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Molecular characteristics of a 16SrII subgroup C phytoplasma strain associated with camelthorn witches'-broom disease in Iran

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Abstract

Camelthorn (*Alhagi* spp.) is an ecologically significant perennial weed in Iran. A recent survey indicated a potential phytoplasma-induced disease affecting its populations. Since 2017, symptoms such as the little leaf, yellowing, internode shortening, witches'-broom, and stunting have been noted in camelthorn plants in Chahgeer (Abarkooh, Yazd Province). To identify the causal agent, total DNA was extracted from symptomatic and asymptomatic plants and analyzed using nested PCR with P1/P7 and R16F2n/R16R2 primer pairs. Phytoplasma DNA was detected exclusively in symptomatic plants. Sequence analysis confirmed a high degree of identity with previously characterized 16SrII-C subgroup phytoplasma strains. Additionally, simulated RFLP, pairwise homology, and phylogenetic analyses further validated its classification within the 16SrII-C subgroup. The identified phytoplasma is closely related to pathogens causing witches' broom disease in regional crops, including *Prunus armeniaca*, *Daucus sativa*, *Prunus persica*, and *Medicago sativa*, suggesting its possible role in disease epidemiology. This study provides the first molecular evidence linking camelthorn witches'-broom to 16SrII-C phytoplasma, highlighting its significance in the spread of phytoplasma-associated diseases in the region.

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Introduction

Phytoplasmas are bacteria that lack cell wall and cause significant economic losses in a wide range of plant species worldwide. The primary symptoms of phytoplasma infections include phyllody, virescence, witches'-broom, abnormal proliferation of shoots and roots, leaf yellowing or reddening, reduced leaf size, smaller fruit, overall decline, and stunting (Bertaccini et al., 2014). These pathogens are transmitted by phloem-feeding insects such as leafhoppers, plant hoppers, and psyllids that play a crucial role in their epidemiology (Weintraub & Beanland, 2006). Currently, phytoplasmas are classified within the provisional genus *Candidatus* Phytoplasma and organized into various ribosomal groups and subgroups based primarily on 16S rRNA gene sequence analysis. To date, 53 *Candidatus* Phytoplasma species have been identified through 16S rRNA gene sequencing (IRPCM, 2004; Bertaccini et al., 2014, 2022; Rodrigues Jardim et al., 2023). Camelthorn (*Alhagi maurorum*, *Fabaceae*) is a significant perennial weed in Iran that is traditionally used as an herbal medicine and has recently been cultivated as a forage crop. In Iran, short reports have documented the association of phytoplasma strains from the 16SrII-C and 16SrXII-A subgroups with the camelthorn plant (Esmaeilzadeh-Hosseini & Salehi, 2021; Salehi et al., 2023). Given its wide distribution in fields and gardens and the expansion of its cultivation, investigating the molecular characteristics of these phytoplasma strains is essential, particularly considering the occurrence of similar strains in fruit trees and other crops.

Materials and Methods

Source of the disease

Twenty camelthorn plants, in the pastures of Chahgeer (Abarkooh, Yazd Province), exhibiting similar symptoms indicative of phytoplasma infection, were selected for DNA extraction, PCR, cloning and molecular analyses including phylogenetic and virtual RFLP analyses.

DNA extraction and polymerase chain reaction (PCR)

Total DNA was extracted from 0.2 g tissues of symptomatic camelthorn using the method described by Zhang et al. (1988). Total DNA extracted from five asymptomatic and a symptomatic periwinkle plant infected with parsley witches'-broom phytoplasma, a 16SrII-D strain (Salehi et al., 2016) were used as negative and positive controls, respectively. DNA samples were tested for phytoplasma presence by direct PCR using P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995) and nested PCR using R16F2n/R16R2 primer pair (Gundersen & Lee, 1996). PCR was performed in a 25 µl reaction volume containing 50 ng DNA, 0.4 µM of each primer, 0.25 mM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 units of *Taq* DNA polymerase (CinnaGen, Iran) in the buffer supplied by the manufacturer. Amplification was carried out in a thermal cycler (Bio-Rad T100, USA) for 35 cycles as follows: 45 sec denaturation at 94°C (3 min for the first cycle), 45 sec annealing at 55°C and 2 min of extension at 72°C. In the final cycle the extension step was extended to 10 min. PCR conditions for the nested PCR were the same except that the annealing temperature was 58°C. PCR products were separated in 1% agarose gels in 1X TBE buffer (108 g Tris-HCl, 55 g boric acid, 40 ml EDTA 0.5 M, pH 8.0). DNA bands were stained with ethidium bromide and visualized with a UV transilluminator. The molecular weight of the PCR products was estimated by comparison with 100 bp. DNA ladder (Fermentas, Vilnius, Lithuania).

Cloning and DNA sequencing of PCR products

Six R16F2n/R16R2 and four P1/P7 primed PCR products from naturally infected camelthorn plants were used for cloning and sequencing. These amplicons were ligated into the pTZ57R/T vector and cloned into *Escherichia coli* DH5α using the Ins T⁄A clone PCR Product Cloning Kit (Sinaclone, Tehran, Iran) according to the manufacturer's instructions. The presence of the correct insert was confirmed with *Eco*RI and *Pst*I restriction endonucleases. Plasmid DNA from recombinant colonies was purified using the GF-1 PCR Clean-Up Kit (Vivantis, Malaysia, HQ). Sequencing was performed by Macrogen (South Korea) in both directions using forward and reverse M13 primers. R16F2n/R16R2 primed rDNA product and SR region were selected for further analysis. The representative of phytoplasma sequences was then submitted to NCBI GenBank.

Sequence homology and phylogenetic analyses

The obtained partial 16S rDNA sequence from six amplicons of the 16S rRNA gene from naturally infected camelthorn plants was compared with 16S rDNA sequences of phytoplasmas in GenBank using BLASTn from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). F2nR2 16S rDNA sequences of 22 phytoplasmas, including camelthorn witches'-broom phytoplasma strain were aligned using the Clustal W program and a phylogenetic tree was constructed using the neighbor-joining plot option of MEGA7 software (Kumar et al., 2016). *Acholeplasma laidlawii* was used as an outgroup to root the tree. Bootstrapping was performed 100 times to estimate the stability and support for the branches.

Virtual RFLP analysis of sequences

Virtual RFLP analysis using *i*PhyClassifier (Zhao et al., 2009) was used to determine subgroup affiliation of camelthorn strains. RFLP profile of 1,246 bp R16F2n/R16R2 primed sequences of camelthorn phytoplasma (GenBank accession number: MT248271) was compared to those of 16SrII-subgroup phytoplasmas. Each aligned DNA fragment was digested *in silico* with the 17 restriction enzymes witch have been used for the phytoplasma 16S rDNA RFLP analysis (Lee et al., 1998).

Results

Symptomatology of sources of phytoplasmas employed in this study

Characteristic symptoms of camelthorn plants grown in 500 hectares of pastures of Chahgeer (Abarkooh, Yazd Province) were: little leaf, yellowing, internode shortening, witches'-broom and stunting (Fig. 1). The disease incidence was up to 2%.

Fig. 1. Little leaf, yellowing, internode shortening, witches'-broom and stunting observed in camelthorn plants (A) compared to a symptomless one (B) in Chahgeer (Abarkooh, Yazd Province).

Polymerase chain reaction and nucleotide sequence analyses

DNA fragments of approximately 1800 and 1246 bp were amplified in direct and nested PCR respectively, from 16 symptomatic plants and positive control. No amplification was observed in DNA samples from symptomless plants in direct and nested PCR assays. All R16F2n/R16R2 primed PCR products showed similar RFLP patterns comparable with 16SrII-C subgroup strains (Lee et al., 1998) after restriction with *Alu*I, *Hha*I, *Hin*fI, *Hpa*II, *Mse*I and *Rsa*I enzymes (Data not shown). The obtained 16S rDNA nucleotide sequences (R16F2n/R2 fragment, 1246 bp.), amplified from symptomatic plants were identical to each other (sequence identity 100%) and one 1246 bp DNA fragment was submitted to GenBank data base under accession number MT248271**.** The BLAST search showed that this strain had 100 % identity with alfalfa, carrot and peach witches'-broom, members of the peanut witches'-broom phytoplasma group (16SrII). Phylogenetic analysis using the neighbour-joining method (MEGA software version 7.0) confirmed that the camelthorn phytoplasma clustered within the 16SrII phytoplasma group clade, most closely related to the faba bean phyllody (X83432) representative of subgroup 16SrII-C (Fig. 2). Pairwise homology (%) of 16S rRNA gene sequences and 16S-23S intergenic spacer region (SR) between alfalfa witches'-broom Chahgeer (CAWB) and camelthorn (*Alhagi maurorum*) phytoplasma strain (ALWB) showed 100% homology (Tables 1 and 2).

Virtual RFLP analysis

After virtual digestion, the RFLP pattern of the 1246 bp 16S rDNA of camelthorn phytoplasma strain (ALWB), was similar to the reference pattern of the 16Sr group II, subgroup C (GenBank accession number: AJ293216), with a pattern similarity coefficient of 0.99. The phytoplasma under study was a variant of 16SrII-C (Fig. 3).

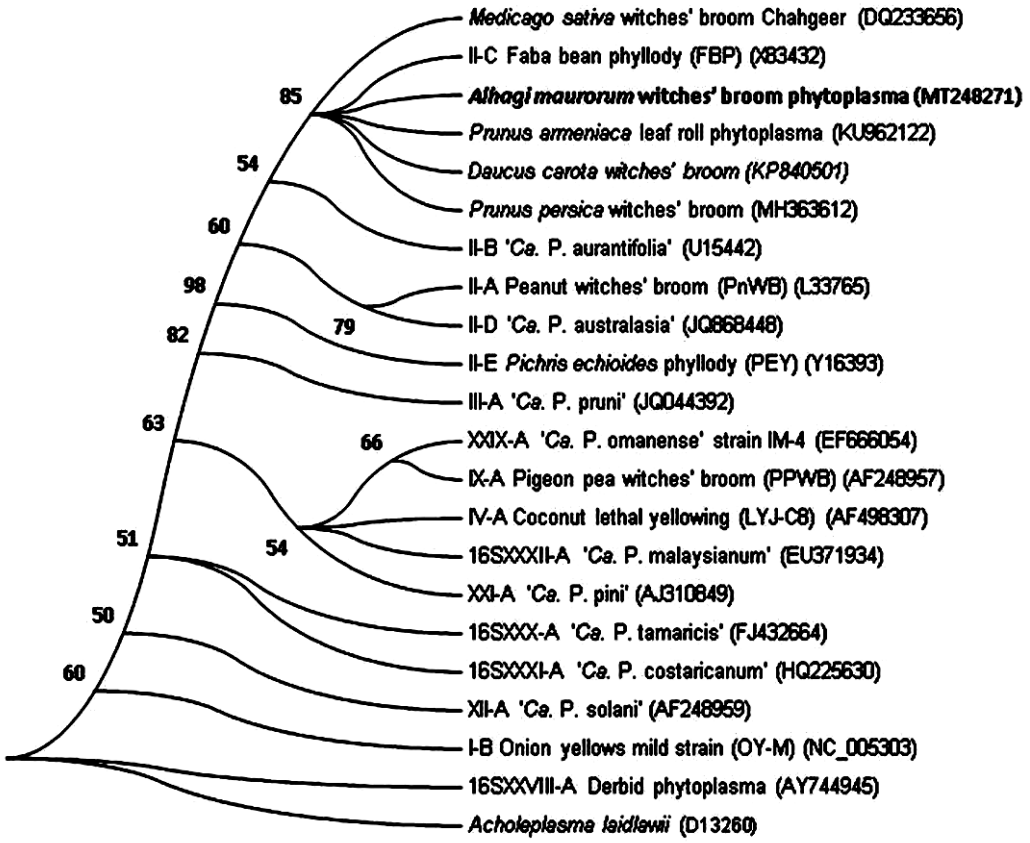

Fig. 2. Phylogenetic tree constructed by the Neighbor-Joining method of 1,246 bp of *16S rRNA* gene sequences from 22 phytoplasmas and *Acholeplasma laidlawii*, as outgroup. *'Ca.* P.': '*Candidatus* Phytoplasma'. Ribosomal grouping is indicated (16Sr) before phytoplasma name or acronym. GenBank accession numbers for sequences are given in parentheses after the phytoplasma acronyms. Numbers at the nodes are bootstrap (confidence) values based on 1,000 repetitions (only values above 40 are shown).

Table 1. Pairwise homology (%) among CAWB and AlWB and phytoplasmas in group 16SrII using 16S rRNA gene sequences.

	CAWB	**AlWB**	**CWBP YN20**	**CWBP YN01**	**FBP**	**'*Ca*. P. aurantifolia'**	**PnWB**	**'*Ca*. P. australasia'**	**'*Ca*. P. asteris'**
CAWB	100								
AlWB	100	100							
CWB YN20	100	100	100						
CWBP YN01	100	100	100	100					
FBP	100	100	100	100	100				
'*Ca*. P. aurantifolia'	99.4	99.4	99.4	99.4	99.4	100			
PnWB	98.4	98.4	98.4	98.4	98.4	98.5	100		
'*Ca*. P. australasia'	98.5	98.5	98.5	98.5	98.5	98.7	99.8	100	
'*Ca*. P. asteris'	90.4	90.4	90.4	90.4	90.4	90.7	90.3	90.3	100

Abbreviations: CAWB: alfalfa witches'-broom Chahgeer; AlWB*: Alhagi maurorum* witches'-broom; CWB: Cactus witches'-broom; FBP: faba bean phyllody; PnWB: peanut witches'-broom.

Table 2. Pairwise homology (%) among 16S-23S intergenic spacer region (SR) of CAWB (KF607107), AlWB (PV704771) and phytoplasmas in group 16SrII.

	AlWB	**CAWB**	**FBP**	**ShP**	**'*Ca*. P. australasia'**	**'*Ca*. P. aurantifolia'**	**'*Ca*. P. trifolii'**
AlWB	100						
CAWB	100	100					
FBP	99.6	99.6	100				
ShP	98.8	98.8	98.4	100			
'*Ca*. P. australasia'	98.8	98.8	98.4	100	100		
'*Ca*. P. aurantifolia'	98.4	98.4	97.9	97.1	97.1	100	
'*Ca*. P. trifolii'	80.2	80.2	80.2	79.8	79.8	79.4	100

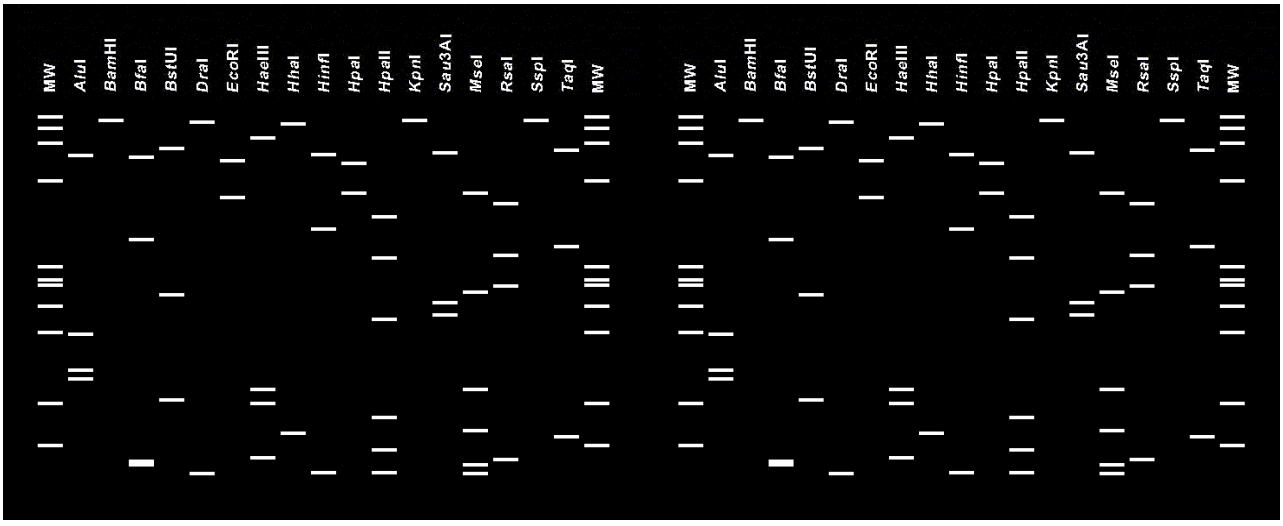

Fig. 3. Virtual RFLP profiles using *i*PhyClassifier tool. Left**,** camelthorn witches'-broom phytoplasma strain and right, reference pattern of the 16Sr group II, subgroup C (GenBank accession number: AJ293216).

Discussion

Based on disease symptoms and PCR using specific primers, camelthorn witches'-broom symptoms are caused by a phytoplasma, and based on molecular studies, the associated phytoplasma is a phytoplasma from 16SrII subgroup C. In Yazd Province, a type of little leaf and witches'-broom in camelthorn has been observed (Fig. 4), which based on PCR, is not caused by phytoplasmas and is probably caused by mites or other agents. DNA fragments of approximately 1800 and 1246 bp were amplified in direct and nested PCR respectively, from 16 symptomatic plants and no amplification was observed in DNA samples from 4 samples with little leaf and witches'-broom symptoms (Fig. 4).

Previously, carrot witches'-broom, alfalfa witches'-broom, apricot leaf twist, plum decline and peach witches'-broom due to 16SrII subgroup C have been identified in Yazd Province and camelthorn witches'-broom phytoplasma may role in epidemiology of these phytoplasma diseases. Among them, alfalfa witches'-broom (AWB) is very important and distributed all over the country especially in central, southern and east parts of Iran, and its natural transmission by *Orosius albicinctus* and *Neoaliturus haematoceps* was confirmed (Salehi et al., 1993a, 1995; Esmailzadeh Hosseini et al., 2015a, b).

Fig. 4. Little leaf and witches'-broom in camelthorn occur without infection by phytoplasma disease.

16SrII subgroup C were predominant strains in destructive epidemics of AWB in Chahgeer (Abarkooh, Yazd Province) where the AWB disease reduced alfalfa production to less than one-third, alfalfa plantations were destroyed completely, and farmers switched on to other crops (Esmailzadeh Hosseini et al., 2015c). As this plant is widely distributed in fields and gardens as an important weed, and also its cultivation is also expanding, management against the camelthorn plant is necessary.

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Conflict of interest

The authors declare that there are no conflicts of interest present.

CRediT author statement

S. A. Esmaeilzadeh-Hosseini: Obtaining resources, conducting the project, writing the manuscript. **M. Salehi:** Methodology, Data curation, Review, and Editing.

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