local Georgian varieties ('Buera', 'Goruli Mtsvane', Saperavi, and 'Saperavi Pachkha') (Fig. 1C and D); and mild symptoms were observed in 22 local Georgian varieties and 1 international variety (Moscato Bianco) (Fig. 1E and F; Table 1). Leaf samples were collected from 25, 19, and 37 plants of varieties showing severe, moderate, and mild symptoms, respectively (Table 1). Leaf samples were also collected from six bindweed plants showing yellowing, reddening, dwarfism, and leaf malformation (Fig. 2).

GY phytoplasma identification. PCR-based amplification of the 16S rRNA gene showed that 55 of 81 examined grapevines were infected by phytoplasmas. In detail, phytoplasmas were detected in all grapevine plants showing severe symptoms and in 58% (11 of 19 plants) and 51% (19 of 37 plants) of grapevine plants showing moderate and mild symptoms, respectively (Table 1). Moreover, all six bindweed samples have been found infected by phytoplasmas. DNA amplification was obtained from periwinkles infected by phytoplasma reference strains STOL, AY1, and EY1. Reliability of the results was supported by the absence of DNA amplification in the reactions of healthy periwinkle and the negative control (PCR mixture devoid of DNA). *Alu*I-, *Bfa*I-, *Bst*UI-, and *Mse*I-digestion analysis showed that all the phytoplasma strains identified in grapevine and bindweed belonged to the species '*Ca*. P. solani', taxonomic subgroup 16SrXII-A, because they had restriction patterns indistinguishable from one another and from the patterns characteristic of the STOL (16SrXII-A) reference strain (data not shown).

'*Ca*. P. solani' strain characterization by *vmp1* and *stamp* gene sequence analysis. The *vmp1* gene fragment was amplified from 43 of 55 infected grapevines, and from all six infected bindweeds. In detail, amplification was obtained in 22, 9, and 12 grapevine plants showing severe, moderate, and mild symptoms, respectively (Table 1). Enzymatic digestions of the 49 *vmp1* amplicons showed the presence of six *Rsa*I profiles among BNp strains from Georgia. Such profiles were confirmed by virtual RFLP analysis carried out on *vmp1* nucleotide sequences amplified from BNp strains representative of each actual RFLP pattern. Virtual RFLP-based comparison of *vmp1* RFLP profiles obtained in this study with profiles reported in literature showed that Georgian BNp strains showed previously described (V1, V14, and V15) and new (undescribed [und]1, und2, and und3) restriction patterns (Fig. 3). Strains showing profiles V1, V14, and und2 were prevalent within BNp strain populations in Georgia and were identified with significantly different distribution in grapevine varieties showing severe, moderate, and mild symptoms in both the Khaketi and Shida Kartli regions ($\chi^2 = 16.671$, df = 10, $P = 0.029$; Fig. 4). On the other hand, strains showing profile und1 were found only in Chardonnay showing severe symptoms and in symptomatic bindweed from the Shida Kartli region, while strains showing profiles V15 and und3 (identified in a single grapevine plant) were found only in varieties showing mild symptoms in the Khaketi region (Table 1; Fig. 4).

Nucleotide sequence analysis, performed on 15 BNp strains from Georgia selected on the basis of the diversity found through RFLP analysis of *vmp1* gene amplicons, revealed the presence of 12 and 7 genetic variants of *vmp1* (here designated as VmGe1 to VmGe12) and *stamp* (here designated as StGe1 to StGe7) genes, respectively (Table 2). Average sequence identity among genetic variants was 92.3 and 97.8% for *vmp1* and *stamp* genes, respectively. Eleven Georgian BNp *vmp/stamp* types were described as the combination of *vmp1* and *stamp* genetic variants (Table 2). Two of such types (VmGe8/StGe3 and VmGe12/StGe7) were identified more frequently (5 of 15 BNp strains); the other types were found in single plant samples.

Table 2. Bois noir phytoplasma types identified in Georgia by nucleotide sequence analyses of the membrane protein coding genes *vmp1* and *stamp*

Strain	Plant host	Location	Profile[a]	Genetic variant (accession number)		*vmp/stamp* type
				vmp1	*stamp*	
Carv1	*Convolvulus arvensis* (+++)	Mukhrani	2 (V14)	VmGe1 (KT184867)	StGe1 (KT184879)	VmGe1/StGe1
Carv2	*C. arvensis (+++)*	Mukhrani	4 (und1)	VmGe2 (KT184868)	StGe2 (KT184880)	VmGe2/StGe2
Char7	Chardonnay (+++)	Mukhrani	1 (V1)	VmGe3 (KT184869)	StGe3 (KT184881)	VmGe3/StGe3
Char8	Chardonnay (+++)	Mukhrani	2 (V14)	VmGe4 (KT184870)	StGe4 (KT184882)	VmGe4/StGe4
Sape19	Saperavi (++)	Mukhrani	2 (V14)	VmGe5 (KT184871)	StGe4[d]	VmGe5/StGe4
GoMt25	Goruli Mtsvane (++)	Mukhrani	2 (V14)	VmGe6 (KT184872)	StGe4[d]	VmGe6/StGe4
Kisi38	Kisi (+++)	Kondoli	5 (und2)	VmGe7 (KT184873)	StGe3[e]	VmGe7/StGe3
Rkat47	Rkatsiteli (+)	Kondoli	1 (V1)	VmGe8 (KT184874)	StGe3[e]	VmGe8/StGe3
Sape51	Saperavi (++)	Kondoli	1 (V1)	VmGe8[b]	StGe3[e]	VmGe8/StGe3
Sape62	Saperavi (++)	Shida Kartli	1 (V1)	VmGe8[b]	StGe3[e]	VmGe8/StGe3
Khik70	Khikhvi (+)	Kindzmaraulis	5 (und2)	VmGe9 (KT184875)	n.d.[f]	n.d.
Amla77	Amlakhy (+)	Kindzmaraulis	6 (und3)	VmGe10 (KT184876)	StGe5 (KT184883)	VmGe10/StGe5
Sabu84	Saperavi Budeshuri (+)	Kindzmaraulis	5 (und2)	VmGe11 (KT184877)	StGe6 (KT184884)	VmGe11/StGe6
Tsol89	Tsolikuri (+)	Kindzmaraulis	3 (V15)	VmGe12 (KT184878)	StGe7 (KT184885)	VmGe12/StGe7
Kiqu94	Kikhvi Loladzis (+)	Kindzmaraulis	3 (V15)	VmGe12[c]	StGe7[e,g]	VmGe12/StGe7

[a] Restriction fragment length polymorphism (RFLP) *vmp1* profile. Designation of V1, V14, and V15 *Rsa*I-RFLP profiles according to the SEE-ERANET nomenclature (X. Foissac, INRA, Bordeaux, France); und1, und2, and und3 are new, previously undescribed restriction patterns.
[b] Strains Sape51 and Sape62 share 100% *vmp1* sequence identity with strain Rkat47.
[c] Strains Kiqu94 shares 100% *vmp1* sequence identity with strain Tsol89.
[d] Strains Sape19 and GoMt25 share 100% *stamp* sequence identity with strain Char8.
[e] Strains Kisi38, Rkat47, Sape51 and Sape62 share 100% *stamp* sequence identity with strain Char7.
[f] Abbreviation: n.d. = not done.
[g] Strains Kiqu94 shares 100% *stamp* sequence identity with strain Tsol89.

Table 3. '*Candidatus* Phytoplasma solani' *vmp*/*stamp* types identified by collective nucleotide sequence analyses of the membrane protein coding genes *vmp1* and *stamp*

Strain[a]	Host	Location	Profile[b]	Accession number				*vmp/stamp* type
				vmp1	Variant	*stamp*	Variant	
Vv17	*Vitis vinifera*	Serbia	V2-TA	KC703032	Vm10	KC703018	St2	Vm10/St2
Rqg31	*Reptalus quinquecostatus*	Serbia	V2-TA	KC703031	Vm10	KC703017	St2	Vm10/St2
STOL	*Capsicum annuum*	Serbia	V2-TA	AM992103	Vm10	FN813261	St4	Vm10/St4
Vv21	*V. vinifera*	Serbia	V2-TA	KC703026	Vm10	KC703012	St4	Vm10/St4
Vexp Rpm5	*R. panzeri*	Serbia	V2-TA	KC703028	Vm10	KC703014	St4	Vm10/St4
Vexp Rpg11	*R. panzeri*	Serbia	V2-TA	KC703027	Vm10	KC703013	St4	Vm10/St4
Rpm34	*R. panzeri*	Serbia	V2-TA	KC703024	Vm10	KC703010	St4	Vm10/St4
Rpg39	*R. panzeri*	Serbia	V2-TA	KC703023	Vm10	KC703009	St4	Vm10/St4
Rqg60	*R. quinquecostatus*	Serbia	V2-TA	KC703025	Vm10	KC703011	St4	Vm10/St4
LA6_I_C	*Convolvulus arvensis*	Germany	V2-TA	JQ977735	Vm11	JQ977720	St5	Vm11/St5
GGY	*V. vinifera*	Germany	V2	AM992102	Vm12	FN813256	St5	Vm12/St5
Vv12_Kn6	*V. vinifera*	Austria	V2-TA	KJ469734	Vm14	KJ469724	St2	Vm14/St2
Vv12_751	*V. vinifera*	Austria	V2-TA	KJ469734[c]	Vm14	KJ469723	St3	Vm14/St3
Vv12_754	*V. vinifera*	Austria	V2-TA	KJ469734[c]	Vm14	KJ469721[e]	St5	Vm14/St5
60/11	*V. vinifera*	Italy	V3	KJ145346	Vm15	KJ145345	St9	Vm15/St9
Aa25	*V. vinifera*	Italy	V3	HM008614	Vm15	KJ145387	St9	Vm15/St9
Ho13_1006	*Hyalesthes obsoletus*	Austria	V3	KJ469727	Vm15	KJ469718	St9	Vm15/St9
Ho13_838	*H. obsoletus*	Austria	V3	KJ469729	Vm18	KJ469720	St8	Vm18/St8
CrHo13_1183	*H. obsoletus*	Austria	V3	KJ469728	Vm23	KJ469719	St19	Vm23/St19
MK44	*V. vinifera*	Macedonia	V3	KF957605	Vm25	KF957607	St6	Vm25/St6
Aa16	*V. vinifera*	Italy	V4	HM008602	Vm27	KJ145380	St21	Vm27/St21
Rpg47	*R. panzeri*	Serbia	V4	KC703034	Vm28	KC703020	St1	Vm28/St1
Rqg50	*R. quinquecostatus*	Serbia	V4	KC703033	Vm28	KC703019	St1	Vm28/St1
CrAr12_722_2	*Anaceratagallia ribauti*	Austria	V4	KJ469735	Vm28	KJ469722	St1	Vm28/St1
Vv12_752	*V. vinifera*	Austria	V4	KJ469735[d]	Vm28	KJ469721[e]	St5	Vm28/St5
Aaq1	*V. vinifera*	Italy	V4	HM008601	Vm28	KJ145383	St18	Vm28/St18
166/11	*V. vinifera*	Italy	V4	KJ145355	Vm28	KJ145343	St20	Vm28/St20
136/11	*V. vinifera*	Italy	V4	KJ145354	Vm28	KJ145340	St20	Vm28/St20
P10/11	*V. vinifera*	Italy	V4	KJ145353	Vm28	KJ145342	St20	Vm28/St20
Mca21	*V. vinifera*	Italy	V4	HM008599	Vm28	KJ145382	St20	Vm28/St20
CrHo12_601	*H. obsoletus*	Austria	V4	KJ469730	Vm33	KJ469721	St5	Vm33/St5
19-25	*V. vinifera*	Germany	V5	AM992101	Vm34	FN813267	St11	Vm34/St11
PO	*H. obsoletus*	France	V7	AM992095	Vm35	FN813270	St10	Vm35/St10
Rqg42	*R. quinquecostatus*	Serbia	V7-A	KC703030	Vm36	KC703016	St31	Vm36/St31
LG	*Solanum lycopersicum*	France	V8	AM992097	Vm38	FN813259	St10	Vm38/St10
78/11	*V. vinifera*	Italy	V9	KJ145349	Vm39	KJ145334	St5	Vm39/St5
Mp49	*V. vinifera*	Italy	V9	HM008607	Vm40	KJ145376	St32	Vm40/St32
Mvercer2	*V. vinifera*	Italy	V12	HM008612	Vm44	KJ145375	St22	Vm44/St22
P136/11	*V. vinifera*	Italy	V12	KJ145358	Vm45	KJ145336	St5	Vm45/St5
P75/11	*V. vinifera*	Italy	V12	KJ145357	Vm45	KJ145333	St5	Vm45/St5
315/11	*V. vinifera*	Italy	V12	KJ145360	Vm45	KJ145330	St5	Vm45/St5
353/11	*V. vinifera*	Italy	V14	KJ145352	Vm46	KJ145338	St1	Vm46/St1
115/11	*V. vinifera*	Italy	V14	KJ145350	Vm46	KJ145337	St1	Vm46/St1
Mp46	*V. vinifera*	Italy	V14	HM008606	Vm46	KJ145379	St1	Vm46/St1
Ag4a	*V. vinifera*	Italy	V14	HM008605	Vm46	KJ145377	St1	Vm46/St1
Vv5	*V. vinifera*	Serbia	V14	KC703035	Vm46	KC703021	St1	Vm46/St1
Rpm35	*R. panzeri*	Serbia	V14	KC703029	Vm46	KC703015	St3	Vm46/St3
287/11	*V. vinifera*	Italy	V14	KJ145351	Vm46	KJ145332	St5	Vm46/St5
Vv24	*V. vinifera*	Serbia	V14	KC703036	Vm46	KC703022	St30	Vm46/St30
Ca13_RF	*Convolvulus arvensis*	Austria	V14	KJ469732	Vm50	KJ469721[e]	St5	Vm50/St5
P7	*Catharanthus roseus*	Lebanon	V15	AM992100	Vm53	FN813258	St15	Vm53/St15
P42/11	*V. vinifera*	Italy	V15	KJ145356	Vm54	KJ145341	St20	Vm54/St20
CrHo12_721	*H. obsoletus*	Austria	V17	KJ469731	Vm57	KJ469722[f]	St1	Vm57/St1
MK94	*V. vinifera*	Macedonia	V18	KF957606	Vm59	KF957609	St11	Vm59/St11
CrHo12_650	*H. obsoletus*	Austria	V18	KJ469725	Vm60	KJ469716	St11	Vm60/St11
Vv12_274	*V. vinifera*	Austria	V23	KJ469726	Vm61	KJ469717	St29	Vm61/St29
425/11	*V. vinifera*	Italy	unreported	KJ145348	Vm62	KJ145335	St5	Vm62/St5
Vv12_Ill6	*V. vinifera*	Austria	unreported	KJ469733	Vm63	KJ469722[f]	St1	Vm63/St1

[a] Reference strains of each *vmp*/*stamp* type are presented in bold.
[b] For gene *vmp1*, designation of "V" *Rsa*I restriction fragment length polymorphism profiles according to the SEE-ERANET nomenclature (X. Foissac, INRA, Bordeaux, France).
[c] Phytoplasma strains sharing the same *vmp1* sequence of the strain Vv12_Kn6.
[d] Phytoplasma strains sharing the same *vmp1* sequence of the strain CrAr12_722_2.
[e] Phytoplasma strains sharing the same *stamp* sequence of the strain CrHo12_601.
[f] Phytoplasma strains sharing the same *stamp* sequence of the strain CrAr12_722_2.

Sequence identity calculation among nucleotide sequences of *vmp1* and *stamp* genes of '*Ca*. P. solani' strains retrieved from the NCBI GenBank highlighted the presence of 63 *vmp1* genetic variants (here designated as Vm1 to Vm63) of 105 analyzed sequences and 35 *stamp* genetic variants (here designated as St1 to St35) of 89 analyzed sequences. Average sequence identity among genetic variants was 90.5 and 95.5% for *vmp1* and *stamp* genes, respectively. Collective analysis of *vmp1* and *stamp* nucleotide sequences, available in the NCBI GenBank for 58 '*Ca*. P. solani' strains, allowed the identification of 38 *vmp/stamp* types, here designated as the combination of *vmp1* and *stamp* genetic variants (Table 3). Comparison with representative strains of each variant from GenBank nucleotide sequences showed that BNp strains from Georgia showed 11 *vmp1* (VmGe1 to VmGe7 and VmGe9 to VmGe12) and 6 *stamp* (StGe1 to StGe6) novel genetic variants, previously unreported. Only BNp phytoplasma strains Tsol89 and Kiqu94 (VmGe12/StGe7) shared 100% sequence identity with *vmp1* and *stamp* genes of '*Ca*. P. solani' strain P7 (Vm53/St15; Table 3), identified in periwinkle in Lebanon (Cimerman et al. 2009). Nucleotide sequences of one BNp strain from Georgia, representative of each *vmp1* and *stamp* genetic variant, were deposited at the NCBI GenBank (accession numbers shown in Table 2).

Phylogenetic analysis and selective pressure of '*Ca*. P. solani' strains from Georgia and other geographical regions. The *vmp1* gene phylogenetic tree showed that BNp strains identified in the present study grouped within four of the eight main clusters identified. The majority of BNp strains from Georgia (8 of 15), belonging to genetic variants VmGe3, VmGe7, VmGe8, VmGe9, and VmGe12, grouped within the cluster *vmp1*-5 along with genetic variants Vm53 (identical to VmGe12), Vm56, and Vm57 (Fig. 5). The *stamp* gene phylogenetic tree showed that BNp strains identified in the present study grouped within three of the four main clusters identified. The majority of BNp strains from Georgia (12 of 14), belonging to genetic variants StGe1, StGe2, StGe3, StGe4, and StGe7, clustered within the cluster *stamp*-4 along with genetic variants St3, St4, St15 (identical to StGe7), and St28 (Fig. 6).

Based on phylogenetic analysis of concatenated nucleotide sequences of the genes *vmp1* and *stamp*, five *vmp/stamp* clusters were identified. The cluster *vmp/stamp*-4 included '*Ca*. P. solani' strains associated with the *U. dioica*-related biological cycle, while the other four clusters (*vmp/stamp*-1, -2, -3, and -5) included '*Ca*. P. solani' strains associated with the *Convolvulus arvensis*-related biological cycle. The majority of Georgian BNp strains (13 of 14) grouped within *Convolvulus arvensis*-related clusters *vmp/stamp*-3 and *vmp/stamp*-5, whereas only strain Amla77 grouped within the *U. dioica*-related cluster *vmp/stamp*-4 (Fig. 7). In detail, Georgian BNp strains identified in grapevine and bindweed, grouped within cluster *vmp/stamp*-3, were found to be closely related to strain P7, previously identified in a naturally infected periwinkle plant (*Catharanthus roseus*) in Lebanon (Fig. 7).

The genetic variability and selective pressure in the *vmp1* and *stamp* genes were estimated for the '*Ca*. P. solani' strains according to the abundance of nonsynonymous mutations. The overall ratio between the nonsynonymous and synonymous mutations (dN/dS) was >1.0 for *vmp1* ($dN/dS = 4.567$, $P = 0.000$) and *stamp* ($dN/dS = 2.436$, $P = 0.008$) (Table 4). These high values of dN/dS (i.e., >1) indicated detection of a high number of nonsilent (dN) mutations. For the *stamp* gene, less intensive positive selective pressure (dN/dS) was found within '*Ca*. P. solani' strains from Euro-Mediterranean populations ($dN/dS = 2.711$, $P = 0.004$) in comparison with BNp strains from Georgia ($dN/dS = 2.953$, $P = 0.002$). An opposite trend was seen for *vmp1* with respect to *stamp*, where the dN/dS ratio decreased from 4.637 ($P = 0.000$, Euro-Mediterranean population) to 3.618 ($P = 0.000$, Georgia) (Table 4).

Discussion

The results obtained in this study confirmed the evidence from previous research indicating the presence of BN disease of grapevine in Georgia (Quaglino et al. 2014). In fact, molecular analyses showed a strong association between specific GY disease symptoms and

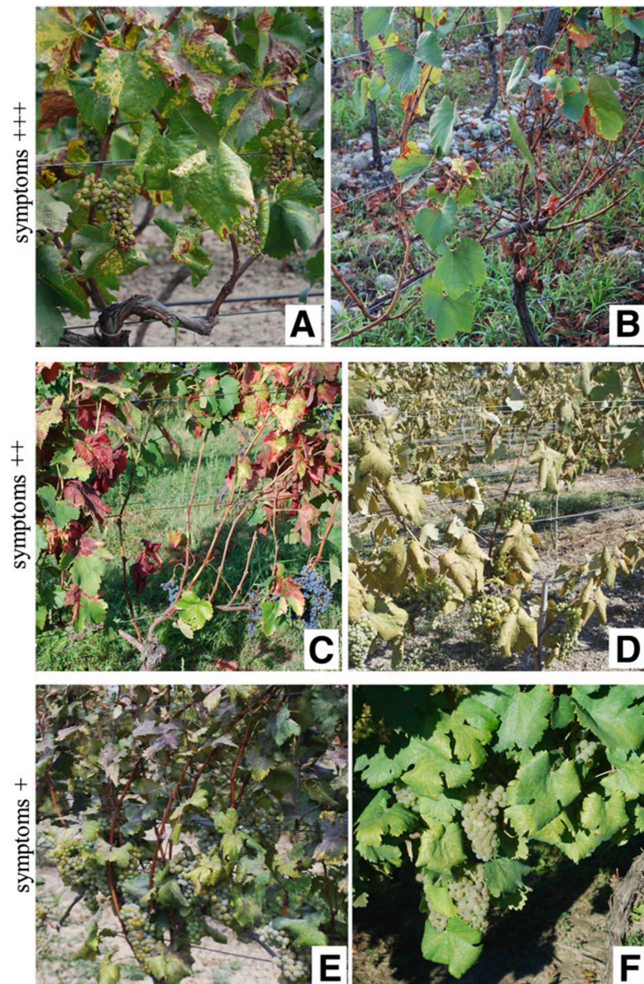

Fig. 1. Intensity of grapevine yellows symptoms observed in Georgian vineyards. Severe symptoms on cultivars **A,** Chardonnay and **B,** Kisi; moderate symptoms on **C,** Saperavi and **D,** Goruli Mtsvane; and mild symptoms on **E,** Rkatsiteli and **F,** Tsitska.

Fig. 2. Symptoms observed on bindweed (*Convolvulus arvensis* L.) plants inside the Georgian vineyard located in Mukhrani. Main symptoms are dwarfism, color alteration, and malformation of the leaves.

grapevine plant infection by BNp ('*Ca*. P. solani' strains) within the examined vineyards. On the other hand, the presence of PCR-negative plants of grapevine cultivars showing moderate and mild symptoms could be connected with the low titer or sporadic distribution of phytoplasmas in symptomatic plant tissues (Constable et al. 2003), or with the uncertain attribution of unclear symptoms to GY. Moreover, RFLP analysis of 16S rDNA amplicons excluded the presence of phytoplasmas genetically distinct from '*Ca*. P. solani' associated with other diseases (i.e., Flavescence dorée) of the GY complex (Belli et al. 2010; Laimer et al. 2009).

Surprisingly, in contrast with what was reported from GY surveys in European countries (Belli et al. 2010; Kosovac et al. in press; Mori et al. 2015), in the present study, BNp-infected bindweed in Georgian vineyards was found showing typical symptoms of phytoplasma infection. Interestingly, asymptomatic bindweed from the same vineyards was negative when tested for PCR-based BNp detection (data not shown). This result could suggest the possibility of monitoring symptoms on bindweed, naturally present in Georgia, as a reporter of the presence of '*Ca*. P. solani' in vineyards where grapevines are not showing typical GY symptoms (i.e., tolerant varieties or plantlets for propagation materials), improving the possibility of containing the disease by preventing movement of symptomless infected material.

In recent years, multiple gene analysis was proposed and employed to describe phytoplasma species distinguished by evident molecular diversity and representing ecologically separated populations. Moreover, this approach was also applied to the investigation of the genetic diversity among phytoplasmas associated with several diseases in order to identify strain-specific molecular markers useful for improving the understanding of complex phytoplasma ecologies (Davis et al. 2013; Quaglino et al. 2013). In order to gain an insight into the genetic diversity among BNp strains in Georgia, nucleotide sequence analysis was performed on two genes (*vmp1* and *stamp*) coding for membrane proteins putatively involved in the recognition and interaction of BNp with its hosts (Cimerman et al. 2009; Fabre et al. 2011). In the present study, such genes were selected because they have higher sequence variability than the *tuf* gene, classically used to distinguish two genetically divergent BNp strain types (*tuf*-a and *tuf*-b) involved in two diverse epidemiological cycles of BN (Langer and Maixner 2004); thus, they are more useful than the *tuf* gene to accurately describe the genetic diversity among BNp strain populations (Aryan et al. 2014; Cvrković et al. 2014; Kosovac et al. in press; Kostadinovska et al. 2014).

Based on *Rsa*I-RFLP digestions of *vmp1* gene amplicons, the profiles V1, V14, and the previously unreported und2 were prevalent among the analyzed BNp strains (Table 1). These data confirmed the specific association of pattern V14 with eastern Europe (Foissac et al. 2013), and highlighted an unexpected diffusion of type V1, reported as the prevalent type in bindweed- and nettle-related BN host systems in Italy, France, and Germany (Foissac et al. 2013) in the Caucasian geographic regions. This evidence, along with the prevalence of type V1 in the international

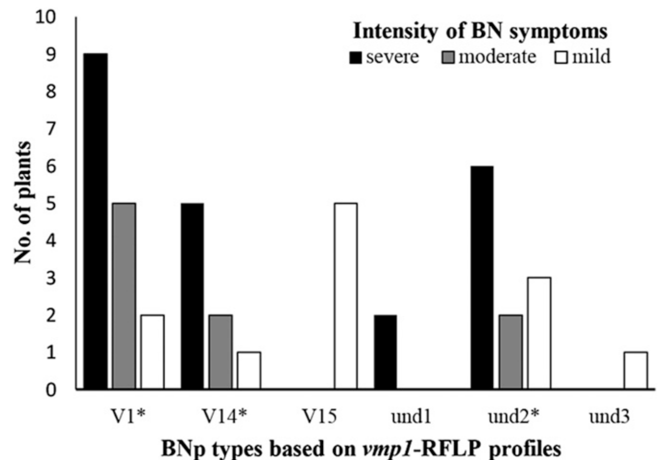

Fig. 4. Distribution of Georgian bois noir (BN) attributed to phytoplasma (BNp) types, determined by restriction fragment length polymorphism (RFLP) analysis of *vmp1* gene amplicons, in grapevine cultivars exhibiting severe, moderate, and mild symptoms. Asterisk (*) indicates that the counts of plants showing different BN symptoms within the *vmp1*-RFLP profile are different at a $P < 0.1$ (χ^2 test).

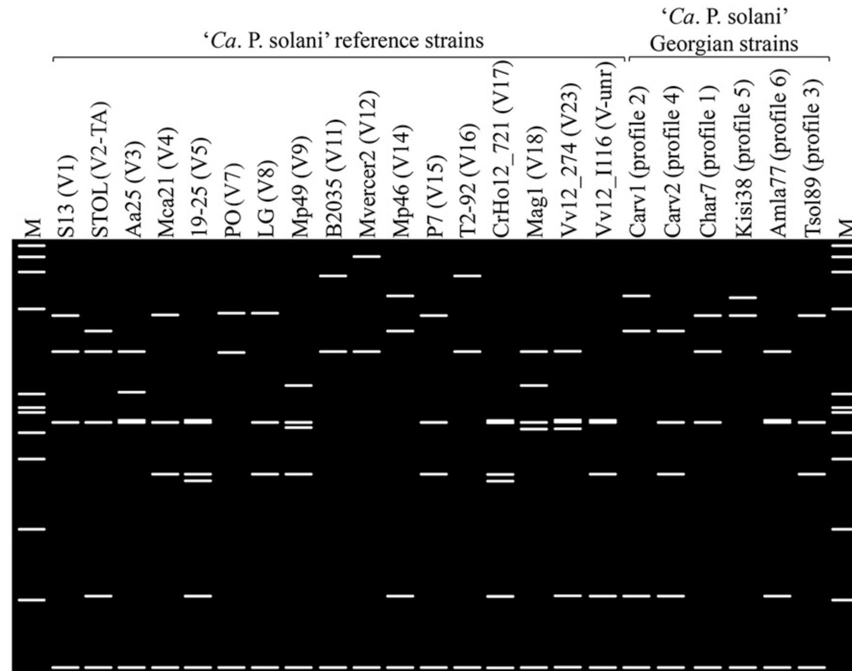

Fig. 3. Virtual *Rsa*I restriction fragment length polymorphism (RFLP) profiles of *vmp1* gene fragments obtained from bois noir attributed to phytoplasma (BNp) strain populations in Georgia. Attribution of *vmp1* restriction patterns of Georgian BNp strains (Carv1, Carv2, Char7, Kisi38, Amla77, and Tsol89) was carried out through comparison with virtual *Rsa*I-RFLP profiles of '*Candidatus* Phytoplasma solani' reference strains (S13, STOL, Aa25, Mca21, 19-25, PO, LG, Mp49, B2035, Mvercer2, Mp46, P7, T2-92, CrHo12_721, Mag1, Vv12_274, and Vv12_I116). Designation of *vmp1 Rsa*I-RFLP profiles is according to the SEE-ERANET nomenclature (X. Foissac, INRA, Bordeaux, France). Profile V-unr (strain Vv12_I116): V = unreported and M = marker Φx174 digested with the enzyme *Hae*III.

cultivar Chardonnay, could suggest the nonindigenous origin of this type, possibly introduced in Georgia through import of planting material from central or western Europe.

As largely reported for phytoplasma-associated diseases of stone fruit trees (i.e., apple proliferation and European stone fruit yellows), symptom intensity observed in infected plants can be influenced by

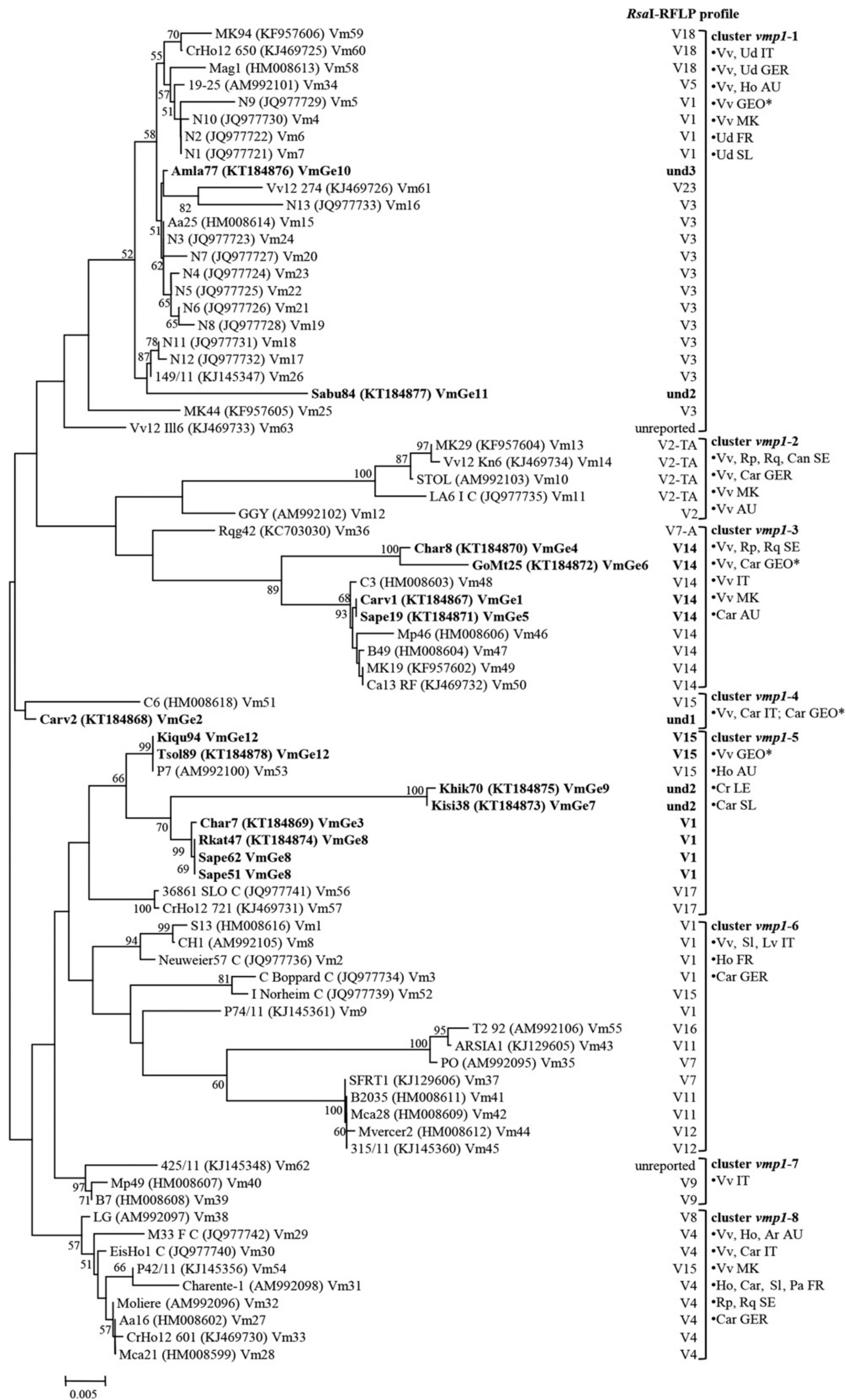

Fig. 5. Unrooted phylogenetic tree inferred from '*Candidatus* Phytoplasma solani' strain nucleotide sequences of gene *vmp1*. Minimum evolution analysis was carried out using the neighbor-joining method and bootstrap replicated 1,000 times. Names of phytoplasma strains included in phylogenetic analysis 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. Clusters are shown as delimitated by parentheses. Acronyms within clusters indicated phytoplasma hosts and origin. Hosts: Ar, *Anaceratagallia ribauti*; Can, *Capsicum annuum*; Car, *Convolvulus arvensis*; Cr, *Catharanthus roseus*; Ho, *Hyalesthes obsoletus*; Lv, *Linaria vulgaris*; Pa, *Prunus avium*; Rp, *Reptalus panzeri*; Rq, *R. quinquecostatus*; Sl, *Solanum lycopersicum*; Ud, *Urtica dioica*; and Vv, *Vitis vinifera*. Origin: AU, Austria; FR, France; GEO, Georgia; GER, Germany; IT, Italy; LE, Lebanon; MK, Makedonia; SE, Serbia; and SL, Slovenia.

both the virulence of the pathogen and the susceptibility level of the plant host (Kison and Seemüller 2001; Seemüller and Schneider 2007; Seemüller et al. 2011). In the case of phytoplasma diseases of grapevine, several studies have investigated the susceptibility of *V. vinifera* cultivars with different approaches (Margaria et al. 2014; Roggia et al. 2014) but genetic diversity and virulence of phytoplasma strains were not considered accurately. In fact, it is reasonable to hypothesize that genetically distinct BNp strains also could show a variable range of virulence. In Georgia, severe symptoms, typically associated with international cultivars (i.e., Chardonnay) in Europe, have been observed on the same cultivars and in the local variety Kisi. Interestingly, the majority of autochthonous Georgian grapevine cultivars were found to be mildly symptomatic, maintaining complete berry production (Table 1; Fig. 1). Intriguingly, the presence of BNp strains showing *Rsa*I-RFLP profiles V1, V14, and und2 of the gene *vmp1* (Table 1; Fig. 4) in grapevine cultivars exhibiting severe,

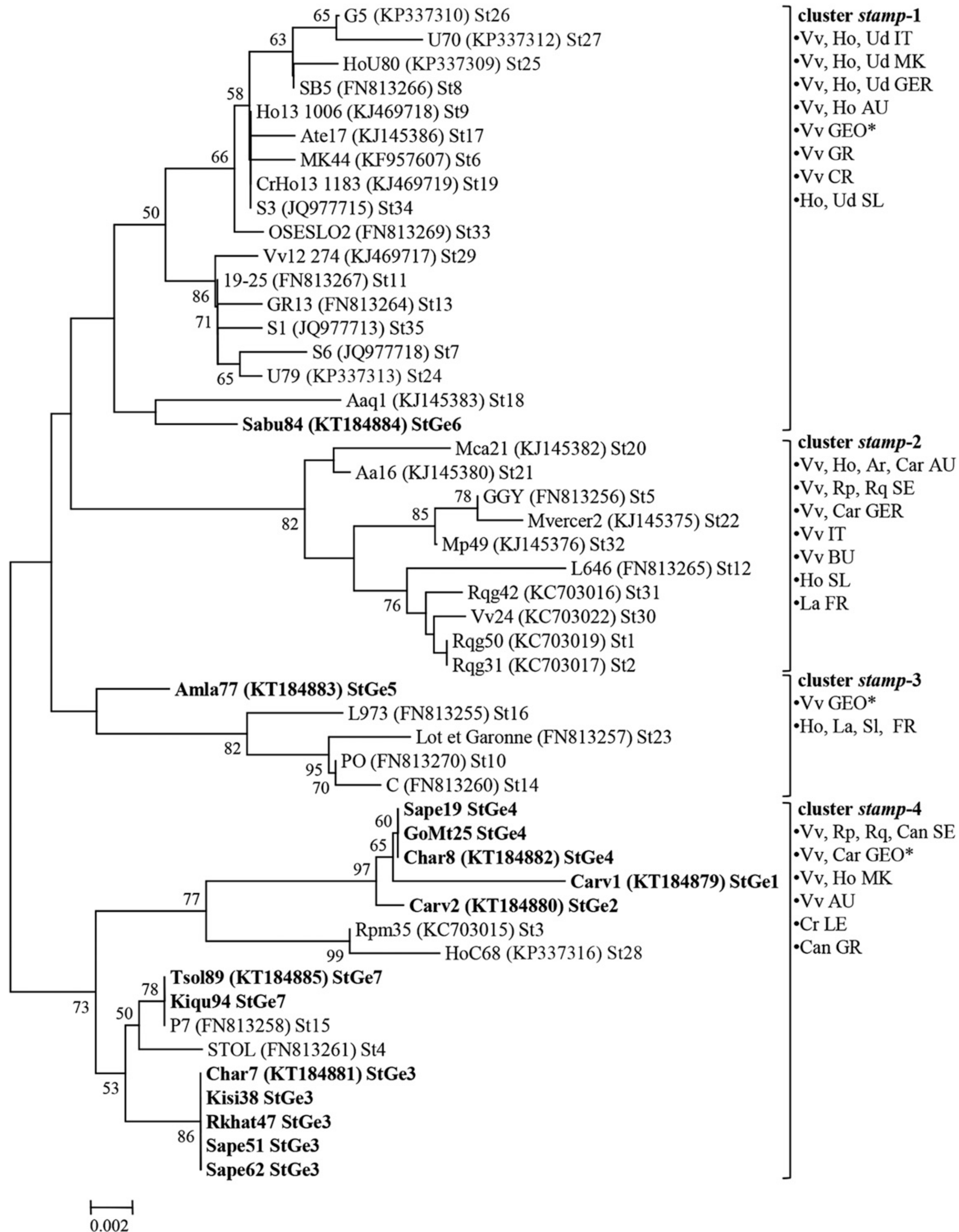

Fig. 6. Unrooted phylogenetic tree inferred from '*Candidatus* Phytoplasma solani' strain nucleotide sequences of gene *stamp*. Minimum evolution analysis was carried out using the neighbor-joining method and bootstrap replicated 1,000 times. Names of phytoplasma strains included in phylogenetic analysis 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. Clusters are shown as delimitated by parentheses. Acronyms within clusters indicated phytoplasma hosts and origin. Hosts: Ar, *Anaceratagallia ribauti*; Can, *Capsicum annuum*; Car, *Convolvulus arvensis*; Cr, *Catharanthus roseus*; Ho, *Hyalesthes obsoletus*; La, *Lavandula angustifolia*; Lv, *Linaria vulgaris*; Pa, *Prunus avium*; Rp, *Reptalus panzeri*; Rq, *R. quinquecostatus*; Sl, *Solanum lycopersicum*; Ud, *Urtica dioica*; Vv, and *Vitis vinifera*. Origin: AU, Austria; BU, Bulgaria; CR, Croatia; FR, France; GEO, Georgia; GER, Germany; GR, Greece; IT, Italy; LE, Lebanon; MK, Makedonia; SE, Serbia; and SL, Slovenia.

moderate, and mild symptoms suggested a different susceptibility of the cultivars to these BNp strains. On the other hand, the mutually exclusive presence of BNp strains showing *Rsa*I-RFLP profiles V15 and und3 in cultivars showing mild symptoms and *Rsa*I-RFLP profile und1 in cultivars showing severe symptoms suggested the hypo- and hypervirulence, respectively, of such BNp strains. The diverse susceptibility of Georgian grapevine cultivars to BNp strains highlighted in the present work needs to be confirmed in further research.

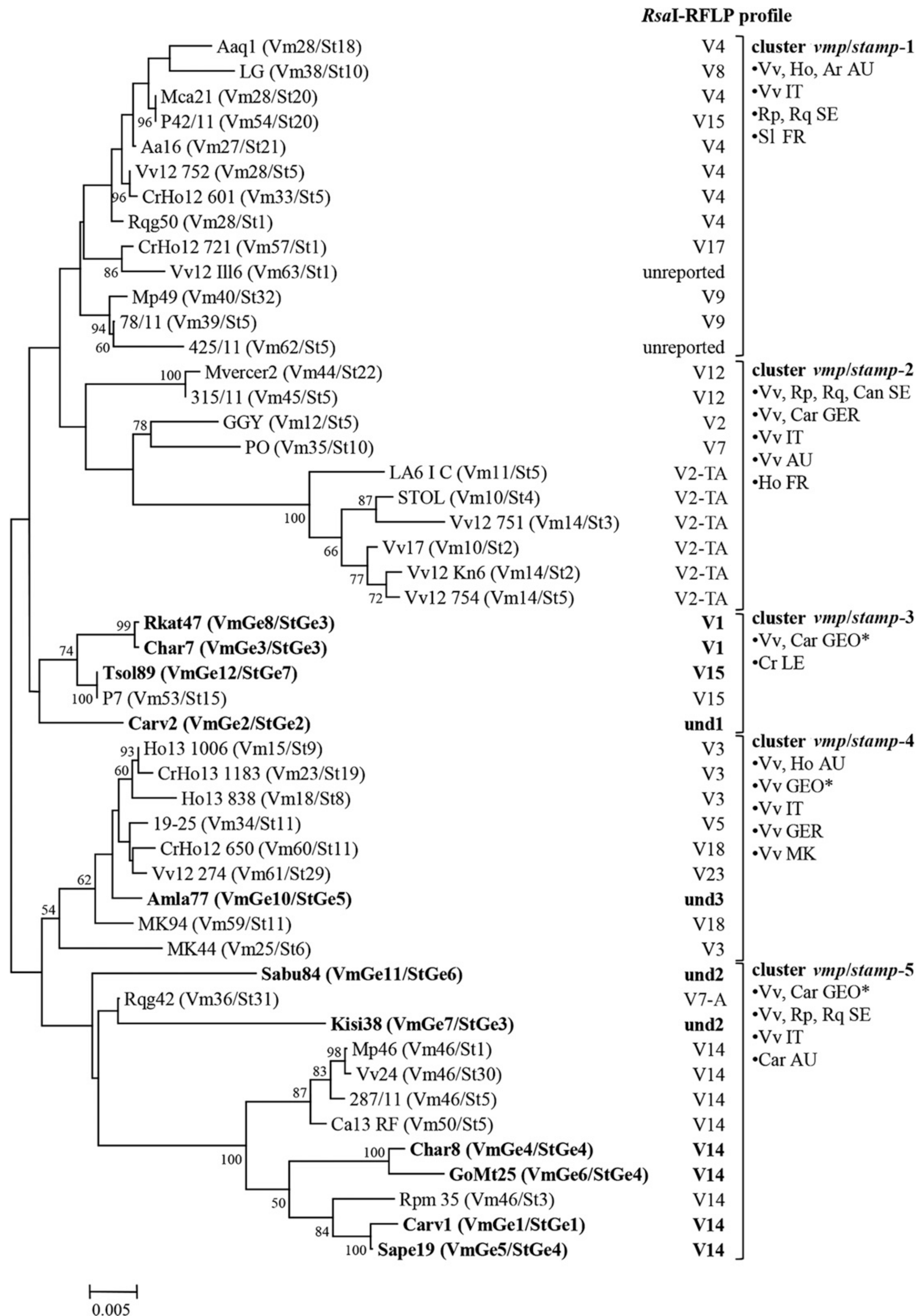

Fig. 7. Unrooted phylogenetic tree inferred from '*Candidatus* Phytoplasma solani' strains based on concatenated nucleotide sequences of the genes *vmp1* and *stamp*. Minimum evolution analysis was carried out using the neighbor-joining method and bootstrap replicated 1,000 times. Names of phytoplasma strains, included in phylogenetic analysis, and their *vmp*/*stamp* types are written on the tree image. gene sequences obtained in the present study are indicated in bold characters. Clusters are shown as delimitated by parentheses. Acronyms within clusters indicated phytoplasma hosts and origin. Hosts: Ar, *Anaceratagallia ribauti*; Can, *Capsicum annuum*; Car, *Convolvulus arvensis*; Cr, *Catharanthus roseus*; Ho, *Hyalesthes obsoletus*; La, *Lavandula angustifolia*; Lv, *Linaria vulgaris*; Pa, *Prunus avium*; Rp, *Reptalus panzeri*; Rq, *R. quinquecostatus*; Sl, *Solanum lycopersicum*; Ud, *Urtica dioica*; Vv, and *Vitis vinifera*. Origin: AU, Austria; BU, Bulgaria; CR, Croatia; FR, France; GEO, Georgia; GER, Germany; GR, Greece; IT, Italy; LE, Lebanon; MK, Makedonia; SE, Serbia; and SL, Slovenia.

Molecular characterization carried out by nucleotide sequence analysis of *vmp1* and *stamp* genes showed that BNp populations in Georgia consist mainly of previously unreported strains. In fact, only Georgian BNp strains Tsol89 and Kiqu94 shared 100% sequence identity with the sequences of the '*Ca*. P. solani' strain P7 (*vmp*/*stamp* type Vm53/St15), identified in naturally infected periwinkle in Lebanon in 2001 (Cimerman et al. 2009).

Phylogenetic analysis showed that *vmp1* genetic variants identified in this and previous studies within the same *Rsa*I-RFLP-based types (V1) can be grouped in separate clusters distinctly associated with nettle- and bindweed-related BN host systems (Fig. 5), confirming that *vmp1* genetic variants of the same *Rsa*I-RFLP-based type can be associated with different BN host systems (Murolo and Romanazzi 2015; Kosovac et al. in press; Kostadinovska et al. 2014). Moreover, clustering of several '*Ca*. P. solani' strains was different in phylogenetic trees built on the basis of *vmp1* and *stamp* genes, decreasing the chance to obtain a comprehensive data interpretation. To improve the significance of sequence analyses, we performed phylogenetic analysis of the concatenated nucleotide sequences of *vmp1* and *stamp* genes, as reported in other studies focused on phytoplasmas (Durante et al. 2012).

Phylogenetic analysis of *vmp1* and *stamp* gene concatenated sequences revealed that the majority of BNp Georgian strains (7 of 14) identified in grapevine cultivars and bindweed grouped along with the Lebanese strain P7 within the cluster *vmp*/*stamp*-3 (Fig. 7). Interestingly, this cluster is clearly distinct from other *vmp*/*stamp* clusters, including bindweed- and nettle-related BN strains previously identified in central and southern Europe (Aryan et al. 2014; Cvrković et al. 2014; Kosovac et al. in press; Murolo and Romanazzi 2015). Other Georgian BNp strains from grapevine and bindweed were associated with the cluster *vmp*/*stamp*-5, including bindweed-related BNp strains previously identified in grapevine in Serbia and Italy (Cvrković et al. 2014; Murolo and Romanazzi 2015). Only one BNp strain (Amla77) from Georgia grouped with nettle-related cluster *vmp*/*stamp*-4. Such evidence suggested the coexistence of the BN host systems related to nettle and bindweed in Georgia, with a high prevalence of bindweed as the main host plant of '*Ca*. P. solani' strains associated with BN.

As shown by Murolo and Romanazzi (2015), the overall ratio between the nonsynonymous to synonymous mutations showed that *vmp1* and *stamp* genes in '*Ca*. P. solani' strains in Georgia are under a positive selection process but the values of dN/dS ratio indicated a more intensive selection for the gene *vmp1* (3.62) compared with *stamp* (2.95). It should be interesting to investigate more accurately this aspect by comparing the dN/dS ratio values among BNp strain populations identified in different geographic regions and in diverse hosts (grapevine, insect vectors, and weeds) in Georgia.

In conclusion, results from the present study showed that BNp strain populations in Georgia consist mainly of new, unreported '*Ca*. P. solani' strains associated with both nettle- and bindweed-related BN host systems. Moreover, the distribution of BNp strains among grapevine cultivars showing a variable range of symptom intensity suggests a different susceptibility of such local cultivars to BN disease. Further studies are in progress to evaluate this important topic from the perspective of improving breeding programs for the production of novel grapevine cultivars tolerant of or resistant to phytoplasma diseases.

Table 4. Codon-based test of positive selection for the analysis of nucleotide sequences of the genes *vmp1* and *stamp* within '*Candidatus* Phytoplasma solani' strain populations

Gene	Source	Prob[a]	Stat[b]
vmp1	Overall	0.000	4.567
	Euro-Mediterranean area	0.000	4.637
	Georgia	0.000	3.618
stamp	Overall	0.008	2.436
	Euro-Mediterranean area	0.004	2.711
	Georgia	0.002	2.953

[a] Probability of rejecting the null hypothesis of strict-neutrality ($dN = dS$) in favor of the alternative hypothesis ($dN > dS$) (in the Prob column) is shown. Values of $P < 0.05$ are considered significant at the 5% level.

[b] Test statistic ($dN - dS$) is shown in the Stat column. dS and dN are the numbers of synonymous and nonsynonymous substitutions per site, respectively. The variance of the difference was computed using the bootstrap method (1,000 replicates). Analyses were conducted using the Nei-Gojobori method (Nei and Gojobori, 1986). The analysis involved 120 and 103 nucleotide sequences of the genes *vmp1* and *stamp*, respectively. All ambiguous positions were removed for each sequence pair. There were 520 and 164 positions in the final dataset for the genes *vmp1* and *stamp*, respectively. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

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