'Bois noir': new phytoplasma disease of grapevine in Iran

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Abstract: Recently, grapevines showing symptoms suggesting the 'bois noir' phytoplasma disease were observed in vineyards located in several central provinces of Iran. Polymerase chain reaction assays using phytoplasma universal primer pair P1A/P7A followed by primer pair R16F2n/R16R2 in nested PCR, confirmed the association of phytoplasmas with symptomatic grapevines. The results of RFLP analyses using *Hpa*II, *Hinf*I, *Mse*I, *Rsa*I, and *Taq*I restriction enzymes, indicated that grapevine phytoplasma isolates in these regions could be related to the 16SrXII group. Sequence analyses of the partial 16S rRNA gene confirmed that Iranian grapevine phytoplasmas are associated with '*Candidatus* Phytoplasma solani'. This is the first report of the 'bois noir' disease outbreak in Iran.

Key words: 'bois noir', '*Ca.* Phytoplasma solani', grapevine, Iran, leaf reddening

Introduction

'Bois noir' is the most important phytoplasma disease of grapevine (*Vitis vinifera* L.). This disease is caused by '*Candidatus* Phytoplasma solani' and occurs in several regions worldwide (Quaglino *et al.* 2013). The 'flavescence dorée' disease which is associated with '*Ca.* Phytoplasma vitis' (suggested name) in Europe (Schvester *et al.* 1961; Marzorati *et al*. 2006), and Australian grapevine yellows disease which is caused by '*Ca.* Phytoplasma australiense' in Australia (Davis *et al.* 1997) are other phytoplasma diseases of grapevine. The 'bois noir', 'flavescence dorée', and Australian grapevine yellows diseases are defined as common names for grapevine yellows diseases (Weintraub and Jones 2010). Still other phytoplasmas which cause diseases on grapevine around the world are: '*Ca.* Phytoplasma asteris', '*Ca.* Phytoplasma pruni' (Prince *et al.* 1993), and '*Ca.* Phytoplasma fraxini' (Gajardo *et al.* 2009).

Iran is one of the main origins of *V. vinifera* cultivars. There have not been any reports about grapevine phytoplasma diseases in Iran, up to present time. On the other hand, for a long time in several grape-growing areas in Iran there have been observations of grapevines showing symptoms suggesting phytoplasma diseases. Therefore, the aims of this study were to detect and characterise phytoplasmas associated with grapevine, in extensive areas in central Iran.

Materials and Methods

Sampling and DNA extraction

Fifty vineyards in five provinces located in the centre of Iran (Chaharmahal-O-Bakhtiari, Hamadan, Isfahan, Markazi, and Yazd) were investigated from September to November of 2012 and 2013. Grapevine samples showing phytoplasma disease symptoms were collected at this time. The midrib tissues of 73 sampled symptomatic and some asymptomatic grapevines were used for DNA extraction using the Murray and Thompson method (1980). The extracted DNA was stored at –20°C until used.

Polymerase chain reaction (PCR) assays

For phytoplasmas detection, phytoplasma universal primer pair P1A/P7A (Lee *et al.* 2004) was used in first PCR round. Then, 30-fold diluted PCR products were used for nested PCR assays using universal primer pair R16F2n/R16R2 (Lee *et al*. 1993).

The following were the PCR parameters for primer pair P1A/P7A: 2 min at 95°C for the first denaturation, 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 75 sec, and extension at 72°C for 90 sec, followed by 10 min at 72°C as the final step. For primer pair R16F2n/R16R2, a pre-denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 75 sec, extension at 72°C for 90 sec, and a final extension step at 72°C for 10 min were done. As negative controls in all the experiments, DNA of symptomless grapevines was used.

Direct and nested PCR reactions were performed with Techne-TC-512 thermocycler. In all PCRs total volume of 20 µl, the PCR mixtures contained about 20 ng DNA, 0.2 mM each of dNTPs (Cinnagen, Iran), 0.5 µl of each primer pair (20 pmol · μl^{-1}), 2 mM $MgCl_2$, 1.5 U of *Taq* DNA polymerase (Cinnagen), and 1X polymerase buffer. Finally, reaction mixtures were analysed by electrophoresis. To facilitate electrophoresis, 1.2% (w/v) agarose gel (stained

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with 5 μg · ml^{-1} ethidium bromide) in Tris-borate-EDTA (TBE) buffer was used. Visualisation of DNA bands were done using an ultra-violet (UV) transilluminator.

Restriction fragment length polymorphism (RFLP) analysis

Five restriction enzymes: *Hpa*II, *Hinf*I, *Mse*I, *Rsa*I, and *Taq*I (Fermentas, Vilnius, Lithuania) were selected for RFLP analysis. About 200 ng of 1,239 bp R16F2n/R16R2-amplified products were used in single digestion analysis for the initial differentiation among Iranian grapevine phytoplasma isolates. Plum phytoplasma isolate PJ15 (accession FJ409624) and sweet cherry phytoplasma isolate PCh8 (accession FJ204397), which were previously collected from the central regions of Iran, were used as reference strains of 16SrXII and 16SrI phytoplasma groups. Restriction profiles were separated with electrophoresis through 16% polyacrylamide gel and stained with silver nitrate.

Cloning and phylogenetic analyses

Nine representative 1,239 bp R16F2n/R16R2-amplified fragments were selected for cloning and sequencing. The fragments were ligated into pTZ57R/T vector using InsT/Aclone PCR product cloning Kit™ (Fermentas) and cloned in *Escherichia coli* MC1061 as competent cells. The recombinant cells containing inserted plasmids were selected with blue/white screening assays. Selected colonies were used for plasmid extraction. The sequences of partial 16S rRNA gene from Iranian grapevine phytoplasmas were deposited in GenBank under the accession numbers: KJ637201 (isolate Faro2), KJ637202 (isolate Gahrud), KJ637203 (isolate GH11), KJ637204 (isolate GH17), KJ637205 (isolate Malayer1), KJ637206 (isolate Malayer2), KJ637207 (isolate PHGH1), KJ637208 (isolate Shahreza), and KJ637209 (isolate Tiran2).

Phylogenetic analyses were also done using MEGA 5.05 software for 16S rRNA gene sequences. Gene fragments (about 1.2 kb 16S rRNA) of nine Iranian grapevine phytoplasmas and 34 phytoplasmas submitted in the GenBank were used to construct a phylogenetic tree. An *Acholeplasma multilocale* isolate was selected as outgroup to root the tree and bootstrap analyses were done in 1,000 replications.

Results

Symptoms of grapevine phytoplasma diseases, and detection of phytoplasmas

During the summer and autumn, grapevine phytoplasma disease symptoms in the investigated regions were almost the same. Symptoms were restricted to the three major types of symptoms: leaf reddening, leaf rolling, and yellowing. In the surveyed regions, the leaf rolling and yellowing symptoms appeared on the white varieties of grapevine, whereas leaf reddening was usually observed on the red varieties.

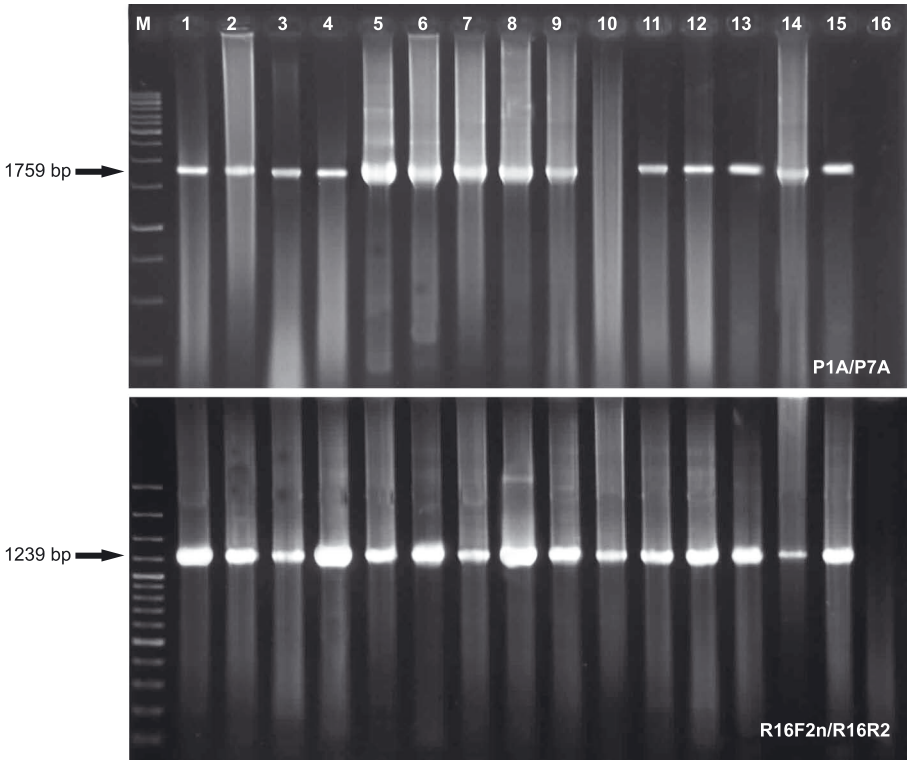

Fig. 1. 1,759 bp phytoplasma 16S rDNA fragments amplified in a PCR assay using primer pair P1A/P7A (top) and 1,239 bp fragments amplified in nested PCR using primer pair R16F2n/R16R2 (bottom) from Iranian grapevine 'bois noir' phytoplasma isolates. Electrophoresis was done through 1.2% agarose gel and stained in ethidium bromide. Lane M is marker III and lane 16 is healthy grapevine. Phytoplasma isolates include: (1) PJ15 (plum), (2) Pch8 (sweet cherry), (3) GH11, (4) GH17, (5) Malayer1, (6) Malayer2, (7) Shahreza, (8) PHGH1, (9) Gahrud, (10) Gahrud2, (11) Gahrud3, (12) Faro1, (13) Faro2, (14) Tiran1, (15) Tiran2. Lane 2 belongs to the 16SrI group and the others belong to the 16SrXII group

For detection of phytoplasmas, the universal primer pair P1A/P7A was used in the first PCR assays, but only six DNA samples amplified the expected 1,759 bp fragment. While using primer pairs R16F2n/R16R2 in nested PCR from the first PCR products, 30 DNA samples amplified 1,239 bp expected fragment (Fig. 1). All of those 30 positive DNA samples were extracted from the red varieties of grapevine.

RFLP analysis

For initial differentiation of grapevine phytoplasmas, the DNAs of all isolates were separately subjected to single digestion with restriction enzymes: *Hpa*II, *Hinf*I, *Mse*I, *Rsa*I, and *Taq*I. Restriction results indicated that when using *Hpa*II, *Hinf*I, and *Rsa*I enzymes, all examined isolates shared identical patterns. However, using the two restriction enzymes *Mse*I and *Taq*I, all grapevine phytoplasma isolates shared patterns similar to Iranian plum phytoplasma isolate PJ15 pattern, a phytoplasma related to '*Ca.* Phytoplasma solani' which was used as a reference strain of the stolbur group. Phytoplasma isolate PJ15 was previously detected on a Japanese plum tree in Isfahan province. The tree had few leaves and had yellowing symptoms (Zirak *et al.* 2009b). The Iranian sweet cherry phytoplasma isolate PCh8, collected from Isfahan, which was used as reference strain of the aster yellows group, shared patterns different from all the other isolates. The *Mse*I and *Taq*I RFLP results indicated that these restriction enzymes could differentiate phytoplasmas related to the 16SrXII group from taxonomically related phytoplasmas – including 16SrI group phytoplasmas (Fig. 2).

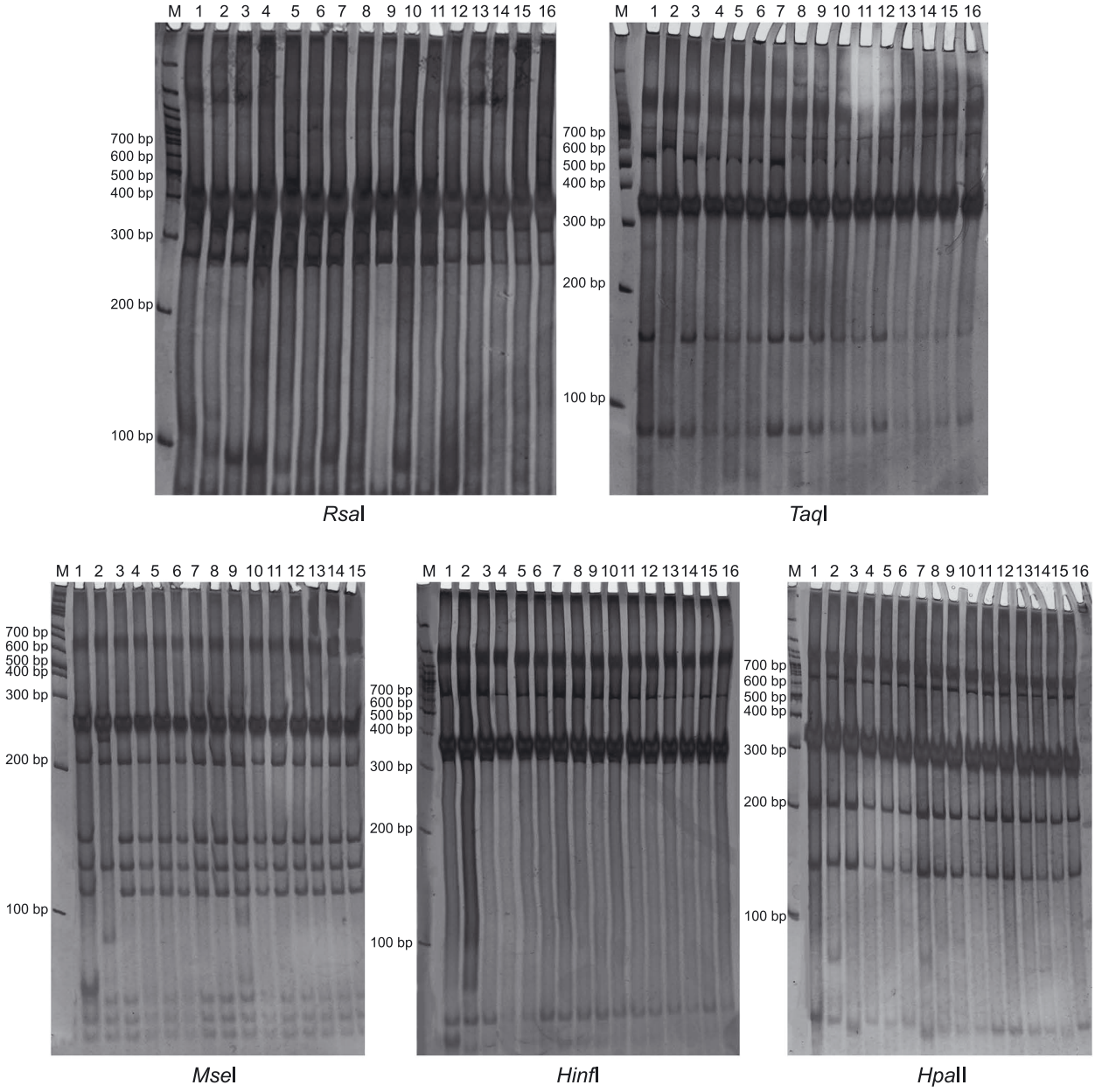

Fig. 2. RFLP analyses of 1,239 bp fragments using restriction enzymes *Hpa*II, *Hinf*I, *Mse*I, *Rsa*I, and *Taq*I from Iranian grapevine 'bois noir' phytoplasmas. Lane M is Ladder 100 plus. The phytoplasma isolates include: (1) PJ15 (plum), (2) Pch8 (sweet cherry), (3) GH11, (4) GH17, (5) Malayer1, (6) Malayer2, (7) Shahreza, (8) PHGH1, (9) Gahrud, (10) Gahrud2, (11) Gahrud3, (12) Faro1, (13) Faro2, (14) Tiran1, (15) Tiran2, (16) Tiran3. Lane 2 belongs to the 16SrI group and the others belong to the 16SrXII group

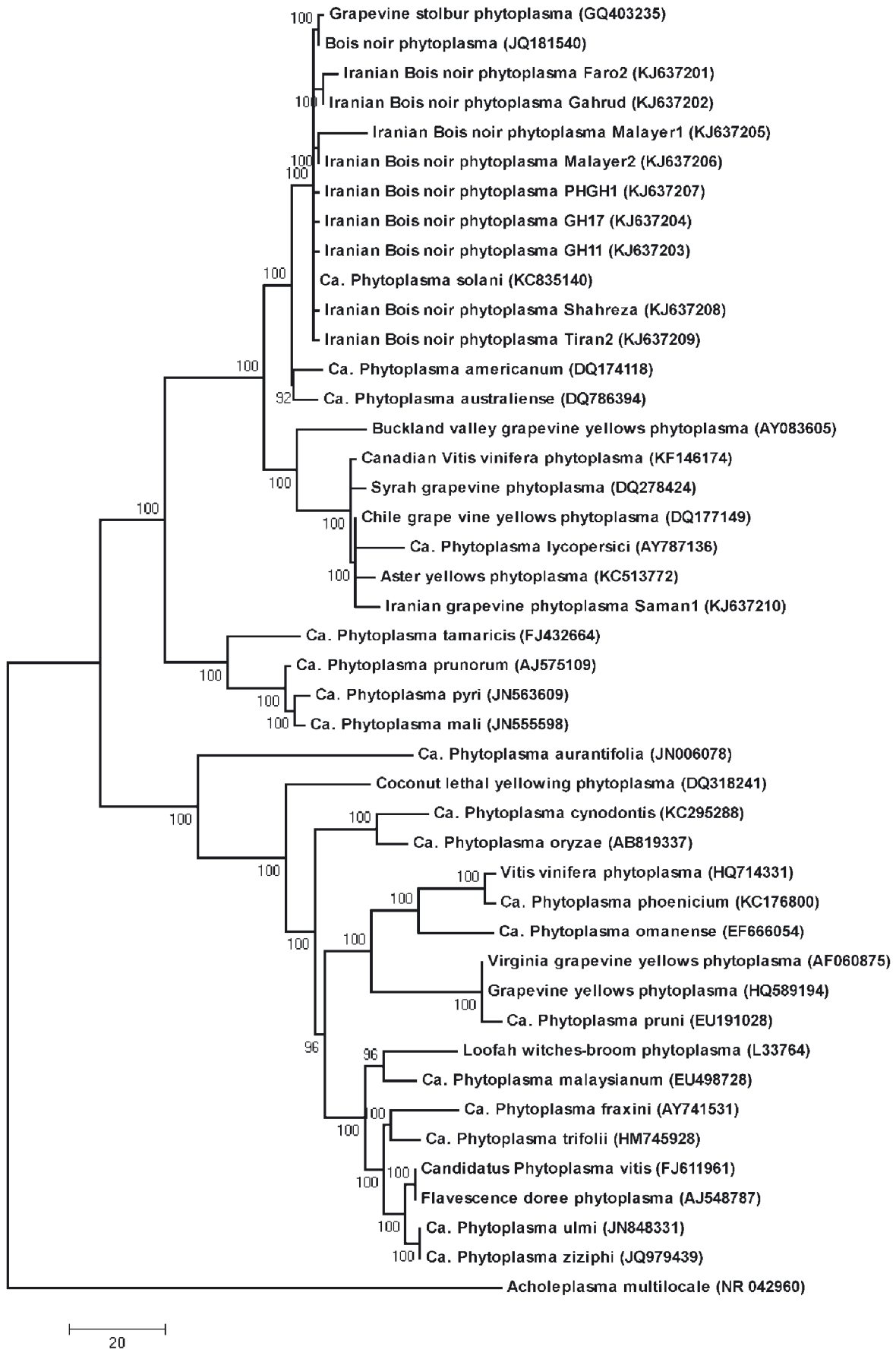

Fig. 3. The phylogenetic tree constructed by maximum parsimony analyses of 1.2 kb 16S rRNA gene fragments of nine Iranian grapevine 'bois noir' phytoplasmas and 34 phytoplasmas in GenBank using MEGA 5.05 software. An *Acholeplasma multilocale* isolate was selected as outgroup to root the tree and bootstrapping was done in 1,000 replications

Phylogenetic analysis

The blast searches of partial 16S rRNA gene sequences indicated that Iranian grapevine 'bois noir' phytoplasma isolates: Faro2, Gharud, GH11, GH17, Malayer1, Malayer2, PHGH1, Shahreza and Tiran2 shared 99% identity with *'Ca.* Phytoplasma solani' isolate 391_05 (EU010006), a 'bois noir' phytoplasma from Italy, and other isolates of *'Ca.* Phytoplasma solani'. The phylogenetic tree constructed using partial 16S rRNA gene sequences shows that all of the Iranian grapevine 'bois noir' phytoplasma isolates were clustered with phytoplasmas belonging to the stolbur group (Fig. 3).

Discussion

The 'bois noir' disease is the most important phytoplasma disease which affects vineyards in Europe and the Mediterranean basin (Boudon-Padieu 2003). The disease was first described as a form of grapevine 'flavescence dorée' disease (Caudwell 1961). Later, it was proved that the 'bois noir' phytoplasma is associated with the stolbur group subgroup A (16SrXII-A) phytoplasmas, which was later described as '*Ca*. Phytoplasma solani' and it could be transmitted by the planthopper *Hyalesthes obsoletus* (Weintraub and Jones 2010; Quaglino *et al.* 2013).

Up until we did our study, there had not been any significant reports about grapevine phytoplasma diseases in Iran. In our present study, during the surveys of vineyards done in central Iran, three major types of symptoms were observed, including leaf reddening, leaf rolling, and yellowing, which appear in grapevines affected with the 'bois noir' disease. Subsequent experiments proved that only red varieties of *V. vinifera* showing leaf reddening were infected with phytoplasmas. Then, '*Ca.* Phytoplasma solani' was recognised as the causal agent of grapevine diseases showing leaf reddening in the surveyed regions. The '*Ca.* Phytoplasma solani' was previously reported from such Middle Eastern countries as Lebanon (Choueiri *et al.* 2002), Turkey (Canik *et al.* 2011), and Jordan (Salem *et al.* 2013) as a causal agent of grapevine diseases. This research, though, is the first significant report on the appearance of grapevine 'bois noir' disease, and in fact, this research notes the first record of '*Ca.* Phytoplasma solani' associated with grapevine in Iran.

The 16SrXII group of phytoplasmas, specifically '*Ca.* Phytoplasma solani', are widespread phytoplasmas in Iran especially in the central provinces where the population of the pathogens and host ranges have been well-studied till now (Zirak *et al.* 2009a, 2009b, 2010; Hosseini *et al.* 2011; Vali Sichani *et al.* 2011). The wide host ranges of the diseases imply that these phytoplasmas are transmitted from alternative plant hosts to the main plants by active insect vectors. The polyphagous planthopper *H. obsoletus*, was reported as a natural vector of 'bois noir' phytoplasma. This planthopper feeds on several wild and cultivated herbaceous plants such as bindweed (*Convolvulus arvensis*) and stinging nettle (*Urtica dioica*) growing near the vineyards (Langer and Maixner 2004). These weeds, along with other infected host plants, could be the source of pathogens for vineyard infection. Infection of bindweed by '*Ca.* Phytoplasma solani' (Salehi *et al.* 2005), and activity of *H. obsoletus* in several orchards (Lashkari *et al.* 2009), were previously reported from Iran. Therefore, it could be true, that *H. obsoletus* transmits the '*Ca.* Phytoplasma solani' from infected weeds to vineyards in the investigated regions.

Finally, the results obtained in this study indicated that grapevines in the centre of Iran are affected with the 'bois noir' disease. The alternative host plants, especially weeds which act as distributers, and the activity of insect vectors within the vineyards, play a great role in disease epidemiology. Weed control and control of the vector *H. obsoletus*, could be the most effective methods to be used in grapevine 'bois noir' disease-management, in central Iran.

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