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ORIGINAL ARTICLE

Molecular identification of *Candidatus* Phytoplasma spp. associated with Sophora yellow stunt in Iran

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Abstract

In the spring of 2012, sophora (*Sophora alopecuroides* L.) plants showing symptoms of leaf yellowing, little leaves and stunting were observed in Firooz-kuh (Tehran province), Sari (Mazandaran province) and Urmia (West Azerbaijan province) in Iran. Symptomatic plants from the three locations were subjected to nested polymerase chain reaction (PCR) to amplify 16SrRNA using primer pair P1/P7 followed by primer pair R16F2n/R16R2. The amplicons were purified, sequenced and the nucleotide sequences were analyzed by virtual restriction fragment length polymorphism (RFLP). The phytoplasmas associated with the yellows disease were identified as members of the 16SrIX group (*Candidatus* Phytoplasma phoenicium) and the 16SrXII group (*Candidatus* Phytoplasma solani). The two phytoplasmas were placed in 16SrIX-C and 16SrXII-A subgroups, respectively, in constructed phylogenetic trees. This is the first report on sophora yellows associated with *Candidatus* Phytoplasma phoenicium.

Key words: *Candidatus* Phytoplasma phoenicium, nested-PCR, Sophora yellows

Introduction

Phytoplasmas are important phloem-limited, insect--transmitted agents associated with approximately a thousand plant diseases, many of which are lethal in hundreds of plant species including weeds throughout the world (Marcone *et al.* 1997). Plants infected with phytoplasma exhibit an array of symptoms that suggests profound disturbances in the balance of growth regulators (Duduk and Bertaccini 2011). Typical symptoms include virescence, phyllody, sterility of flowers, proliferation of axillary buds resulting in witches broom growth, elongation of internodes and generalized stunting (Bertaccini *et al.* 1996, 2014). Phytoplasmas cause diseases in several weeds which may act as alternative natural hosts facilitating the spread of phytoplasmas to economically important plants and thereby increasing economic losses. So far more than 100 weed species have been reported to have phytoplasma infections in many regions of the world. Phytoplasmas are able to survive in many economical crops and the insect vectors are capable of transmitting phytoplasmas from weeds to crops which are known as phytoplasma hosts. The chance of transmission in the future seems high, given the large phytoplasma reservoirs already existing and the propensity of new phytoplasma strains to evolve (Mall *et al.* 2010). Phytoplasma identity is esssential for phytoplasma disease management, especially in providing information about the mode of disease spread (insect vectors, propagation material, etc.). *Sophora alopecuroides* L. (sophora) is one of several herbaceous species of Leguminosae (Fabaceae), native to southeast Europe, southern Asia, Australasia, the Pacific Islands, western South America and Puerto Rico and possesses medicinal properties. It grows along the edges of fields, banks and less frequently on sand dunes, from sea level to 1750 m (Chamberlin 1970). However, in many areas of Iran it is regarded as a rather noxious weed species necessitating control. This plant grows around and in orchards, especially in

plum, peach and nectarine orchards, showing phytoplasma-like symptoms. Its infectivity by *Candidatus* Phytoplasma solani was verified previously (Allahverdipoor *et al*. 2013a, b) and it exhibits phytoplasma-like symptoms including leaf yellowing, little leaf and phyllody. Since weeds can serve as reservoirs for phytoplasmas the aim of the present study was to verify the presence of phytoplasma diseases in symptomatic sophora plants in three different geographical regions of Iran using polymerase chain reaction (PCR) assay. The phytoplasmas detected were characterized and classified using sequence analysis of PCR-amplified 16S rDNA and virtual restriction fragments length polymorphism (RFLP).

Fig. 1. Sophora with yellowing symptoms, left – healthy plant, right – infected plant

Materials and Methods

During the spring season of 2012, leaves and shoots from 24 symptomatic *S. alopecuroides* plants showing typical symptoms of leaf yellowing, little leaf and phyllody were collected in Firooz-kuh (Tehran province), Sari (Mazandaran province) and Urmia (West Azerbaijan province) regions of Iran (Figs. 1, 2) around and in plum, peach and nectarine orchards showing phytoplasma-like symptoms. At least eight symptomatic sophora plants were collected from each region. Asymptomatic sophora plants were also collected and used in molecular analysis as negative controls. Total DNA was extracted from 0.25 g of leaves and midribs using the CTAB extraction method (Maixner *et al*. 1995) and DNeasy Plant Mini kit (Qiagen, UK) and maintained at −80°C until use. Total DNA of healthy plants was extracted and used as negative controls. The universal primer pair P1/P7 (Schneider *et al*. 1995) was employed in direct PCR to amplify a 1.8 kbp fragment of 16S rDNA. A 30-fold dilution of the direct PCR product was used as templates for nested PCR using primer pair R16f2n/R16R2 which amplifies an internal fragment of 1.2 kbp from the 16S rDNA species in the genus *Candidatus* Phytoplasma (Lee *et al*. 1993). A total volume of 20 µl PCR reaction mixtures contained 20 ng DNA template, 0.2 mM of each dNTPs (Cinnagen, Iran), 1.6 mM $MgCl_2$, 1U of *Taq* DNA polymerase (Cinnagen, Iran), 0.5 µl of each primer pair (20 pmol · μl^{-1}) and 1X PCR buffer was used. The reaction mixtures were subjected to 35 cycles under

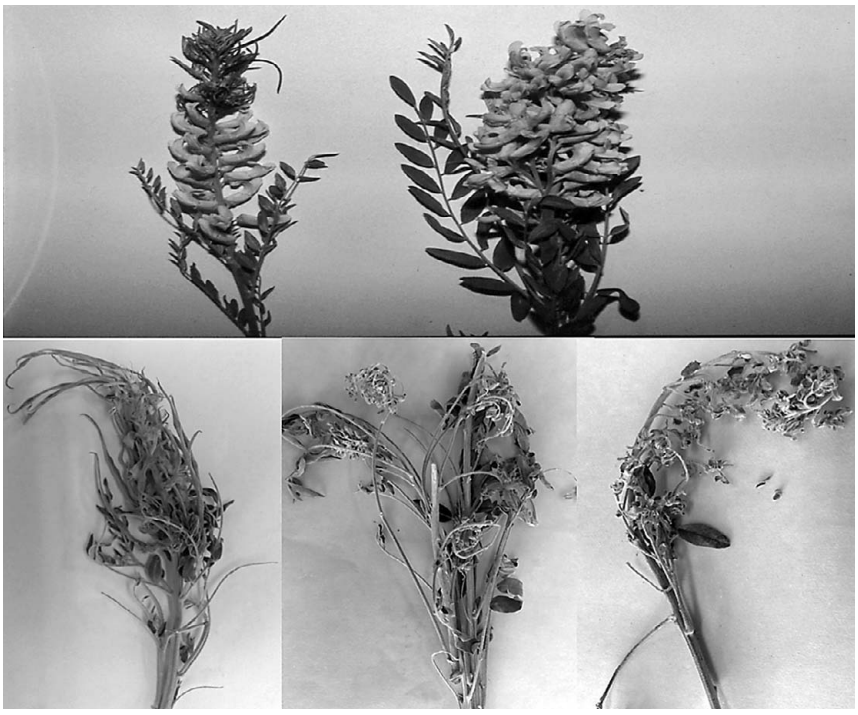

Fig. 2. Sophora with phyllody symptoms, healthy plant is on the top

Table 1. Strains of *Candidatus* Phytoplasma isolated from *Sophora alopecuroides* with yellow stunt and phyllody symptoms from various regions of Iran with GenBank accession numbers of their DNA spacer sequence

Strain	Location	GenBank accession No.
Candidatus Phytoplasma solani strain S141	Sari-Mazandaran province	KX172137
Candidatus Phytoplasma solani strain S135	Firoozkuh-Tehran province	KF923878
Candidatus Phytoplasma solani strain S139	Firoozkuh-Tehran province	KX172136
Sophora alopecuroides phyllody Phytoplasma isolate S191	Sari-Mazandaran province	KJ001834
Sophora alopecuroides phyllody Phytoplasma isolate S194	Urmia-West Azarbaijan province	KX172134
Sophora alopecuroides phyllody Phytoplasma isolate S196	Sari-Mazandaran province	KX172135

the following conditions: 1 min (2 min for the first cycle) denaturation step at 94°C, 2 min for annealing at 50°C and 3 min (10 min for the last cycle) for primer extension at 72°C. PCRs were performed in a thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). The PCR products were analyzed by electrophoresis in 1% agarose gel and stained with ethidium bromide. An ultraviolet (UV) trans-illuminator was used to visualize DNA bands. PCR products of nested PCR were purified and directly sequenced. Sequencing was performed by Macrogen (South Korea) on both strands with three replicates. Sequences were compared with the phytoplasmas sequences deposited in GenBank database using BLASTn. Nucleotide sequence similarity and multiple alignment and phylogenetic tree construction of phytoplasma 16S rDNA gene sequences were carried out using the neighbor-joining (NJ) method and bootstrap analysis replicated 1,000 times employing MEGA6 software (Tamura *et al.* 2011). *Acholeplasma laidlawii* was used as an outgroup. Assignment of phytoplasmas to 16Sr group/subgroup was done by *in silico* RFLP analyses of F2n/R2 amplicons using the software iPhyClassifier (Zhao *et al.* 2009). RFLP profiles of the test phytoplasmas were compared to those of 16SrIX-subgroups A to G using: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I recognition sites.

Results

Sophora plants with phytoplasma symptoms were collected from different regions of Iran including Firooz-kuh, Sari and Urmia during 2012 and DNA fragments of approximately 1.8 kbp and 1.25 kbp were amplified using phytoplasma universal primer pairs, P1/P7 and R16f2n/R16R2 in direct and nested PCR, respectively from all 24 symptomatic plants. No DNA was amplified form asymptomatic plants with either of the PCR methods. Comparison of 1,200 bp of 16S rDNA sequences of six of these phytoplasmas with those present in GenBank revealed that the three phytoplasma

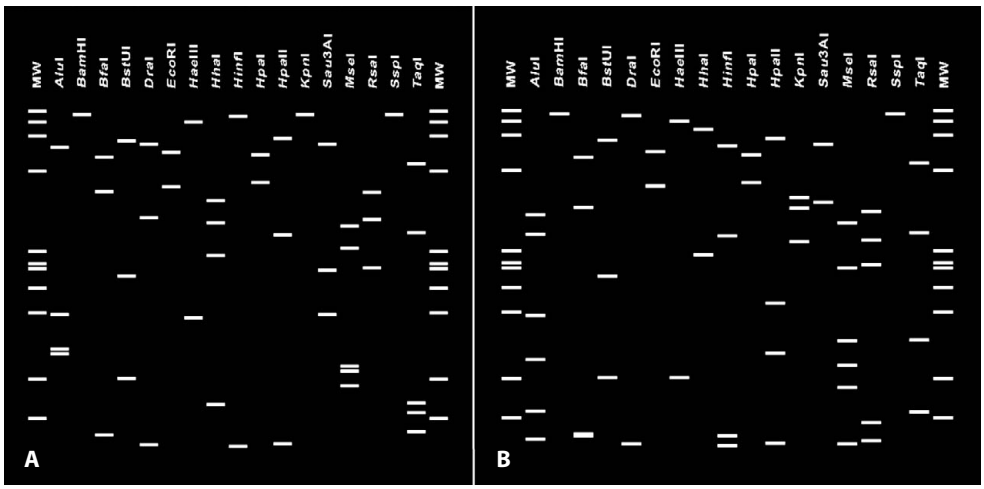

Fig. 3. Virtual restriction fragment length polymorphism (RFLP) pattern of $R_{16}F_{2n}/R_{16}R_2$ PCR product sequence from Sophora. A – *Candidatus* Phytoplasma solani strain S135, B – *Sophora alopecuroides* phyllody phytoplasma isolate S191. Restriction sites for the 17 restriction enzymes used in the simulated digestions: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I

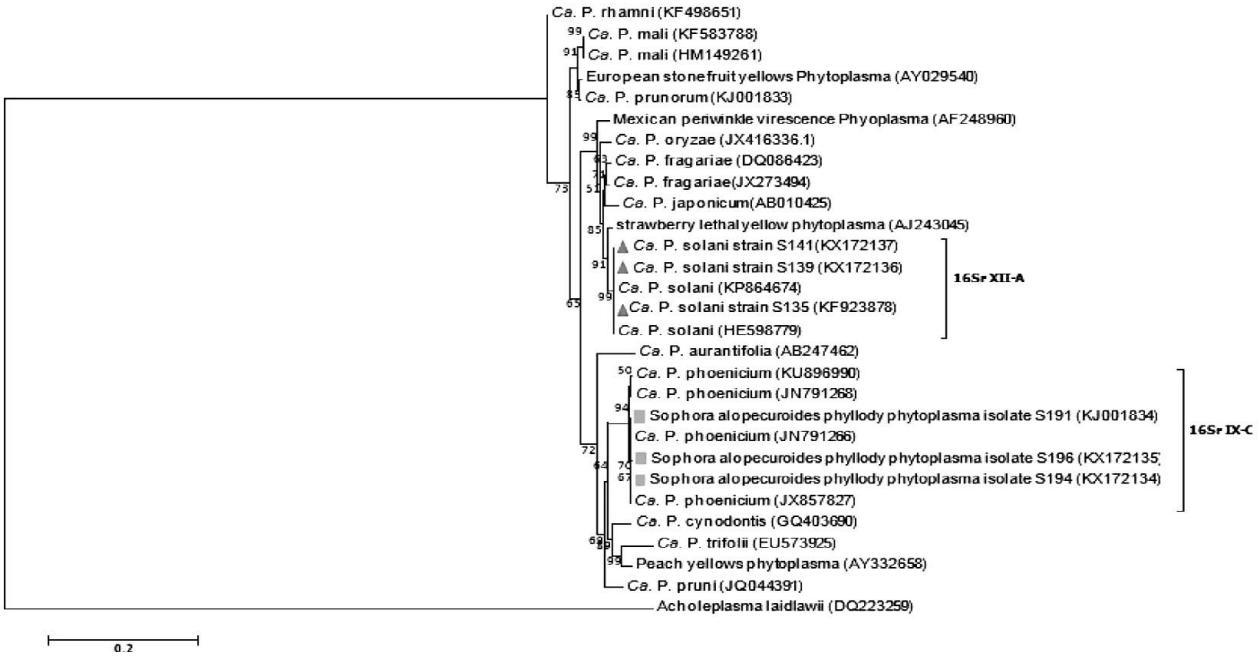

Fig. 4. Phylogenetic tree constructed by the neighbor joining method of 16S rRNA gene sequences from 22 phytoplasma and the strains isolated from Sophora plants. *Acholeplasma laidlawii* was used as outgroup. Numbers at the nodes are bootstrap values based on 1,000 repetitions. GenBank accession numbers for sequences are given in parentheses. Ca. P. – *Candidatus* Phytoplasma

strains under study shared 99-100% sequence identity with *Candidatus* Phytoplasma phoenicium (accession number AF515636), ribosomal RNA group IX and the other three strains had 100% identity to *Candidatus* Phytoplasma solani (accession number AF248959), ribosomal RNA group XII. Nucleotide sequences determined in the present study were deposited in the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers: KJ001834, KX172134, KX172135, KX172136, KX172137 and KF923878. Computer-simulated restriction analyses were carried out on R16f2n/R16R2 amplified sequences from sophora together with 22 reference phytoplasmas and representative strains belonging to 16SrIX and 16SrXII subgroups. Comparison of virtual gel images revealed that RFLP patterns of sophora shared a virtual RFLP similarity coefficient >98% with *Candidatus* Phytoplasma phoenicium strains of subgroup IX-C and *Candidatus* Phytoplasma solani strains of subgroup XII-A (Fig. 3). Phylogenetic analyses of sequences presented in this survey with those of phytoplasmas present in GenBank clearly demonstrated that the three phytoplasmas (with accession numbers: KJ001834, KX172134 and KX172135) clustered with the strains belonging to group 16SrIX, and the other three strains (with accession numbers: KX172136, KX172137 and KF923878) belonged to group 16SrXII and were well separated from the members of other phytoplasma groups (Fig. 4). Furthermore, these phytoplasmas clustered together with the strains of *Candidatus* Phytoplasma phoenicium (subgroup 16SrIX-C) and *Candidatus* Phytoplasma solani (subgroup 16SrXII-A), verifying their close similarity to the above-mentioned subgroups.

Discussion

Sophora plants with little leaf, yellowing and phyllody symptoms were collected around and in phytoplasma-infected plum, peach and nectarine orchards. Approximately 50% of the plants from the three sampled regions were symptomatic at the time of collection. Sequence analyses and virtual RFLP of Sophora phytoplasma strains clearly showed that three of the studied phytoplasma strains belonged to phytplasma group 16SrIX-C (*Candidatus* Phytoplasma phoenicium) and three other strains belonged to the 16SrXII-A group (*Candidatus* Phytoplasma solani). Phytoplasmas of ribosomal group 16SrIX (pigeon pea witches' broom group) are associated with diseases affecting crops and wild plants in different geographic areas worldwide (Kenyon *et al.* 1998; Verdin *et al.* 2003; Khan *et al.* 2007; Davis *et al.* 2010). Several strains of *Candidatus* Phytoplasma phoenicium have previously been reported from a number of plants including *Lactuca serriola, L. sativa, Solanum lycopersicon, Sonchus* sp. [16SrIX-E], *Carthamus tinctorius*, *Chrysanthemum morifolium* cv. Paniz and *Prunus amygdalus* [16SrIX-B] from Iran (Salehi *et al.* 2006, 2011; Bayat *et al.* 2013). It has also been shown that *Candidatus* Phytoplasma

phoenicium is associated with a lethal disease of almond, peach and nectarine, called almond witches' broom disease (AlmWB) (Abou-Jawdah *et al*. 2009). Molino Lova *et al*. (2011) showed that Iranian phytoplasma strains associated with AlmWB and almond brooming shared >99% similarity with phytoplasmas of subgroup IX-C.

Phytoplasmas classified in the 16S rRNA gene RFLP group 16SrXII infect a wide range of wild and cultivated plants including grapevine (where it induces Bois noir, BN), potato, and strawberry worldwide (Davis *et al*. 1997; Valiunas *et al*. 2006; Quaglino *et al*. 2013). Our study in Iran also showed that the 16SrXII group of phytoplasmas, especially *Candidatus* Phytoplasma solani, is widespread in Iran and has been reported on different crops such as potato (Hosseini *et al*. 2011) plum, peach, nectarine (Zirak *et al*. 2009a, b, 2010; Allahverdipoor *et al*. 2013a, b) and grapevine (Mirchenari *et al*. 2015).

Previous reports of phytoplasmas affecting sophora species include a 16SrXII phytoplasma associated with *S. japonicum* yellows (Duduk *et al*. 2010) in China. Other phytoplasmas have also been identified on *S. japonicum* including *Candidatus* Phytoplasma ziziphi in China associated with witches' broom disease and a 16SrI *Candidatus* Phytoplasma asteris associated with sophora yellows (Yu *et al*. 2012; Chen *et al*. 2013). Allahverdi *et al*. (2014) reported a 16SrXII phytoplasma association with *S. alopecuroides* from Firooz-kuh (Iran) with leaf yellowing, little leaf and stunting symptoms. The association of a phytoplasma belonging to 16SrVI (*Candidatus* Phytoplasma trifolii) was previously established in sophora exhibiting yellowing and witches' broom symptoms in China (Li *et al*. 2013) and West Azarbaijan province of Iran (Zibadoost and Rastgou 2016).

Phytoplasma infections have been documented in more than 100 weed plant species thus far (Singh and Upadhyaya 2013). Sophora is widely distributed as a volunteer plant and grows frequently in or around farms and orchards in the studied regions. Our results suggest the potential of sophora acting as a significant alternative host plant for *Candidatus* Phytoplasma phoenicium and *Candidatus* Phytoplasma solani, posing a threat to crops such as almond, nectarine, plum, peach and grapevine in geographical regions where these plants are main crops. The wide host ranges of these phytoplasmas implicate them as alternative hosts in which the phytoplasmas are maintained, perpetuated and transmitted by insect vectors to commercial crops and they can be a severe threat to them. This is, to our knowledge, the first report of a phytoplasma of the 16SrIX group member infecting sophora. The emergence of this phytoplasma in the sophora represents ongoing evolution in the adaptation of *Candidatus* Phytoplasma phoenicium to a new ecological niche (Arocha-Rosete *et al*. 2011). Additional studies are needed to determine the distribution, insect vectors, and economic impact of *Candidatus* Phytoplasma solani and *Candidatus* Phytoplasma phoenicium on plum, peach and nectarine orchards and other crops in the studied regions.

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