Received: 14 March 2018 | Revised: 23 July 2018 | Accepted: 13 October 2018

DOI: 10.1111/jph.12769

ORIGINAL ARTICLE

WILEY Journal of Phytopathology

Molecular study of two distinct phytoplasma species associated with streak yellows of date palm in Iran

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Abstract

A disease with symptoms similar to palm lethal yellowing was noticed in the early 2013 in Khuzestan Province (Iran) in date palm (*Phoenix dactylifera*). Infected trees displaying symptoms of streak yellows and varied in the incidence and severity of yellowing. A study was initiated to determine whether phytoplasma was the causal agent. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) methods using universal phytoplasma primers pairs R16mF1/mR1 and M1/M2 were employed to detect putative phytoplasma(s) associated with date palm trees. Nested PCR using universal primers revealed that 40 out of 53 trees were positive for phytoplasma while asymptomatic date palms from another location (controls) tested negative. RFLP analyses and DNA sequencing of 16S rDNA indicated that the presence of two different phytoplasmas most closely related to clover proliferation (CP) phytoplasma (group 16SrVI) and ash yellows (AY) phytoplasma (group 16SrVII). Sequence analysis confirmed that palm streak yellows phytoplasmas in each group were uniform and to be phylogenetically closest to "*Candidatus* P. fraxini" (MF374755) and "*Ca*. P. trifolii" isolate Rus-CP361Fc1 (KX773529). Result of RFLP analysis of *sec*A gene of positive samples using *Tru*I and *Taq*I endonuclease is in agreement with rDNA analysis. On this basis, both strains were classified as members of subgroups 16SrVI-A and 16SrVII-A. This is the first report of a phytoplasma related to CP and AY phytoplasma causing date palm yellows disease symptoms.

KEYWORDS

"*Ca.* Phytoplasma trifolii", "*Candidatus* Phytoplasma fraxini", date palm, streak yellows

1 | INTRODUCTION

Iran is one of the leading dates producing and exporting countries in the world. Palm tree cultivation was severely affected by various pathogens such as fungi, viruses and phytoplasma worldwide (Abdullah, Lopez Lorca, & Jansson, 2010; Harries, 1977; Howard, Norris, & Thomas, 1983). Phytoplasma diseases of date palms have been reported from Egypt (Ammar, Amer, & Rashed, 2005), Kuwait (Al-Awadhi, Hanif, Suleman, & Montasser, 2002), Saudi Arabia (Alhudaib, Rezk, & Alsalah, 2014; El-Zayat, Shamloul, Abdulsalam, Djerbi, & Hadidi, 2002) and Sudan (Al Khazindar, 2014; Cronjé, Dabek, Jones, & Tymon, 2000).

Phytoplasmas belong to the class Mollicutes (Bai et al., 2006; Bertaccini, 2007; Kirkpatrick, 1992; Lee, Davis, & Gundersen-Rindal, 2000) and reside in the phloem tissue of the plant, and many are known to be transmitted by phloem-feeding insects such as leafhoppers and planthoppers (Lee & Davis, 1992). According to the most recent classification scheme, phytoplasmas are differentiated into major groups and subgroups of strains based on RFLP analysis of the 16S rRNA gene (Lee, Gundersen-Rindal, Davis, & Bartoszky, 1998; Martini et al., 2007; Wei, Davis, Lee, & Zhao, 2007).

The phytoplasma groups implicated in phytoplasma diseases of date palms have been identified as group 16SrIV (America; Harrison

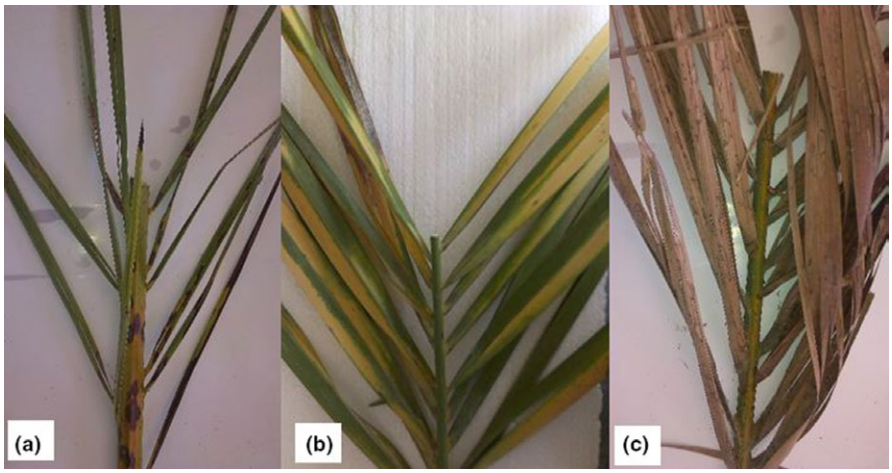

FIGURE 1 Date palm plant showing elongated internodes and abnormal branches (a), plant leaves with streak yellows (b) and date leaf drying (c) [Colour figure can be viewed at wileyonlinelibrary.com]

and Jone, 2004), group 16SrI (Egypt and Saudi Arabia; Al Khazindar, 2014; Alhudaib, Arocha, Wilson, & Jones, 2008), 16SrII (Saudi Arabia; Alhudaib et al., 2014) and 16SrXIV (Sudan; Cronjé et al., 2000).

A streak yellows and decline disease affecting date palms was first recognized in Ahvaz, Khuzestan Province of Iran, during 2013. Symptoms observed on affected palms include leaf streak yellows, tiny narrow leaves and leaf drying (Figure 1). The present study was undertaken from 2015 to determine the aetiology of Iran date palm streak yellows. Presently, at several locations, symptomatic palms have been identified, but not all palms within the plantings are necessarily affected. We report the detection of 16SrVII and 16SrVI group phytoplasmas in the symptomatic date palms sampled in Khuzestan, Bushehr and Kerman Provinces of Iran. Detected phytoplasmas were most closely related to two distinct Phytoplasma species, "*Candidatus* Phytoplasma fraxini" and "*Candidatus* Phytoplasma trifolii."

2 | MATERIALS AND METHODS

2.1 | Plant samples and DNA extraction

A total of 53 date palm samples were collected in 2015 and 2016 from symptomatic and asymptomatic date palm trees in infected date palm orchard located in Khuzestan, Bushehr and Kerman Provinces of Iran. Also, five wood samples were collected from symptomatic date palms. Potential insect vectors also collected from Khuzestan gardens by sticky yellow cards. Total nucleic acid was extracted from leaf tissue, wood from interior basal trunks of five symptomatic Iran date palms and collected leafhoppers as described previously (Doyle & Doyle, 1987). The precipitated DNA was re-dissolved in 50 µl of sterile distilled water. The DNA samples were analysed in 1% agarose gels and adjusted to a suitable concentration for further usage.

2.2 | Polymerase chain reaction analysis

Amplifications were performed in 25 µl final reaction volumes each containing 50 ng of DNA template, 50 ng of each primer P1/Tint (Deng & Hiruki, 1991; Schneider, Seemüller, Smart, & Kirkpatrick, 1995), 125 µM of each dNTP, 1 U of Taq DNA polymerase (Sinaclone, Tehran, Iran) and standard polymerase chain reaction (PCR) buffer with 1.5 mM $MgCl_2$. PCR was performed for 35 cycles in a programmable thermal controller (Bio-Rad, USA) and previously described thermal cycling conditions (Schneider et al., 1995). Products of P1/Tint-primed PCR were diluted 1:30 with sterile deionized water and 2 µl of each dilution then used as template during 35 cycles of PCR with nested rRNA primer pair R16mF1/mR1 (Gundersen & Lee, 1996) and 6R758f/16R1232r (=M1/M2) (Gibb, Padovan, & Mogen, 1995). For nested PCR, the following thermal cycling parameters were used: denaturation for 30 s (5 min for first cycle) at 94°C, annealing for 2 min at 56 and 50°C, respectively, for nested primers, and extension for 2 min and 30 s at 72°C. Reactions were terminated by a 10-min extension step and cooled to 4°C. Palm DNAs were also assayed by PCR (40 cycles) employing nonribosomal primers SecAfor1/SecArev3 and SecAfor2/SecArev3 as previously described (Hodgetts, Boonham, Mumford, Harrison, & Dickinson, 2008). Aliquots (6 µl) of each final reaction mixture were electrophoresed through 1.2% agarose gels using TAE (40 mM Tris-acetate, 1 mM EDTA) as running buffer. DNA products in gels were stained with ethidium bromide (EtBr), visualized by UV transilluminator and photographed.

2.3 | Restriction fragment length polymorphism analysis of PCR products

Products of M1/M2- and SecAfor2/SecArev3-primed PCR were digested separately with 10 U of restriction endonuclease *Mse*I and *Taq*I (Vivantis, Malaysia) at 65°C, for a minimum of 16 hr. Digests were separated by electrophoresis through 3% agarose gels using TAE as running buffer. Products in gels were visualized and recorded as described above.

Virtual restriction fragment length polymorphism (RFLP) patterns were performed for the partial sequences of 16S rDNA bounded by the two conserved nucleotide blocks corresponding to

the annealing sites for the phytoplasma universal 16S rRNA primer pair R16F2n/R16R2 (Gundersen & Lee, 1996), and computer-simulated virtual RFLP patterns were generated by using an i*Phy*Classifier (Zhao et al., 2009). Each 16S rDNA fragment was digested in silico with 17 distinct restriction enzymes (*Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I) that were used for the phytoplasma 16S rDNA RFLP analysis (Lee et al., 1998). The virtual RFLP patterns were compared, and a similarity coefficient (F) was calculated for each pair of phytoplasma strains by using a Perl program developed by Wei, Lee, Davis, Suo, and Zhao (2008).

2.4 | Sequencing and phylogenetic analysis

Selected eight R16mF1/mR1 (1,500 bp)- or M1/M2 (500 bp)-primed rDNA products amplified from same samples of date palm were sequenced directly by commercial service (Macrogen Inc., Seoul) in both directions. The obtained sequences were deposited in the NCBI and aligned using Clustal W (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) from the Molecular Evolutionary Genetics Analysis program-MEGA6 (Tamura et al., 2011). Phylogenetic analyses were done with maximum parsimony (MP) analysis by using the close-neighbour-interchange algorithm, using 16S rDNA sequence from Iran date palm samples and from 2 "*Candidatus* phytoplasma" strains by using *Acholeplasma laidlawii* as the out-group. The analysis was replicated 100 times. A bootstrap analysis was performed to estimate the stability and support for the inferred clades (Tamura, Nei, & Kumar, 2004).

3 | RESULTS

3.1 | Detection of phytoplasma

In total, 53 leaf samples and nine population of leafhoppers were collected that 40 out of leaf samples were symptomatic. Phytoplasma was detected with nested PCR in all symptomatic date palm leaves, woods and two leafhopper population. No amplification was obtained from the negative controls without DNA template and from the asymptomatic date palms. Comparative nested PCR assays using universal primer pair R16mF2n/R16R1 in the second reaction yielded product for DNA from streak yellows date palm and lime witches' broom samples as positive control. None of positive samples have shown specific band in direct PCR stage. Also, 80% of samples collected in winter were positive in nested PCR, but only 20% of samples collected in spring were positive. This may have related to seasonal and spatial variation in phytoplasma distribution and titre in trees. *Sec*A gene amplification using SecAfor2/SecArev3 was resulted in all CP-positive samples.

3.2 | RFLP analysis of PCR products

The collective in silico 16S rDNA RFLP patterns from phytoplasma-positive date palm indicate that two different phytoplasmas most closely related to clover proliferation (CP) phytoplasma (group 16SrVI) and ash yellows (AY) phytoplasma (group 16SrVII) associated with streak yellow date palm (SYDP) symptoms in Iran (Figure 2).

The SYDP phytoplasmas are identical to one another (representative RFLP patterns shown in Figure 3). The virtual RFLP pattern derived from the query 16S rDNA R16F2n/R16R2

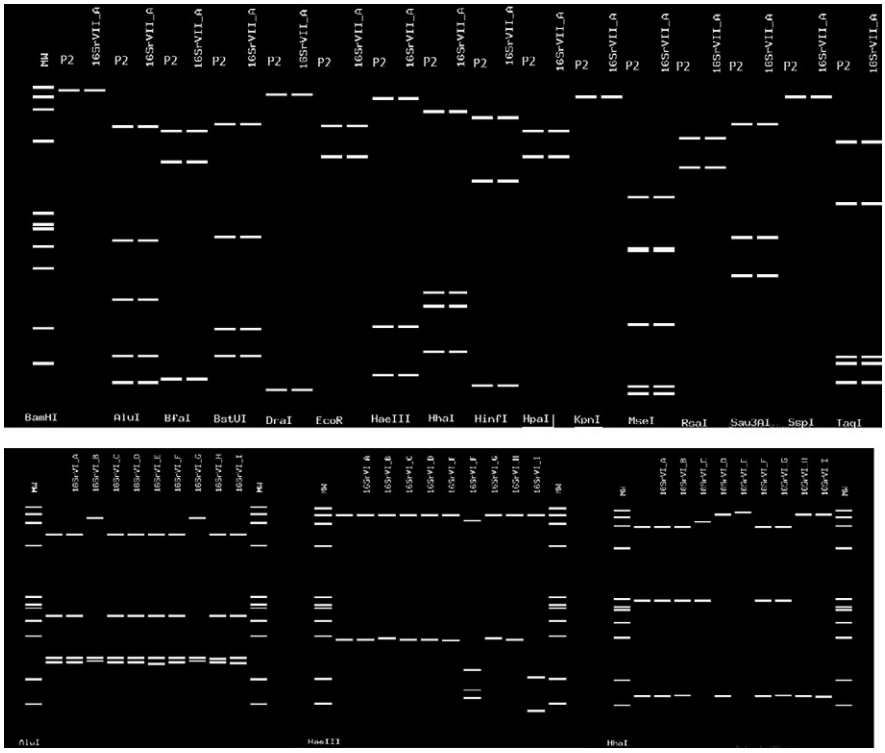

FIGURE 2 In silico RFLP analyses of phytoplasmal ribosomal protein DNA, amplified by PCR with primer pair R16F2n/R16R2, from date palms. Up: comparative RFLP pattern of AY-related strain P2 with "*Ca*. P. fraxini" strain ASHY-7 (HQ589190) (subgroup 16SrVI-A). Down: comparative RFLP pattern of CP-related strain P34 with CP major subgroups using restriction enzymes, *Alu*I, *Hae*III, *Hha*I. RFLP: restriction fragment length polymorphism

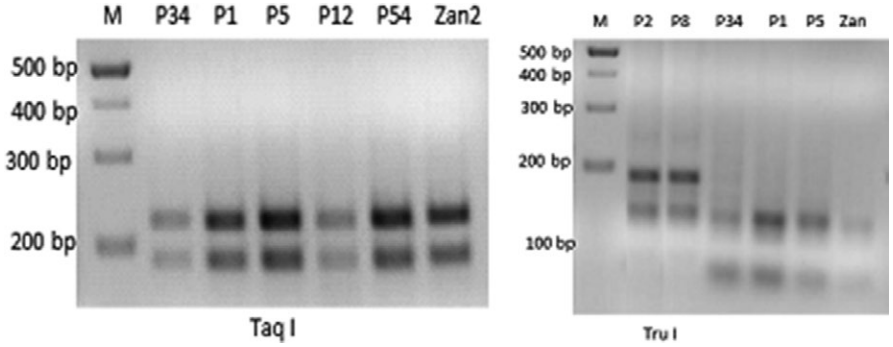

FIGURE 3 RFLP analyses of phytoplasma ribosomal protein DNA amplified by PCR with primer pair M1/M2 from date palms affected by CP- and AY-related phytoplasmas. PCR products were digested with *Mse*I and *Taq*I. M is DNA size marker 100 bp (Fermentas). RFLP: restriction fragment length polymorphism

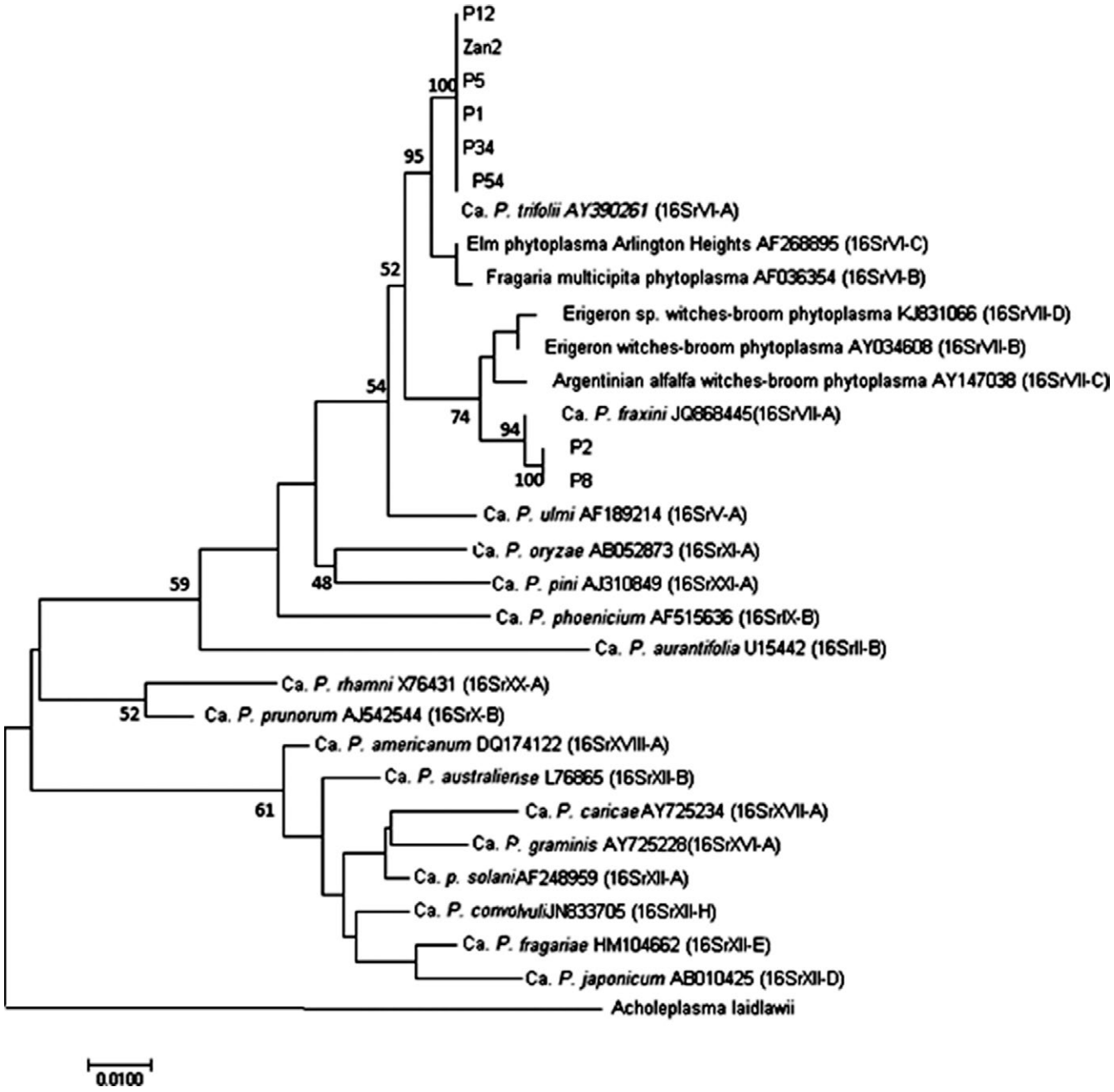

FIGURE 4 The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model (Tamura et al. 1993). Bootstrap values are shown on the branches

fragment of positive samples P1 (MH023532), P5 (MH023533), P12 (MH023534), P34 (MH023531), P54 (MH023535) and leafhopper population 2 (Zan2 (MH023530)) is identical (similarity coefficient 1.00) to the reference pattern of 16Sr group VI, subgroup A (GenBank accession: AY390261). The phytoplasma under study is a member of 16SrVI-A, and those from samples P2 (MH023528) and P8 (MH023529) are identical (similarity coefficient 1.00) to the reference pattern of 16Sr group VII, subgroup A (GenBank accession: AF092209).

Streak yellow date palm phytoplasmas (P1, P5, P12, P34 and P54) related to from AY (group 16SrVII) can be differentiated from other subgroups by seven restriction enzymes, *Alu*I, *Hae*III, *Hha*I, *Hinf* I, MseI, *Sau*I and *Taq*I, and those related to CP phytoplasma (P2 and P8) can be differentiated from other subgroups by *Alu*I, *Dra*I, *Hae*III, *Hha*I, *Mse*I, *Rsa*I and *Taq*I (Figure 2).

3.3 | Sequencing and phylogenetic analysis

The BLAST comparison of SYDP phytoplasma 16S rDNA sequences data from five leaf and wood samples P1 (MH023532), P5 (MH023533), P12 (MH023534), P34 (MH023531), P54 (MH023535) and leafhopper population 2 (Zan2 (MH023530)) indicated that SYDP phytoplasma has approximately 99% homology to "*Ca*. P. trifolii" isolate Rus-CP361Fc1 (KX773529) and 16S rDNA sequences data from two leaf samples show 99% identity to "*Ca*. P. fraxini" strain ASHY-7 (HQ589190) (subgroup 16SrVII-A). Incidence of CP-related phytoplasmas in SYDP samples is four times more than AY related samples.

The obtained aligned sequences of the above phytoplasma cluster with 22 phytoplasmas were classified into 16SrVI-A and 16SrVII-A subgroups (Figure 4). The tree with the highest log

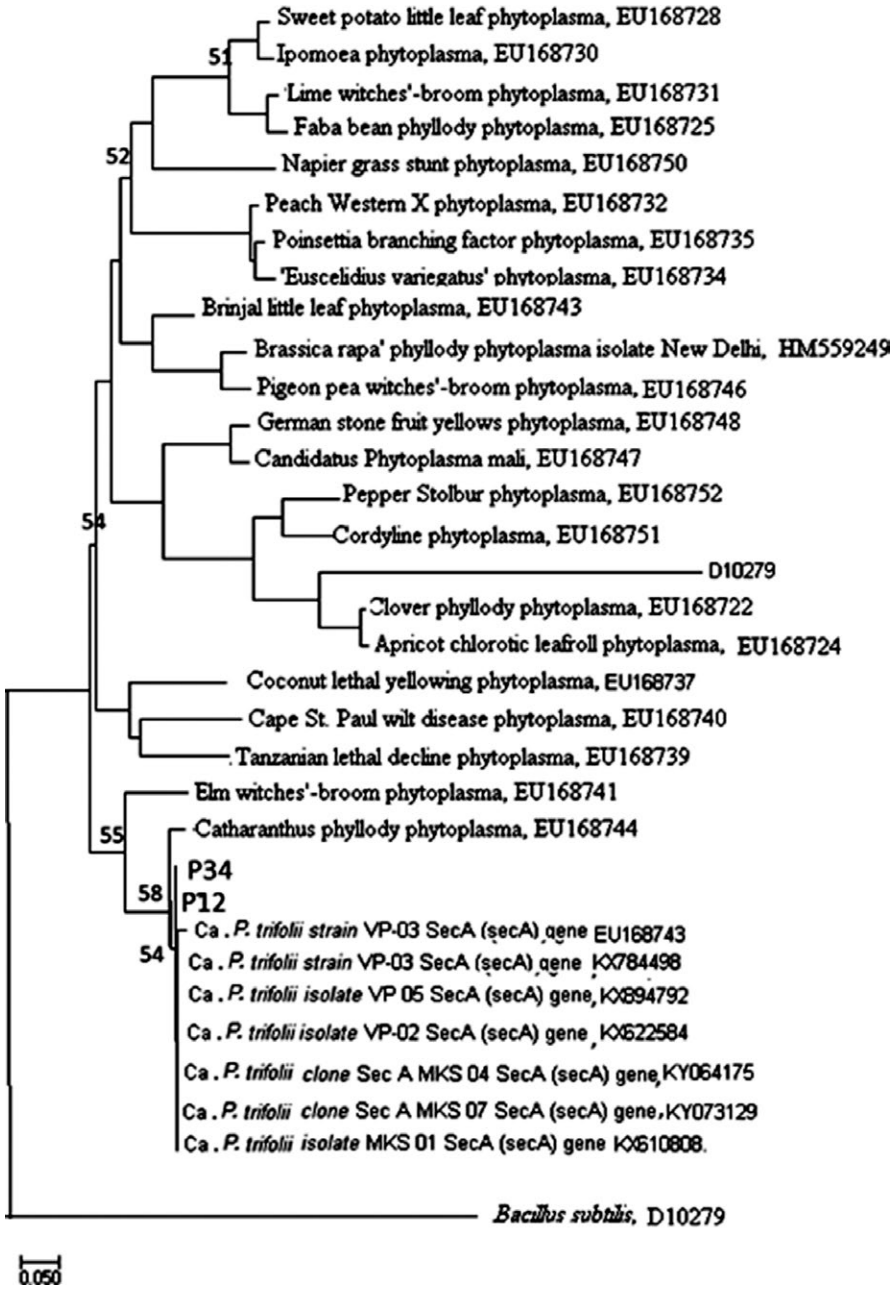

FIGURE 5 The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). Bootstrap values are shown on the branches

likelihood (−4,560.15) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 31 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1,038 positions in the final data set. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, & Tamura, 2016). Inside the group VI branch, the five SYDP phytoplasma strains identified in this study were clustered along with the previously characterized "*Ca*. P. trifolii" (AY390261), and inside the group VI branch, the two SYDP phytoplasma strains (P2 and P8) identified in this study were clustered along with the previously characterized "*Ca*. P. fraxini" (JQ868445).

*Sec*A phylogeny analysis of CP-positive samples was clustered along with the previously characterized "*Ca*. P. trifolii" (EU168743) (Figure 5). The tree with the highest log likelihood (−5,621.21) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 33 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 435 positions in the final data set. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

4 | DISCUSSION

The presence of SYDP phytoplasma is well correlated with the development of streak yellows syndrome in Iran date palm. The appearance of the disease is similar to that reported as palm yellowing (Cronjé et al., 2000; Al-Awadhi et al., 2002; Harrison, Helmick, & Elliott, 2008, Harrison, Helmick, & Elliott, 2009). However, certain symptoms reported to be diagnostic for palm yellowing, namely, streak yellowing of leaves were expressed with variable incidence and severity in Iran date palm growing areas in Khuzestan, Bushehr and Kerman Provinces.

Until now, phytoplasmas of the 16SrIV, 16SrI, 16SrII and 16SrXIV have been reported with diseases in date palm such as white tip die-back, slow decline and yellowing in America, North Africa and Kuwait, respectively (Al-Awadhi et al., 2002; Cronjé et al., 2000; Harrison, Womack, & Carpio, 2002). The RFLP and DNA sequencing data indicate that SYDP phytoplasma is most closely related to CP and AY phytoplasma, the members of the taxonomic groups 16SrVI and 16SrVII that to best of our knowledge it is first time a phytoplasma from these groups reported from date palm in the world.

Streak yellow date palm phytoplasma was present in low titre in our study, and positive samples were detected just in nested PCR stage. Seasonal and spatial variation in phytoplasma distribution and titre is known to exist in trees and other perennial hosts (Lee, Gundersen, Hammond, & Davis, 1994). An improved understanding of this variation and variation associated with different tissues in date palm would assist in phytoplasma detection efforts.

The predominant phytoplasma DNA amplified was that of CP-related strains. The importance of mixed infections in this disease is unknown, but they occur commonly in other phytoplasma diseases (Lee et al., 1994). Typically, although not always, the predominant phytoplasma is the primary pathogen. Still, low titre strains may influence disease expression, especially in perennial hosts (Lee et al., 1994).

The CP phytoplasma is considered a pathogen of herbaceous plants, but it has reported from Salix (M. Ghayeb Zamharir, *unpublished data*) in Iran. The host range is presumed to be similar to that of vector-transmitted agent (Golino, Oldfield, & Gumpf, 1989). We did not identify to species most of the insects collected on sticky cards, but some of those assayed were carriers of CP-related phytoplasma.

Discovering that the phytoplasma in symptomatic date palm is related to CP phytoplasma suggests that vectors of the CP-related phytoplasma may also be vectors of AY related phytoplasma. Although speculative, it is possible that the presence of leafhoppers in large numbers in Khuzestan, Bushehr and Kerman Provinces during these years resulted in the current outbreak of SYDP, and studies continue to assess the infectivity of local leafhopper populations and the efficacy of using systemic insecticides to deter potential vectors from feeding and spreading this disease.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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How to cite this article: Ghayeb Zamharir M, Eslahi MR. Molecular study of two distinct phytoplasma species associated with streak yellows of date palm in Iran. *J Phytopathol*. 2019;167:19–25. https://doi.org/10.1111/jph.12769