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Association of *'Candidatus* Phytoplasma trifolii' related strain with white willow proliferation in Iran

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Abstract

A phytoplasma was identified in white willow trees exhibiting symptoms of proliferation, growing in urban areas in Hamedan province of Iran. The association of a phytoplasma with this disease was confirmed by nested PCR of the 16S rRNA gene using universal phytoplasma-specific primer pair P1/P7 and R16mF2n/R2. Results of nested-PCR amplified a product of expected size (1.4 Kb). Comparison of the sequencing results with those available in GenBank and phylogenetic analysis allowed classification of the phytoplasma into '*Candidatus* phytoplasma trifolii' (16SrVI) taxonomic group. To the best of our knowledge, this is the first report on the molecular detection and identification of a clover proliferation group phytoplasma in willow trees in Iran.

Keywords Proliferation · '*Candidatus* phytoplasma trifolii' · White willow

Salix alba is a species of willow, native to Europe and central Asia. The name derives from the white tone to the underside of the leaves (Meikle 1984). In Iran, *S. alba* is a traditional tree normally grown in urban areas. White willows are fast-growing, but relatively short-lived, being susceptible to several diseases, including phytoplasma diseases (Ghayeb Zamharir 2017; Ghayeb Zamharir and Taheri 2017). During a survey in Hamedan province (Iran), symptoms of proliferation were observed in white willows in Bahar (Fig. 1) typical of diseases caused by phytoplasmas.

The present study was initiated to determine the group affiliations of Hamedan province, Iran white willow proliferation-associated phytoplasmas. Knowledge of group affiliation is significant in order to facilitate the future identification of their insect vectors and alternate plant hosts.

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Leaf samples from eight white willow trees with proliferation symptoms were collected in 2016 from Hamedan province, Iran. Asymptomatic samples of white willow and positive samples of lime witches broom disease were also included in the assay as negative and positive controls, respectively. Total DNA was extracted as described Doyle and Doyle (1990). A nested-PCR was performed using the universal phytoplasma primers P1/P7 (Deng and Hiruki 1991) followed by R16MF2n/R16MR2 (Gundersen and Lee 1996). Direct sequencing was carried out in both directions from two of eight positive samples (SPH1 and SPH2). Sequences were aligned using the clustalX software (Thompson et al. 1997) and used for searching against the database of National Center for Biotechnology Information (NCBI) by BLASTn. Published phytoplasma sequences were retrieved from GenBank; 29 sequences of 16S rRNA gene from different groups. Phylogenetic trees were built by maximum parsimony (16S rRNA) methods using the neighbor joining algorithm, with a 100-replicate bootstrap search using MEGA6.

Fragments of the expected size (1.4 kb) were only amplified from all symptomatic samples (8 samples) and positive controls. No amplification was produced from

Fig. 1 Proliferation in a white willow tree from Hamedan (Iran) (left) compared with a healthy willow tree (right)

healthy samples or water controls. R16MF2n/R2 sequences of SPH1 and SPH2 strains were submitted to the GenBank database and assigned accession numbers MF540615 and MF540616. These showed 100% identity to each other and 99% sequence identity to the reference isolate of '*Ca*. *P. trifolii*' (AY390261) and is therefore identified as a 'Ca. *P. trifolii*'-related strain. Phylogenetic analysis with selected reference strains and sequence analysis using the iPhyClassifier tool (Zhao et al. 2009) indicated that the phytoplasma clustered together with member strains of 'Ca. *P. trifolii*' (16SrVI) (Fig. 2).

Salix spp. are found throughout the world and are associated with phytoplasmas belonging to different taxonomic groups including '*Ca. P. asteris*' (16SrI) in China (Mou et al. 2014), clover proliferation group in Canada (Khadhair and Hiruki 1995), stolbur phytoplasma (Alfaro-Fernandez et al. 2011) and '*Ca*. P. phoenicum' and '*Ca*. *P. solani*' in Iran (Ghayeb Zamharir 2017; Ghayeb Zamharir and Taheri 2017). However, Clover proliferation group were reported from *Salix bebbiana* Sarg., *S. discolor* Muhl., *S. exigua* Nutt, and *S. petiolaris* in Canada, but there are no sequence data to compare Canadian isolates with Iranian ones. Also it appears that isolates related to group 16SrVI of phytoplasmas are associated with disease in different *Salix* species in Iran and Canada. To the best of our knowledge this is the first report of a '*Ca*. *P. trifolii*'-related strain associated with *Salix alba* in Iran.

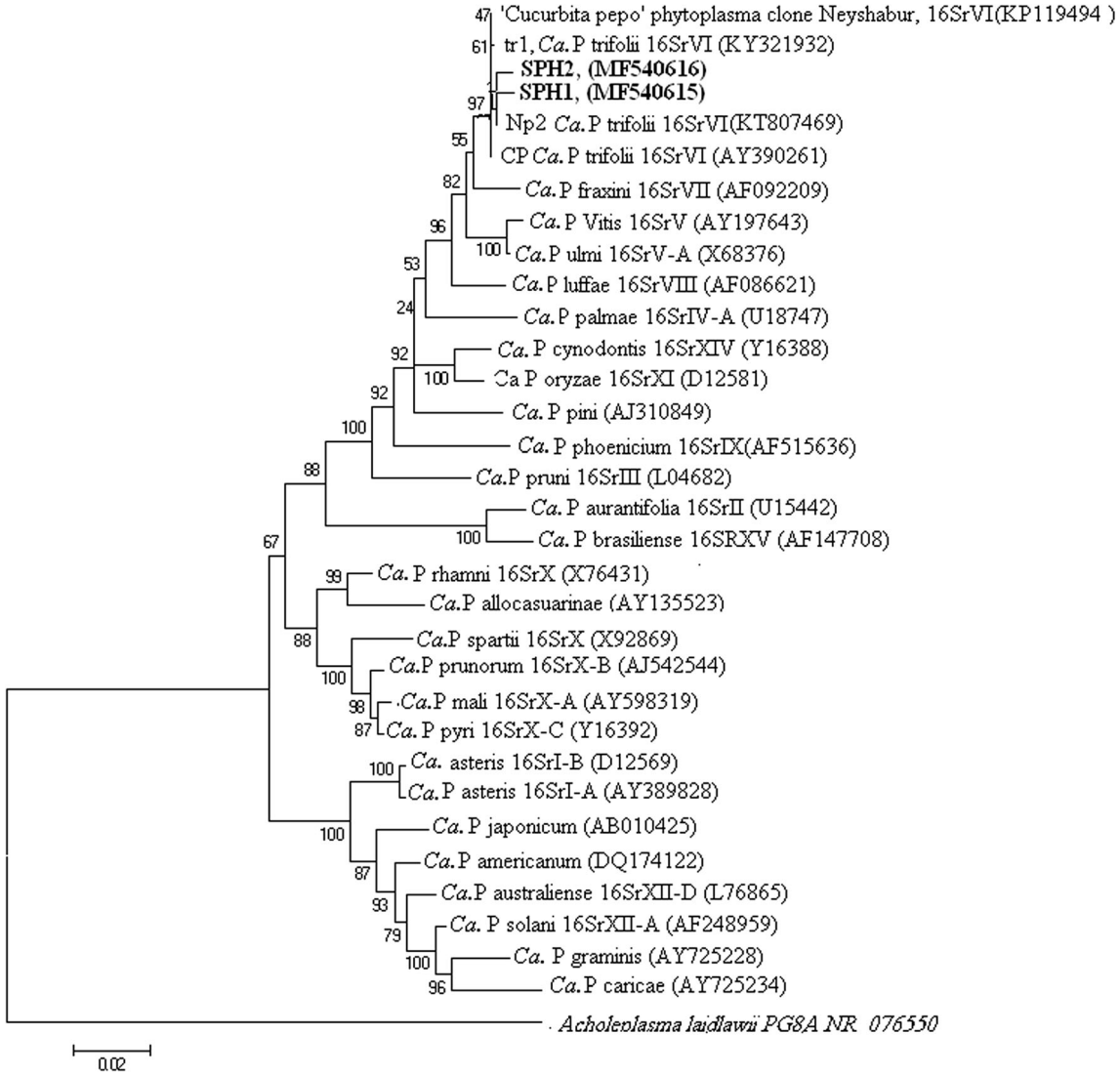

Fig. 2 Evolutionary analyses conducted in MEGA6 (Tamura et al., 2013) using the neighbor joining method on 29 phytoplasma strains with the strains SPH1 and SPH2 of *Salix alba* proliferation from Hamedan province. *Acholeplasma laidlawii* was used as outgroup. '*Candidatus* Phytoplasma' (Ca. P.) names, 16Sr groups and strain names are indicated on the branches followed by the GenBank number of the sequence employed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches

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