Australasian Plant Disease Notes (2018) 13: 40
https://doi.org/10.1007/s13314-018-0321-6

"*Candidatus* Phytoplasma trifolii" related strain affecting *Salix babylonica* in Iran

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Received: 2 May 2018 / Accepted: 12 October 2018 / Published online: 27 October 2018

Abstract

Over the last few years, in the northwest of Iran, symptoms of witches' broom have been observed in Babylon willow trees (*Salix babylonica*). Samples from symptomatic and symptomless trees were tested using nested PCR with universal primer pair P1/P7 followed by R16F2n/R2. Phylogenetic and virtual RFLP analysis showed that the phytoplasma associated with willow proliferation is related to the16SrVI-A subgroup.

Keywords Clover proliferation · Phytoplasma · P1/P7 · R16F2n/R2 · *Salix babylonica*

Salix babylonica, commonly known as Babylon willow, is a medium to large sized deciduous ornamental tree commonly grown in most regions of Iran. During the last few years, symptoms of witches' broom, little leaf, and yellowing, resembling those often caused by phytoplasmas, have been observed in Babylon willow trees in Zanjan and West Azerbaijan provinces (Fig. 1). A total of 14 samples from both symptomatic (12 samples) and symptomless (2 samples) trees were collected from different areas in Zanjan and West Azerbaijan provinces and transported in cold condition to the laboratory and stored at −20 °C until used. Total DNA extraction was performed using the procedure of Murray and Thompson (1980).

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The extracted DNA was used as template for PCR with universal primers P1/P7, which amplify a 1784 bp DNA fragment of the phytoplasma 16S/23S rRNA region (Deng and Hiruki 1991; Smart et al. 1996).

Nested-PCR for amplification of the 16S rDNA was conducted using phytoplasma universal primer sets R16F2n/R16R2 (Gundersen and Lee 1996) in the second round. The expected amplicon of 1.2 kb was amplified in all 12 samples from symptomatic trees. Partial sequencing of the 16SrRNA region of four strains from different sampling areas was conducted with universal primer pair R16F2n/R2. The nucleotide sequences were assembled using Bioedit sequence alignment editor, version 7.0.9.0 and compared with available sequences in GenBank using blast alignment. Phylogenetic and molecular analyses were conducted by the neighbor-joining method in MEGA6 software (Tamura et al. 2013).

The obtained nucleotide sequences were deposited in the GenBank database under accession numbers MG437258, MG437259, MG437260 and, MG437261. In Blast software, the sequences showed 99% identity with some strains affiliated with '*Candidatus* Phytoplasma trifolii' in GenBank (KX773529,

Fig. 1 Witches' broom symptoms on Babylon willow trees from northwest of Iran. (**a**) Symptoms of Phytoplasma in Babylon willow tree, West Azerbaijan province, (**b**) Zanjan province, Iran

KP864671 and, KP864669). In the resulting phylogenetic tree constructed, based on the 16S rDNA sequences, all phytoplasma strains associated with Babylon willow witches' broom (BWWB) were clustered in the 16SrVI-A subgroup (Fig. 2).

Virtual RFLP of the sequences obtained (1.2 kbp) was performed with 17 typical restriction enzymes, *Alu*I, *BamH*I, *Bfa*I, *BstU*I (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Mse*I, *Rsa*I, *Sau3A*I (*Mbo*I), *Ssp*I, and *Taq*I, used for RFLP analyses of phytoplasma 16S rDNA genes (Lee et al. 1998) by iPhyClassifier (https://plantpathology.ba.ars.usda.gov/cgi.bin/resource/iphyclassifier.cgi) (Zhao et al. 2009). After in silico restriction digestion a virtual gel electrophoresis image was plotted and captured and putative restriction site maps were compared with the pattern of strains deposited in GenBank. The restriction patterns revealed that the detected strains in the present study, SB21U, SB22U, SB23Z and SB24Z shared respectively 98.2%, 98.0%, 98.5 and 97.6% similarity with "*Candidatus* Phytoplasma trifolii" reference strain (AY390261) belonging to the 16SrVI-A subgroup. According to the iPhyClassifier online software, SB21U and SB23Z have the closest pattern with reference strain (AY390261) (Fig. 3).

Several phytoplasmas were previously reported from Salix trees including '*Ca.* Phytoplasma asteris' (16SrI-C) and '*Ca. P. trifolii*' (16SrVI-A) in China (Wei et al. 2009; Zhang et al. 2012), clover proliferation (16SrVI) and stolbur group in Spain (Alfaro-Fernández et al.

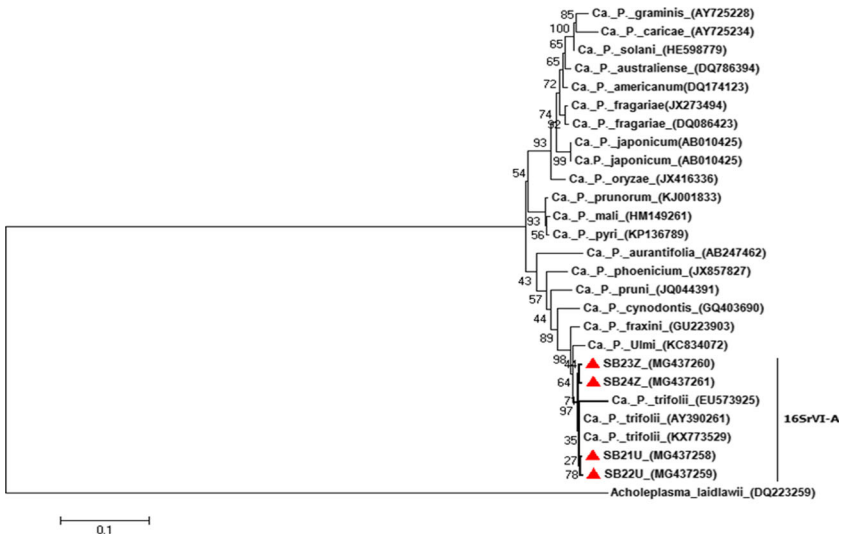

Fig. 2 The neighbor-joining tree generated using 16S rDNA sequences of isolated phytoplasma strains and 23 selected '*Candidatus* Phytoplasma species' from GenBank. The numbers next to nodes are confidence values of bootstrap (1000 replicates). *Acholeplasma laidlawii* was used as the out-group. The scale at the bottom represents genetic distance in nucleotide substitutions per site. Ca. P. sp.: '*Candidatus* Phytoplasma' sp.

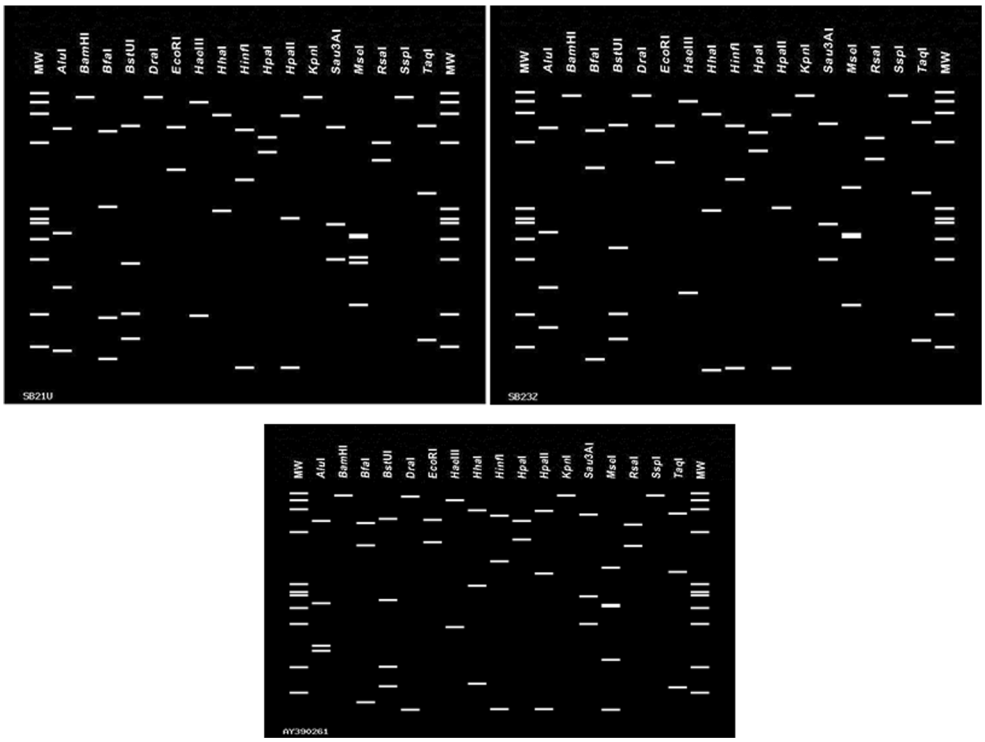

Fig. 3 Virtual RFLP patterns from in silico digestion of about 1200 bp nucleotide sequence of 16S rRNA gene of phytoplasmas associated with Babylon willow witches' broom (SB21U and SB23Z), and reference strain ('*Ca*. *P. trifolii*'; AY390261) using 17 restriction enzymes. (*Alu*I, *BamH*I, *Bfa*I, *BstU*I (*Tha*I), *Dra*I, *EcoR*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau3A*I (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I) in iPhyClassifier software. MW: ΦX174 DNA-*Hae*III digested size marker

2011; Khadhair and Hiruki 1995), '*Ca*. P. phoenicium' (16SrIX) and Ca. *P. solani*' (16SrXII) in Iran (Ghayeb Zamhari 2017; Ghayeb Zamharir and Taheri 2017). Clover proliferation phytoplasma group (16SrVI) causes diseases in numerous plant species in different geographic areas worldwide (Raj et al. 2009; Choueiri et al. 2007; Salehi et al. 2008). However, to the authors' knowledge, there is no previous report of the 16SrVI phytoplasma group associated with Babylon willow witches' broom from Iran.

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