Molecular Typing of ‘*Candidatus Phytoplasma solani*’ in Iranian Vineyards

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Abstract

Grapevine (*Vitis vinifera* L.) is one of the most important horticultural crops in Iran, with >200,000 ha of cultivated area. Recently, outbreaks of the grapevine yellows Bois noir that is associated with phytoplasma strains related to ‘*Candidatus Phytoplasma solani*’ were recorded in several Iranian regions. This has resulted in severe economic losses. We carried out a survey in 2015, followed by collection of leaf samples from symptomatic grapevines and weeds. Because no information is available on the molecular epidemiology of ‘*Ca. P. solani*’ in Iran, multiple gene analyses were carried out here according to molecular characterization of the *tuf* and *vmp1* genes. From the molecular characterization, all of the samples (i.e., grapevines, weeds) were infected

with *tuf* b type. Detailed molecular characterization of the *vmp1* gene (i.e., PCR–restriction fragment length polymorphism, sequence analysis) defined five molecular types: V1, V4, V10, V15, and V20. The abundance of *Convolvulus arvensis* in vineyards and detection of the same ‘*Ca. P. solani*’ molecular types in grapevines and weeds suggest that *C. arvensis* has a major role in Bois noir epidemiology of Iranian vineyards. Therefore, control strategies should be developed to manage these host plants to reduce inoculum sources of the phytoplasma in vineyards.

Keywords: grapevine, phytoplasma, stolbur, *tuf*, *Vitis vinifera*, *vmp1*

As one of the most important horticultural crops in Iran, grapevine (*Vitis vinifera* L.) cultivation covers >200,000 ha (FAO 2016). Bois noir (BN) is a grapevine yellows disease that is widespread throughout Europe, particularly in the Mediterranean region, as well as in the Middle East (Aryan et al. 2014; Cvrković et al. 2014; Johannesen et al. 2012). As such, BN is known to cause serious economic losses in grapevine production (Ember et al. 2018; Endeshaw et al. 2012).

The causal agent of BN is a phytoplasma that belongs to the 16SrXII-A subgroup: ‘*Candidatus Phytoplasma solani*’ (Quaglino et al. 2013). The epidemiology of phytoplasma-induced diseases is determined by the interactions between the host plant, the pathogen, and environmental conditions (Rotter et al. 2018). For BN, grapevine is an erroneous feeding substrate for *Hyalesthes obsoletus* and *Reptalus panzeri* planthoppers, and consequently grapevine represents a dead-end host for ‘*Ca. P. solani*’ (Cvrković et al. 2014; Johannesen et al. 2012). However, the recent finding of ‘*Ca. P. solani*’ in roots of symptomatic and recovered plants has opened new scenarios for the study of the epidemiology of this phytoplasma (Landi et al. 2019).

According to the characterization of the elongation factor Tu (*tuf*) gene, ‘*Ca. P. solani*’ consists of two main genetically divergent strains of *tuf* a and *tuf* b, which are involved in two separate epidemiological cycles of BN (Langer and Maixner 2004). The *tuf* b type is primarily associated with field bindweed (*Convolvulus arvensis*). This is generally seen to be the major host plant of *H. obsoletus* (Johannesen et al. 2012; Langer and Maixner 2004), which is polyphagous and can also infect a number of other weeds (Cvrković et al. 2014; Johannesen et al. 2012). The *tuf* a strain of ‘*Ca. P. solani*’ is spread via an epidemic cycle that involves the stinging nettle (*Urtica dioica*), which is the only documented herbaceous reservoir of ‘*Ca. P. solani*’ molecular types (Johannesen et al. 2012; Langer and Maixner 2004).

In addition to the epidemiological significance of the *tuf* gene, a further gene encoding variable membrane protein 1 (VMP1) has been proposed for the molecular characterization of ‘*Ca. P. solani*’ (Cimerman et al. 2009). Because *vmp1* has higher sequence variability than the *tuf* genes, it is generally more informative and is thus more useful for epidemiological studies of BN phytoplasma (Aryan et al. 2014; Cvrković et al. 2014; Johannesen et al. 2012; Kostadinovska et al. 2014; Murolo and Romanazzi 2015).

Bois noir has been reported for different viticultural regions of Iran (Ghayeb Zamharir et al. 2017; Karimi et al. 2009; Mirchenari et al. 2015; Salehi et al. 2014). The association of ‘*Ca. P. solani*’ with *C. arvensis* has recently been reported in Iran (Salehi et al. 2019). However, there is a lack of information for Iranian vineyards on ‘*Ca. P. solani*’ molecular typing and the reservoir plants that might be involved in the epidemiological cycle(s) of BN. Therefore, the aim of this study was to examine the *tuf* and *vmp1* genes of the ‘*Ca. P. solani*’ strains in naturally infected grapevines and potential reservoir plants in Iran, to define these epidemiological cycle(s) of BN.

Materials and Methods

Surveys and sample collection. The surveys were carried out from mid-September to the beginning of October 2015 in the provinces of Azarbaijan Gharbi, Azarbaijan Sharghi, Zanjan, Qazvin, Fars, Lorestan, and Khorasan Razavi in Iran (Fig. 1). Symptomatic grapevines were recorded on a two-dimensional map, and 100 leaf samples were collected. Near symptomatic grapevines, 50 *C. arvensis* samples were also collected from plants that showed symptoms related to phytoplasma infection.

DNA extraction. Total nucleic acids were extracted from 0.5 g of leaf petioles from both the grapevine and weed samples. The cetyltrimethylammonium bromide extraction procedure was carried out as described by Angelini et al. (2001). DNA was quantified using a Bio-photometer Plus (Eppendorf, Hamburg, Germany), and samples with absorbency ratios of $A_{260}/A_{280} > 1.8$ and $A_{260}/A_{230} > 1.7$ were considered for further molecular analysis. The concentration of DNA in the samples was standardized to 20 ng/μl.

Molecular identification with universal primers. Amplifications were performed in a final reaction volume of 25 μl, which contained 40 ng of DNA template, Master Mix 2× (Applied Biological

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Materials, Richmond, BC, Canada), and 0.5 μ M of each primer (Sigma-Aldrich, Milan, Italy). The polymerase chain reactions (PCRs) were performed in a programmable cycler (Bio-Rad, Hercules, CA) using the universal P1/P7 phytoplasma primer pair. This was followed by nested PCR with the fStol/rStol specific primer pair for stolbur phytoplasma (Maixner et al. 1995). Five microliters of each PCR product was analyzed by gel electrophoresis with 1.5% agarose (Sigma-Aldrich) gels. The gels were stained with GelRed (Biotium, Fremont, CA), visualized under UV light at 312 nm using a transilluminator, and imaged using an imaging system (Gel Doc XR, Bio-Rad). The expected lengths of the amplified DNA fragments were estimated by comparisons with a 100-bp DNA Opti-Marker (Applied Biological Materials).

Molecular typing of the *tuf* and *vmp1* nonribosomal genes. The samples that were positively amplified with the fStol/rStol primer pair were selected for further molecular analysis using a multilocus sequence analysis approach. This considered the two different nonribosomal genes *tuf* and *vmp1*.

The *tuf* gene was amplified using the Tuf1f/r primer pair and then in the nested PCR using the TufAyf/r primer pair (Schneider et al. 1997). The genetic variability was determined by cleaved amplified polymorphism analysis (Langer and Maixner 2004). The amplicons were digested with 2.5 U of the *Hpa*II endonuclease (ThermoScientific, Waltham, MA) for 4 h at 37°C. The products in the 2% agarose gels were resolved and recorded as described above.

The *vmp1* gene was amplified with the StolH10F1/R1 primer pair (Cimerman et al. 2009), followed by a second round of PCR with the TYPH10F/R primer pair (Fialová et al. 2009). The products of the

nested PCRs were defined by electrophoresis through 1% agarose gels. An aliquot was then digested with 2.5 U of the *Rsa*I restriction enzyme (ThermoScientific) at 37°C, according to the manufacturer's instructions. The digested products were analyzed by electrophoresis on 2.5% agarose gels, which were resolved and recorded as described above. The 100-bp Opti DNA marker (Applied Biological Materials) was used for the reference lengths of the fragments produced. Finally, the pictures captured by the imaging system (Gel Doc XR, Bio-Rad) were analyzed using Quantity One software (Bio-Rad).

Table 1. '*Candidatus* Phytoplasma solani' nucleotide sequences used in the phylogenetic analysis for the *vmp1* genes from the study vineyard

Isolate	Host	Cultivar	Origin	Accession
C4	<i>Vitis vinifera</i>	Qermez Keshmeshi	Zanjan	MK547294
DG12	<i>Vitis vinifera</i>	Qermez Keshmeshi	Qazvin	MK547295
FZ10	<i>Vitis vinifera</i>	Askari	Fars	MK547297
FZ11	<i>Vitis vinifera</i>	Rish-Baba	Fars	MK547298
KG11	<i>Vitis vinifera</i>	Peykani	Khorasan Razavi	MK547299
L3	<i>Vitis vinifera</i>	Sefid keshmeshi	Lorestan	MK547300
DW3	<i>Convolvulus arvensis</i>		Qazvin	MK547296
LW19	<i>Convolvulus arvensis</i>		Lorestan	MK547301

Fig. 1. Location for the sampling of grapevines and weeds in Iran.

Sequence analysis and phylogenetic relationships. According to the molecular characterization carried out on the *tuf* and *vmp1* genes by restriction fragment length polymorphism (RFLP), eight representative samples (grapevines, six; weeds, two) were selected for sequence analysis of the amplicons generated by the *tufAYf/r* and *TYPH10F/TYPH10R* primer pairs at Genewiz Genomics sequencing service (Genewiz UK, Takeley, U.K.; <https://www.genewiz.com/>). To obtain a consensus sequence, the forward and reverse nucleotide sequences were read and edited using Chromas version 2.33 software and assembled using the GAP4 Staden Package (<http://staden.sourceforge.net/>). Bioedit software version 7.0.0 (<http://www.mbio.ncsu.edu/Bioedit/bioedit.html>) was used to cut off 20 to 30 bp of the terminal end sequence. All of the consensus sequences for each gene were used as query sequences in BLAST searches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the nucleotide identities for the ‘*Ca. P. solani*’ strains available in

GenBank (<https://www.ncbi.nlm.nih.gov/>). To verify the accuracy in the determination and recognition of the different RFLP pattern types of *vmp1* obtained in the PCR-RFLP analysis, pDRAW32 software version 1.1.101 (AcaClone software, <http://www.acaclone.com>) was used, with virtual digestion of the nucleotide consensus sequences with *RsaI* endonuclease.

The nucleotide sequences have been submitted to GenBank under the accession numbers given in Table 1. From the NCBI database, 11 nucleotide sequences were downloaded (Table 2), which were used together with the nucleotide sequences generated in this study to reconstruct the phylogenetic relationships according to *tuf* and *vmp1*. The nucleotide sequences were aligned using Clustal X (version 1.83). Mega 6 (<http://www.megasoftware.net/index.html>) was used to calculate the phylogenetic relationships according to the neighbor-joining method with 1,000 bootstrap replicates.

Results

BN symptoms on the grapevine leaves started to appear from the end of July and became more evident as the vegetative season advanced. The plants showed crisp, brittle, downward-rolling leaves, which showed reddening (orange to purple) with the red varieties (Fig. 2A) and yellowing (golden to chlorotic) with the white varieties. The discoloration always also involved the main leaf veins. Sectorial discoloration of the leaf blades occurred in some of the varieties, and flower abortion, berry withering, and shriveling of bunches were seen (Fig. 2B). Moreover, when the symptomatic leaves fell off, the petioles remained on the canes longer. Partial or total lack of lignification was recorded for the canes of the infected grapevines in autumn. In the survey vineyards, there was an abundant presence of *C. arvensis*, which often showed some leaf symptoms and yellowing, which were related to phytoplasma infections (Fig. 2C).

Molecular detection of the phytoplasma. Both positive and negative grapevine and weed samples from the first PCRs were analyzed in nested PCRs with the *fStol/rStol* primer pair. For the grapevine samples, a specific band of ~500 bp was obtained in 45% of the samples analyzed, whereas for the weed samples, 50% were positively amplified. The positive samples were considered in the further analysis for the molecular characterization.

Molecular characterization of the *tuf* gene in the stolbur isolates. An amplicon of ~940 bp was obtained in the nested PCR with the *tufAYf/r* primer pair from 30 infected grapevine samples, as well as

Table 2. ‘*Candidatus* Phytoplasma solani’ nucleotide sequences used in the phylogenetic analysis for the *vmp1* gene available in the NCBI database

Isolate	Host	Origin	Vmp1 type	Accession
Rkat47	<i>Vitis vinifera</i>	Georgia	V1	KT184874
Char7	<i>Vitis vinifera</i>	Georgia	V1	KT184869
N9	<i>Urtica dioica</i>	France	V1	JQ977729
GGY	<i>Vitis vinifera</i>	Germany	V2	AM992102
Aa25	<i>Vitis vinifera</i>	Italy	V3	HM008614
N3	<i>Urtica dioica</i>	Slovenia	V3	JQ977723
Moliere	<i>Prunus mahaleb</i>	France	V4	AM990984
19-25	<i>Vitis vinifera</i>	Germany	V5	AM990980
Mp49	<i>Vitis vinifera</i>	Italy	V9	HM008607
B7	<i>Vitis vinifera</i>	Italy	V9	HM008608
Charente2	<i>Hyalesthes obsoletus</i>	France	V10exV15	AM990977
Mvercer2	<i>Vitis vinifera</i>	Italy	V12	HM008612
Char8	<i>Vitis vinifera</i>	Georgia	V14	KT184870
C3	<i>Vitis vinifera</i>	Italy	V14	HM008603
P7	<i>Catharanthus roseus</i>	Lebanon	V15	AM990978
EGY POT4	<i>Solanum tuberosum</i>	Egypt	V20	Foissac (Personal communication)

Fig. 2. Symptomatic plants. **A**, Crisp, brittle, downward-rolling leaves, with reddening (orange to purple), in the red grapevine varieties. **B**, Sectorial discoloration of the blades in some varieties, with flower abortion, berry withering, and shriveling of grape bunches. **C**, Small and yellowing leaves in infected bindweed.

from eight of the weed samples analyzed. No PCR products were obtained from the healthy control plants. PCR-RFLP analysis revealed the *tufb* type in grapevine samples. The *C. arvensis* plants collected in the vineyards were also infected by the *tufb* type.

Molecular characterization of the stolbur *vmp1* genotypes and phylogenetic analysis. The *vmp1* gene fragment was amplified with the TYPH10F/R primer pair in 30 grapevine samples and in eight of the weed samples. The specific fragments obtained showed a size of ~1,450 bp. For these grapevine samples infected by ‘*Ca. P. solani*’, enzymatic digestions of the *vmp1* amplicons showed four *RsaI* profiles: V1, V4, V10, and V20. For the weed samples, three *vmp1* types were seen: V1, V10, and V15. Based on the RFLP analysis, eight representative isolates were sequenced and the profiles were confirmed by pDRAW32 comparing restriction site analysis with reference strains from previous studies. In particular, the V1 *RsaI* profile was seen for nucleotide positions 118, 547, and 1,051; the V4 for positions 118, 371, 547, 1,051, and 1,125; the V10 and V15 for positions 118, 371, 547, and 1,051; and the V20 for positions 118, 547, 1,051, and 1,125. Based on BLAST analysis, the nucleotide sequences showed high identities for *vmp1* (98 to 100%). The phylogenetic tree was constructed from the analysis of the nucleotide sequences from the present study with the 16 available in GenBank that were representative of *vmp1* reference types. In the resulting dendrogram, the nucleotide sequences generally clustered according to the typology of the RFLP

patterns (Fig. 3): strains with the same RFLP pattern showed high nucleotide similarities for their sequences (>99%).

Discussion

BN is one of the most widespread diseases of grapevines in the Euro-Mediterranean area. The presence of stolbur phytoplasma in Iran was first reported in 2009 (Karimi et al. 2009). In this study, we carried out molecular characterization of ‘*Ca. P. solani*’ in grapevines and weeds, according to the *tuf* and *vmp1* genes. This study thus provides new epidemiological information for BN in Iran.

For the *tuf* characterization, all of the samples putatively infected with ‘*Ca. P. solani*’ showed a restriction pattern identical to the *tufb* references from Lebanon (P7; *Catharanthus roseus*). There was also *tufb* type in the *C. arvensis* samples collected from the vineyards that showed symptoms ascribable to phytoplasma infection. Similar symptoms were recorded in BN-infected bindweed in Georgian vineyards (Quaglino et al. 2016) and sporadically in European countries, where most of the weeds that have been collected showed mild symptoms (Kosovac et al. 2016; Landi et al. 2015). The abundance and infection of *C. arvensis* in Iranian vineyards supports the hypothesis that ‘*Ca. P. solani*’ *tufb* type predominantly spreads via a cycle that involves bindweed (Johannesen et al. 2012; Langer and Maixner 2004). However, in Euro-Mediterranean countries, ‘*Ca. P. solani*’ *tufb* type has also been

Fig. 3. Unrooted phylogenetic tree inferred from the ‘*Candidatus Phytoplasma solani*’ strain nucleotide sequences of the *vmp1* gene. Minimum evolution analysis was carried out using the neighbor-joining method and bootstrap replicated 1,000 times. The names of the phytoplasma strains included in the phylogenetic analysis are given in the tree image. The GenBank accession number of each sequence is given in parenthesis; gene sequences obtained in the present study are indicated with filled circles.

reported for a number of other weedy plants (Cvrković et al. 2014; Johannesen et al. 2012; Marchi et al. 2015; Riedle-Bauer et al. 2006).

To gain more insight into the genetic diversity among the BN strains in Iran, finer characterization was carried out on the *vmp1* gene. We recorded five *vmp1* types in the samples analyzed here: V1, V4, V10, V15, and V20. V1 and V10 were detected in both the grapevines and the weeds, whereas V4 and V20 were detected only in the grapevines and V15 only in *C. arvensis*. The profiles for V1 and V10 were the most prevalent strains recorded among these 'Ca. P. solani'. V1 has been reported for Italy, France, Germany, Georgia, and the Caucasian regions (Foissac et al. 2013; Murolo et al. 2010, 2014; Pacifico et al. 2009; Quaglino et al. 2016). V4 has also been reported for Italy and for Croatia, Serbia, and Bosnia and Herzegovina (Cvrković et al. 2014; Delić et al. 2016; Kostadinovska et al. 2014; Oliveri et al. 2015; Pacifico et al. 2009; Seruga-Music et al. 2011). The V10 and V15 profiles were previously described for Italy, Germany, France, Georgia, and Lebanon (*C. roseus*) (Cimerman et al. 2009; Johannesen et al. 2012; Murolo et al. 2010, 2014; Oliveri et al. 2015; Pacifico et al. 2009; Quaglino et al. 2016). The high variability of the *vmp1* gene has proven to be useful for discriminating 'Ca. P. solani' genotypes.

The close relationships between the 'Ca. P. solani' molecular types recorded in the Iranian vineyards are fundamental to apply future appropriate management strategies based on the control of host plants and the monitoring and control of the vectors.

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