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ORIGINAL RESEARCH ARTICLE

New alternative hosts of '*Candidatus* Phytoplasma australasia' strains in the warm climate of Hormozgan province, southern Iran

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Abstract

Hormozgan province in the south of Iran is one of the main regions in producing tomatoes, eggplants, bell peppers, and other vegetables as well as sesame in the winter season. In a 2021–2022 survey for phytoplasmas in different areas of this province, phytoplasma–type symptoms were observed in tomato, bell pepper, eggplant and sesame crops and many weed plants including *Chenopodium album*, *Taraxacum officinale*, *Erodium cicutarium*, *Physalis angulata, Convolvulus virgatus*, *Tephrosia apollinea*, and *Malva sylvestris*. Nested PCR assays using primers P1/P7 followed by R16mF2/R16mR1 confirmed association of phytoplasma with all symptomatic plants. *16 S rRNA* nucleotide sequencing followed by virtual RFLP analysis showed that all detected phytoplasma strains from different hosts and locations belonged to 16SrII-D subgroup, '*Candidatus* Phytoplasma australasia'. The latter seemed to be the dominant phytoplasma among herbaceous plants in the region. To our knowledge, this is the first world report of natural infection of *T. officinale*, *E. cicutarium*, *P. angulata, C. virgatus*, *T. apollinea*, and *M. sylvestris* by a 16SrII-D phytoplasma. These plants can act as alternative hosts for transmission of the phytoplasma strains to major agricultural plants including tomato, pepper, eggplant, sesame and probably other plants.

Keywords 16SrII · Weed · *Convolvulus* · *Erodium* · *Taraxacum* · *Tephrosia*

Introduction

Vegetables and oilseed plants are short-term crops that grow in different seasons of a year and have high economic returns. Hormozgan province in the south of Iran is one of the main regions in producing tomatoes, eggplants, bell peppers, and other vegetable crops as well as sesame in the winter season. However, the production of these important crops is always under the threat of various pathogens. Phytoplasmas are among the serious pathogens in tomato, eggplant, bell pepper and sesame fields. These pathogens have great importance in the world due to their severe economic damage to herbaceous and woody plants (Chaturvedi et al. 2010). Phytoplasmas are limited to the phloem cells of host plants and are transmitted in nature by phloem feeding insects from the order Hemiptera. They also survive in vectors and perennial plants (Christensen et al. 2005).

Association of phytoplasma strains from five ribosomal groups with crop plants and weeds have been previously reported from Hormozgan province, Iran (Table 1). As shown in Table 1, among different ribosomal groups, members of 16SrII phytoplasma group are the most prevalent in Hormozgan province. In the 16SrII group, members of the subgroup 16SrII-B are important in citrus plantations (e.g., witches'-broom disease of lime), whereas members of 16SrII-D subgroup mainly infect vegetables.

Candidatus Phytoplasma australasia (16SrII-D subgroup) strains are economically important disease agents in the cultivation of vegetables and oilseed crops in southern Iran (Salehi et al. 2014, 2015a, b, 2016, 2017, 2021; Faghihi et al. 2016; Amiri Mazraie et al. 2018a). They infect important crop plants of the Solanaceae family, including tomato,

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Table 1 Phytoplasma groups associated with different plant hosts in Hormozgan province, IRAN

Plant host	Location	Group-subgroup	Reference
Citrus × aurantiifolia	Minab, Roodan, Bandar Abbas and Haji Abad	16SrII-B	Salehi et al. 2002
Citrus reticulata hybrid	Ghale-Ghazi (Bandar Abbas)	16SrII-B	Djavaheri and Rahimian 2004
Citrofortunella × floridana	Hasht Bandi (Minab)	16SrII-B	Faghihi et al. 2017
Citrus × paradisi	Roodan	16SrII-B	Bagheri et al. 2010
Solanum melongena	Bandar Abbas, Minab and Roodan	16SrII-D, 16SrIX	Tohidi et al. 2015; Amiri Mazraie et al. 2018a; Salehi et al. 2021
Solanum lycopersicum	Bandar Abbas and Bandar Khamir	16SrII-D, 16SrVI	Amiri Mazraie et al. 2018a
Cucumis sativus	Bandar Abbas	16SrII-D	Amiri Mazraie et al. 2018a
Vicia faba	Minab	16SrII-D	Amiri Mazraie et al. 2018a
Capsicum annuum	Bandar Abbas	16SrII-D	Faghihi et al. 2016; Amiri Mazraie et al. 2018a
Brassica napus	Bandar Abbas	16SrVI-A	Amiri Mazraie et al. 2018b
Solanum nigrum	Minab	16SrII-B	Samavi et al. 2012
Solanum surattense	Minab	16SrII-B	Samavi et al. 2012
Manilkara zapota	Bandar Abbas	16SrII-B	Bagheri et al. 2017
Cosmos bipinnatus	Bandar Abbas	16SrII-D	Nikooei et al. 2017
Artemisia sieberi	Bandar Abbas	16SrII-D	Hemmati & Nikooei 2019a
Petunia violacea	Bandar Abbas	16SrII-D	Hemmati et al. 2019b
Aerva javanica	Minab	16SrII	Hemmati et al. 2019c
Zinnia elegans	Bandar Abbas	16SrII	Hemmati and Nikooei 2017
Suaeda, aegyptiaca	Hassanlangi (Bandar Abbas)	16SrVI	Askari et al. 2017
Bidens alba	Seyahoo (Bandar Abbas)	16SrIX	Hemmati et al. 2017
Convolvulus glomeratus	Bandar Abbas	16SrIX-J	Nikooei and Hemmati 2018
Conocarpus erectus	Bandar Abbas	16SrIX-A	Hemmati et al. 2021a
Cynodon dactylon	Minab	16SrXIV	Salehi et al. 2009
Periploca aphylla	Seyahoo	16SrX	Faghihi et al. 2010

bell pepper and eggplant (Faghihi et al. 2016; Amiri Mazraie et al. 2018a; Salehi et al. 2021). Infected plants show typical phytoplasma-symptoms including stunting, witches'-broom, big buds, phyllody, virescence, little leaf and yellowing (Faghihi et al. 2016; Amiri Mazraie et al. 2018a; Salehi et al. 2021).

Weeds and wild plants as alternative hosts of phytoplasmas play an important role in the epidemiology and outbreak of phytoplasma diseases (Duduk et al. 2018). Since weeds often grow abundantly in or around fields even in the absence of crops, they can serve as reservoirs for transmission of phytoplasmas to the agricultural plants (Mall et al. 2010). Therefore, in managing phytoplasmal diseases, the alternative hosts and insect vectors are among the critical issues that should be considered. A recent survey of vegetable and sesame fields in Hormozgan province showed a wide range of phytoplasma symptoms in these crops as well as in the weeds in and around the plantations. The present study was conducted to identify crop and wild plant hosts of '*Ca*. P. australasia' (16SrII-D) in this province.

Materials and methods

Sample collection

During October 2021 to April 2022, a regular fortnight survey was carried out to determine phytoplasma diseases in the vegetables (tomato, bell pepper and eggplant) and sesame fields in Hormozgan province (southern Iran). In addition, to identify the alternative hosts of phytoplasmas the weeds inside and around the fields showing symptoms similar to those induced by phytoplasma infection, including witches'-broom, phyllody, virescence, stunting, shortened internodes, yellowing and little leaf as well as several symptomless plants were sampled in the Bandar Abbas, Haji Abad and Bandar Khamir areas in Hormozgan province (Table 2). Depending on the number of infected plants observed, 1–3 samples with and without symptoms were collected from each area and tested by PCR for the presence of phytoplasma. Totally 26 symptomatic samples of sesame (*Sesamum indicum*), tomato (*Solanum lycopersicum*), bell pepper (*Capsicum annuum*), eggplant (*Solanum melongena*), *Chenopodium album*, *Taraxacum officinale*,

Table 2 List of the collected plants and the sampling characteristics

Plant host	Plant family	Sampling location	Sampling date	Number of samples	Sampling field
Sesamum indicum	Pedaliaceae	Shahdadi (Haji Abad)	October 2021	3	Sesame
Physalis angulata	Solanaceae	Shahdadi (Haji Abad)	October 2021	2	Sesame
Solanum lycopersicum	Solanaceae	Kahourestan (Bandar Khamir) Rezvan (Bandar Abbas) Shamil (Haji Abad)	December 2021 February 2021 January 2022	5	Tomato (open field and greenhouse)
Chenopodium album	Chenopodiaceae	Kahourestan (Bandar Khamir)	December 2021	1	Tomato
Capsicum annuum	Solanaceae	Rezvan ((Bandar Abbas) Sarkhon (Bandar Abbas)	February 2021 January 2022	4	Bell pepper
Convolvulus virgatus	Convolvulaceae	Sarkhoun (Bandar Abbas)	December 2021	2	Pepper [a]
Taraxacum officinale	Asteraceae	Sarkhoun (Bandar Abbas)	February 2022	1	Bell pepper
Tephrosia apollinea	Fabaceae	Sarkhoun (Bandar Abbas)	March 2022	2	Pepper [b]
Erodium cicutarium	Geraniaceae	Sarkhoun (Bandar Abbas)	April 2022	1	Bell pepper
Malva sylvestris	Malvaceae	Sarkhoun (Bandar Abbas)	April 2022	2	Bell pepper
Solanum melongena	Solanaceae	Rezvan (Bandar Abbas)	March 2021	3	Eggplant

[a], around; [b], in and around

Erodium cicutarium, *Physalis angulata, Convolvulus virgatus*, *Tephrosia apollinea*, and *Malva sylvestris* were collected and analyzed for phytoplasma presence. In addition, three symptomless plant of each species were collected and used as negative control.

DNA extraction and PCR amplification

To test possible association of phytoplasma with the symptomatic plants, total DNA was extracted from midribs of symptomatic and asymptomatic plants using modified cetyltrimethylammonium bromide (CTAB) method (Zhang et al. 1998). To do so, 0.1–0.2 g of leaf midrib was powdered in liquid nitrogen, transferred to 1.5-ml micro-tubes, and 700 µl CTAB buffer (2% CTAB, 20 mM EDTA, 100 mM Tris-HCl, 1.4 M NaCl, pH: 8, and 0.2% 2-mercaptoethanol) was added. The entire mixture was held in water bath at 65 °C for 30 min. During the incubation, the mixture was briefly inverted every 10 min. After the incubation, 600 µl of chloroform/isoamyl alcohol (24:1) was added, the mixture was vigorously vortexed and centrifuged at 12,000 rpm for 12 min. The supernatant was transferred to a clean 1.5 ml microtube. DNA was precipitated by adding equal volume of ice-cold isopropanol and the mixture was incubated in ice for 20 min, then centrifuged for 12 min at 13,000 rpm after which the supernatant phase was discarded. The pellet was rinsed twice with 300 µl of 80% ethanol (centrifuged for 5 min at 6,000 rpm), air-dried, and dissolved in 100 µl of DPCE water.

The samples were analyzed for phytoplasma DNA by direct or nested PCR using the phytoplasma universal primer pairs P1/P7 (Schneider et al. 1995) and Rl6mF2/R16mR1 (Deng and Hiruki 1991; Gundersen and Lee 1996). The PCR reaction in a 35-µl volume consisted of 2 µl of DNA template, 1 µl (10 µM) of each primer, 18 µl of 2x Taq DNA polymerase Master Mix RED (Ampliqon, Denmark) and 13 µl of sterile distilled water. The standard amplification protocol was 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, annealing temperature for 45 s and 72 °C for 90 s and final extension at 72 °C for 10 min. All reactions were performed in triplicate and each run contained one sterile distilled water as negative control. DNA samples from periwinkle plants infected with a 16SrVI-A phytoplasma strain (accession no. MH430092) were used as positive controls. The PCR products were separated by electrophoresis in a 1% agarose gel containing commercial safe stain (SinaClon, Iran) and visualized under UV light.

Sequencing, phylogenetic and virtual RFLP analyses

The amplicons of the expected size (1434 bp) by Rl6mF2/R16mR1 primer pair were sequenced in both directions (Codon genetic group, IRAN). The sequences were edited and assembled using Geneious Prime Software (version 2019). The obtained partial 16 S rDNA sequences were analyzed by BLAST (http://www.ncbi.nlm.nih.gov/blast).

To identify and classify the phytoplasma strains, the obtained *16 S rRNA* gene sequences were subjected to computer simulated virtual RFLP analysis using the online tool, *i*PhyClassifier with17 key restriction enzymes: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *EcoR*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau3*AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I (Zhao et al. 2009). The phylogenetic relationship of phytoplasma strains under study and those of reference from GenBank were analyzed using the Neighbor-Joining method in MEGA X (Kumar et al. 2018). Multiple sequence alignment was performed using ClustalW option in MEGA X software (Kumar et al. 2018).

Result

Symptomatology

Typical phytoplasma symptoms were observed in various fields and greenhouses of tomato, eggplant, pepper and sesame in Hormozgan province (Table 2). Generally, the most important abnormalities in tomato, pepper and eggplant were big bud, witches'-broom and phyllody, respectively. The symptoms in tomato plants included yellowing, upright branches, big bud, abnormal flowers with large and clumped sepals, deformation of flowers, and virescence (Fig. 1a-e). Additionally, greenhouse tomato variety Dafnis F1 (Syngenta, Switzerland) showed small, asymmetric, hardened seedless fruits and adventitious leaf-like structures on the stems (Fig. 1f-g). Symptoms in pepper and eggplant were similar, including yellowing, witches'-broom, virescence, phyllody and little leaf (Fig. 1h-o). The main disease symptoms in sesame plants were yellowing, virescence, phyllody and witches'-broom (Fig. 1p-r).

At harvesting time, percent incidence of symptomatic plants in field tomato, bell pepper, eggplant, sesame and greenhouse tomato was approximately up to 30, 10, 20, 90 and 0.1, respectively.

Symptoms of witches'-broom, plant stunting, internode shortening, yellowing and little leaf were observed in *Convolvulus virgatus* in and near infected pepper fields (Fig. 2a-c). Furthermore, phytoplasma symptoms were observed in four other weeds, including *Malva sylvestris* (Great mallow), *Tephrosia apollinea*, *Erodium cicutarium*

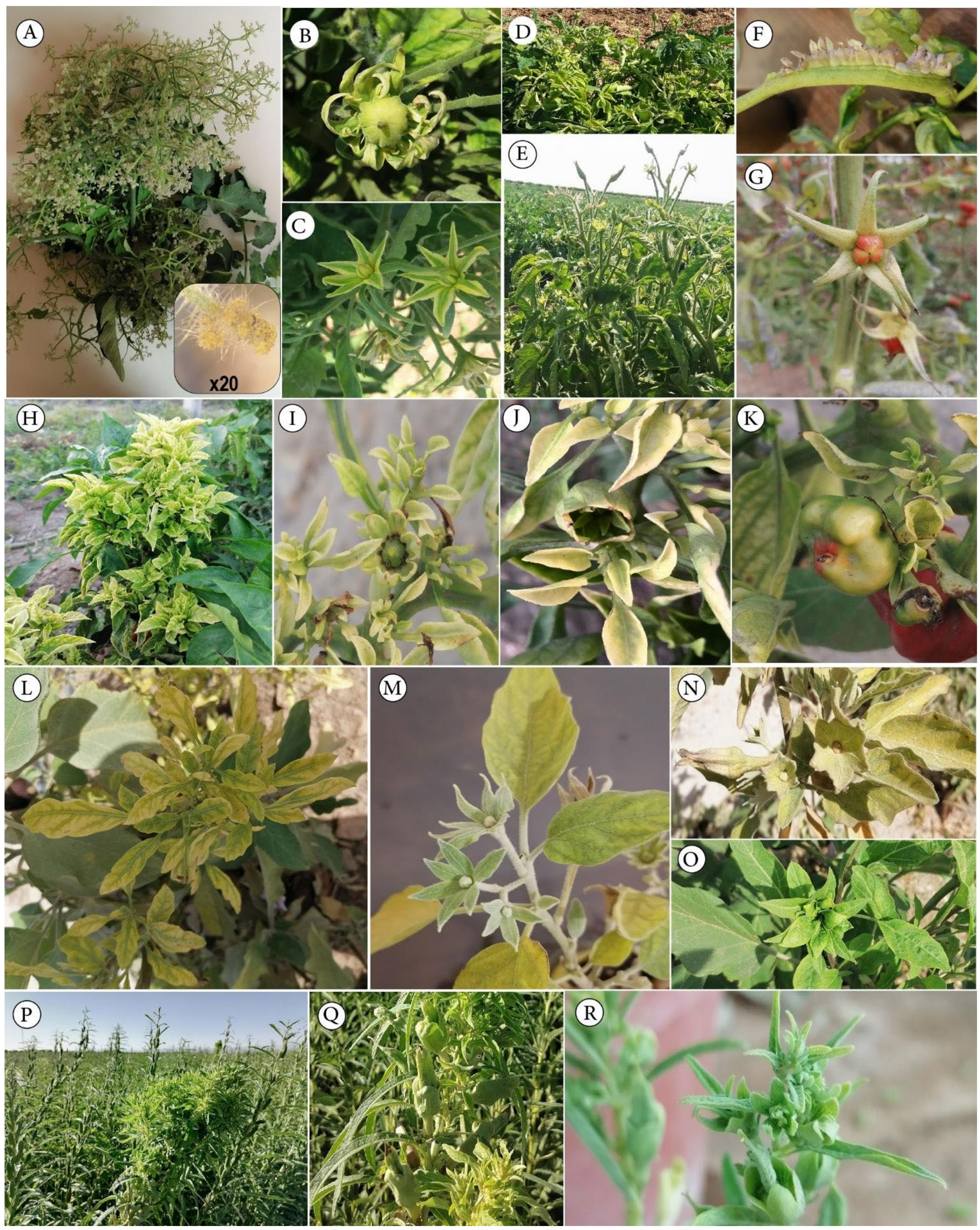

Fig. 1 Symptoms in 16Sr-II-D phytoplasma infected tomato, bell pepper, eggplant and sesame in Hormozgan province, Iran. **A-G**: **(A)** deformation of flowers, **(B)** big buds, **(C)** virescence, **(D)** yellowing and **(E)** upright branches in field tomatoes; **(F)** adventitious leaf-like structures on the stem, **(G)** small, asymmetric, hardened and seedless fruits in greenhouse tomato variety Dafnis F1. **H-K**: **(H)** witches'-broom, **(I)** yellowing, little leaf and virescence, **(J)** phyllody with clumped sepals, and **K**) asymmetric hardened fruits in bell pepper. **L-O**: **L**) yellowing and little leaf and **M-O**) virescence and phyllody in eggplant. **P-R**: **P**) witches'-broom, yellowing, little leaf, **Q** and **R**) phyllody and virescence in sesame

Fig. 2 Witches' broom and little leaf **(A** and **B)** in *Convolvulus virgatus* plants; **C**, a healthy *C. virgatus* plant. Little leaf and witches'-broom symptoms in *Tephrosia apollinea* (2 plants on the right) compared with a healthy plant on the left **(D)**. Upright branching **(E)** and floral proliferation and phyllody in infected *Taraxacum officinale* **(E** and **F)** compared to healthy flower (The top part of F). *Erodium cicutarium* showing little leaf and witches'-broom **(G)**. Little leaf and phyllody symptoms on *Malva sylvestris* **(H**-bottom plant and **I)**. Witches'-broom symptom on *C. album* **(J**-right plant) compared to an asymptomatic plant **(J**-left plant). Witches'-broom, little leaf and phyllody in infected *Physalis angulata* **(K** and **L)** and a healthy (left) and an infected (right) *P. angulat* fruit **(M)**

and *Taraxacum officinale* (Dandelion) in different pepper fields. Little leaf and witches'-broom were the prevalent symptoms in *T. apollinea* and *E. cicutarium* plants (Fig. 2d g), whereas in *T. officinale* and *M. sylvestris* phyllody of flowers was dominant (Fig. 2e-f h-i). Additionally, several *Chenopodium album* (white goosefoot) plants with phytoplasma symptoms including witches'-broom and little leaf were observed in the infected tomato fields (Fig. 2j). In the infected sesame fields of Haji Abad, several cutleaf groundcherry (*Physalis angulata*) plants with typical symptoms of phytoplasma diseases including phyllody, witches'-broom and little leaf were observed (Fig. 2k-m).

PCR detection and molecular analyses

Primer pairs P1/P7 and Rl6mF2/R16mR1 amplified DNA fragments of the expected sizes (1.8 kb and 1.4 kb, respectively) in direct and nested PCR assay from all symptomatic plants as well as positive control but not from the healthy-appearing plants and negative controls. The Rl6mF2/R16mR1 products from one symptomatic plant of each region was sequenced in both directions and after edition and assembling, a sequence from each infected plant was deposited in the GenBank database under the following accession numbers (Table 3); ON908536, ON908537 and ON908538 from tomato, ON908539 and ON908540 from pepper, and ON908541, ON908542, OM975640, OM963128, OM963128, ON908528, ON908530, ON908533 and ON908534 from eggplant, sesame, *Convolvulus virgatus*, *Physalis angulate*, *Malva sylvestris*, *Chenopodium album*, *Tephrosia apollinea*, *Erodium cicutarium*, *Taraxacum officinale*, respectively.

BLASTn search using obtained sequences confirmed the phytoplasma infection and showed that *16 S rRNA* nucleotide sequences of the phytoplasma strains associated with all symptomatic plants in the present study had maximum identity with other phytoplasma strains belonging to the 16SrII group (Table 4).

Phylogenetic and virtual RFLP analyses

Multiple alignment and phylogenetic analysis with all previously described species of the '*Ca*. Phytoplasma' genus (Bertaccini et al. 2022) showed that phytoplasma strains associated with tomato big bud (TBB), bell pepper phyllody (BPP), eggplant phyllody (EPP), sesame phyllody (SP), *C. virgatus* witches'-broom (CVWB), *P. angulata* phyllody (PAP), *M. sylvestris* phyllody (MSP), *C. album* witches'-broom (CAWB), *T. apollinea* witches' broom (TAWB), *E. cicutarium* witches'-broom (ECWB), and *T. officinale* phyllody (TOP) diseases sharing > 98.65% sequence identity of nearly full 16S rRNA gene sequence with two reference strains (GenBank accession numbers: JQ868448 and Y10097) of *Candidatus* Phytoplasma australasia' (Table 4; Fig. 3).

The *i*PhyClassifier revealed that the virtual RFLP patterns derived from the *16 S rRNA* gene sequence of TBB1-3, BPP1 and 2, EPP, SP, CVWB, PAP, MSP, CAWB, TAWB, ECWB and TOP phytoplasmas were identical to the reference pattern of 16Sr group II, subgroup D (Fig. 4).

Discussion

Based on disease symptoms and PCR assays using phytoplasma universal primers, TBB, BPP, EPP, SP, CVWB, PAP, MSP, CAWB, TAWB, ECWB and TOP diseases have phytoplasmal etiology. Phylogenetic and virtual RFLP analyses showed that associated phytoplasmas grouped with 16SrII group, subgroup D ('*Candidatus* Phytoplasma australasia'). No other phytoplasmas were found associated with the diseases of vegetable crops during this survey. Therefore, members of this subgroup appear to be the most prevalent cause of phytoplasma diseases of vegetable crops in Hormozgan province. Association of 16SrII-D subgroup phytoplasmas with vegetable diseases have been previously reported from southern provinces of Iran (Salehi et al. 2014, 2021; Faghihi et al. 2016; Amiri Mazraie et al. 2018a; Esmaeilzadeh-Hosseini et al. 2022). Likewise, phytoplasmas of the 16SrII-D subgroup have been detected in tomato crops in Oman, Iraq, Egypt, Saudi Arabia (Hemmati et al. 2021b), India (Singh et al. 2012) and Pakistan (Akhtar et al. 2018). Eggplant and bell pepper are also common hosts of 16SrII-D phytoplasma (*Ca*. P. australasia) and their infection with this phytoplasma strain has been reported from Australia (Tran-Nguyen et al. 2003), Oman and Egypt (Behiry and Bertaccini 2017), India (Martini et al. 2018; Thorat et al. 2017), Iraq (Alkuwaiti et al. 2017), China (Li et al. 2019) and Iran (Faghihi et al. 2016; Salehi et al. 2021). Also, a 16SrII-D phytoplasma is one of the destructive agents in sesame cultivation. The association of different phytoplasma groups including 16SrII group with sesame plants has been reported in Iran (Salehi et al. 2017).

There is little information about the role of weeds and wild plants in the epidemiology of '*Ca.* P. australasia' related diseases. In previous studies, weeds *Cleome viscosa* (Thorat et al. 2016), *Parthenium hysterophorus* (Thorat et al. 2017),

Table 3 Strain designation and accession number of *16SrRNA* phytoplasma sequences from various crop and weed hosts in Hormozgan province, Iran

Plant host	Phytoplasma strain	Accession number	Phytoplasma Subgroup
Tomato (*Solanum lycopersicum*)	TBB1	ON908536	16SrII-D
	TBB2	ON908537	16SrII-D
Greenhouse tomato (*Solanum lycopersicum*)	TBB3	ON908538	16SrII-D
Pepper (*Capsicum annuum*)	BPP1	ON908539	16SrII-D
	BPP2	ON908540	16SrII-D
Eggplant (*Solanum melongena*)	EPP	ON908541	16SrII-D
Sesame(*Sesamum indicum*)	SP	ON908542	16SrII-D
Convolvulus virgatus	CVWB	OM975640	16SrII-D
Physalis angulata	PAP	OM963128	16SrII-D
Malva sylvestris	MSP	OM963128	16SrII-D
Chenopodium album	CAWB	ON908528	16SrII-D
Tephrosia apollinea	TAWB	ON908530	16SrII-D
Erodium cicutarium	ECWB	ON908533	16SrII-D
Taraxacum officinale	TOP	ON908534	16SrII-D

Table 4 Percent 16 S rRNA gene sequence similarity of phytoplasma strains associated with symptomatic plants in the present study with reference strains of members of the '*Candidatus* Phytoplasma' genus

Candidatus Phytoplasma species	Phytoplasma strains* detected in the vegetable and oilseed fields of Hormozgan, Iran													
	CAWB	CVWB	EPP	ECWB	MSP	BPP1	BPP2	PAP	SP	TOP	TAWB	TBB1	TBB2	TBB3
Ca. P. americanum	88.8	88.94	89.55	88.91	89.05	89.54	89.5	88.88	88.76	88.92	88.91	88.96	89.57	88.91
Ca. P. asteris	89.19	89.41	89.74	89.38	89.45	89.81	89.68	89.34	89.15	89.38	89.38	89.24	89.76	89.19
Ca. P. aurantifolia	98.49	98.48	98.47	98.48	98.47	98.43	98.391	98.49	98.5	98.5	98.48	98.44	98.43	98.15
Ca. P. australasia strain PpYC	100	100	100	100	100	99.91	100	100	100	100	100	100	99.91	99.73
Ca. P. australasia strain TBB	100	100	100	100	100	99.91	100	100	100	100	100	100	99.91	99.73
Ca. P. australiense	89.11	89.39	89.38	89.35	89.38	89.36	89.32	89.32	89.11	89.32	89.36	88.76	89.39	88.73
Ca. P. cirsii	90.33	90.41	90.52	90.38	90.31	90.36	90.54	90.41	90.36	90.44	90.38	90	90.39	89.92
Ca. P. cocostanzaniae	90.41	90.64	90.44	90.61	90.54	90.36	90.46	90.63	90.44	90.67	90.61	89.9	90.39	89.83
Ca. P. convolvuli	89.17	89.24	89.55	89.21	89.28	89.53	89.4	89.17	89.13	89.21	89.21	88.95	89.56	88.9
Ca. P. cynodontis	90.41	90.49	90.35	90.46	90.46	90.19	90.29	90.48	90.44	90.52	90.53	89.81	90.21	89.74
Ca. P. fragariae	89.02	89.16	89.46	89.13	89.2	89.45	89.32	89.09	88.98	89.13	89.13	88.85	89.47	88.82
Ca. P. fraxini	89.74	89.97	90.19	89.93	90.16	90.11	90.21	89.97	89.77	90	90.01	89.73	90.13	89.58
Ca. P. hispanicum	89.09	89.24	89.55	89.21	89.35	89.53	89.49	89.17	89.05	89.21	89.28	88.95	89.56	88.9
Ca. P. japonicum	88.94	89.09	89.29	89.05	89.12	89.27	89.23	89.02	88.9	89.06	89.05	88.76	89.3	88.64
Ca. P. luffae	89.88	90.03	90.6	90.08	90.23	90.53	90.54	90.03	89.91	90.07	90.15	90.09	90.56	90
Ca. P. malaysianum	89.07	89.21	89.60	89.18	89.32	89.93	89.54	89.22	89.1	89.26	89.25	89.37	89.96	89.31
Ca. P. mali	88.64	88.86	89.29	88.83	88.82	89.27	89.15	88.87	88.68	88.91	88.9	88.85	89.3	88.82
Ca. P. meliae	89.03	89.17	89.47	89.14	89.28	89.46	89.41	89.1	88.98	89.14	89.21	88.86	89.48	88.83
Ca. P. noviguineense	89.53	89.75	89.78	89.72	89.65	89.87	89.64	89.76	89.56	89.79	89.72	89.21	89.89	89.16
Ca. P. palmae	90.26	90.41	90.6	90.38	90.38	90.53	90.63	90.41	90.29	90.44	90.38	90.09	90.56	90
Ca. P. phoenicium	90.26	90.41	90.27	90.38	90.38	90.1	90.29	90.41	90.29	90.44	90.38	89.72	90.13	89.65
Ca. P. pini	89.97	90.12	89.94	90.09	90.02	89.94	89.97	90.12	90	90.16	90.09	89.65	89.97	89.5
Ca. P. pruni	91.59	91.83	91.69	91.81	91.90	91.65	91.63	91.82	91.62	91.85	91.81	91.34	91.67	91.13
Ca. P. prunorum	88.72	88.93	89.29	88.9	88.89	89.45	89.15	88.94	88.75	88.98	88.98	88.95	89.47	88.9
Ca. P. pyri	88.94	89.16	89.38	89.13	89.12	89.36	89.23	89.17	88.98	89.21	89.21	88.95	89.39	88.9
Ca. P. rhamni	89	89.22	89.53	89.19	89.26	89.52	89.38	89.23	89.03	89.27	89.26	88.94	89.54	88.88
Ca. P. rubi	88.92	89.14	89.18	89.1	89.25	89.07	89.2	89.14	88.95	89.18	89.18	88.64	89.1	88.61
Ca. P. sacchari	90.63	90.71	90.52	90.68	90.61	90.45	90.54	90.71	90.66	90.74	90.68	90	90.47	89.92
Ca. P. solani	88.72	88.86	89.12	88.83	88.89	89.01	89.06	88.79	88.68	88.83	88.83	88.49	89.04	88.46
Ca. P. trifolii	89.59	89.81	90.02	89.78	89.86	89.93	90.05	89.82	89.62	89.85	89.86	89.55	89.96	89.4
Ca. P. tritici	89.85	90.07	90.48	90.04	90.12	90.41	90.42	90	89.8	90.03	90.04	89.95	90.43	89.87
Ca. P. ulmi	88.92	89.14	89.26	89.1	89.25	89.15	89.2	89.14	88.95	89.18	89.18	88.73	89.18	88.7
Ca. P. ziziphi	89.08	89.02	89.09	89.02	89.07	89.07	89.12	89.1	89.11	89.1	89.1	88.64	89.1	88.61

*CAWB, *Chenopodium album* witches' broom; CVWB, *Convolvulus virgatus* witches' broom; EPP, Eggplant phyllody; ; ECWB, *Erodium cicutarium* witches' broom; MSP; *Malva sylvestris* phyllody; BPP, Bell pepper phyllody; PAP, *Physalis angulata* phyllody ; SP: Sesame phyllody; TOP,*Taraxacum officinale* phyllody; TAWB, *Tephrosia apollinea* witches' broom; and TBB, Tomato big bud

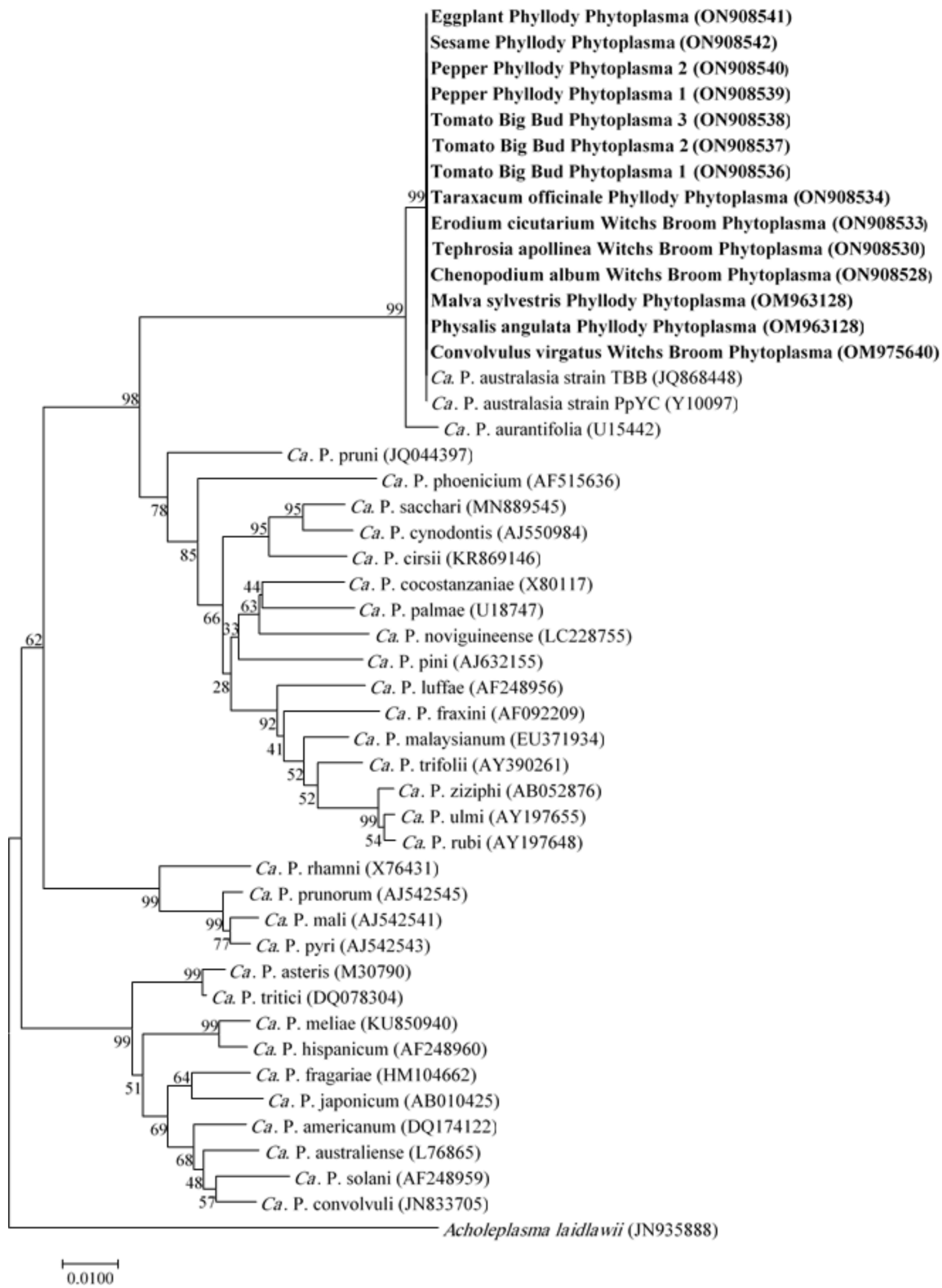

Fig. 3 Phylogenetic tree calculated from the analysis of partial *16 S rRNA* gene sequences with phytoplasma reference sequences (Bertaccini et al. 2022). The tree was constructed by the neighbor-joining method in MEGA X. The isolates characterized in this study are listed in bold. *Acholeplasma laidlawii* served as an outgroup. Bar, 0.01 nucleotide substitutions per site

Phyllanthus niruri (Rao et al. 2019) and *Setaria verticillata* (Mall et al. 2020) from India, and *P. hysterophorus* from Pakistan (Akhtar et al. 2018) have been identified as hosts of '*Ca*. P. australasia' related strains. In the present study *E. cicutarium* and *C. virgatus* (two perennial non-agricultural plants) and five annual weed plants *P. angulata*, *M. sylvestris*, *C. album*, *T. apollinea* and *T. officinale* were identified as secondary hosts of 16SrII phytoplasma. To our knowledge, this is the first report of the natural infection of *C. virgatus* and *T. apollinea* with phytoplasma strains worldwide. Likewise, there is no report about the phytoplasma infection of *E. cicutarium*, except for a piece of information from years ago that reported presence of linear MLO in the phloem of this naturally infected plant by electron microscopy (Graf et al. 1978). Dandelion (*T. officinale*) is one of the most abundant weeds in vegetable fields, and so far, phytoplasmas from 16SrI (Wang and Hiruki 2001), 16SrIII (Firrao et al. 1996; Jomantiene et al. 2002) and 16SrXII (Viczian et al. 1998; Quaglino et al. 2013) groups have been reported in association with the phytoplasma diseases in this weed. Infection of this plant by a 16SrII group phytoplasma is reported in this study. Another common

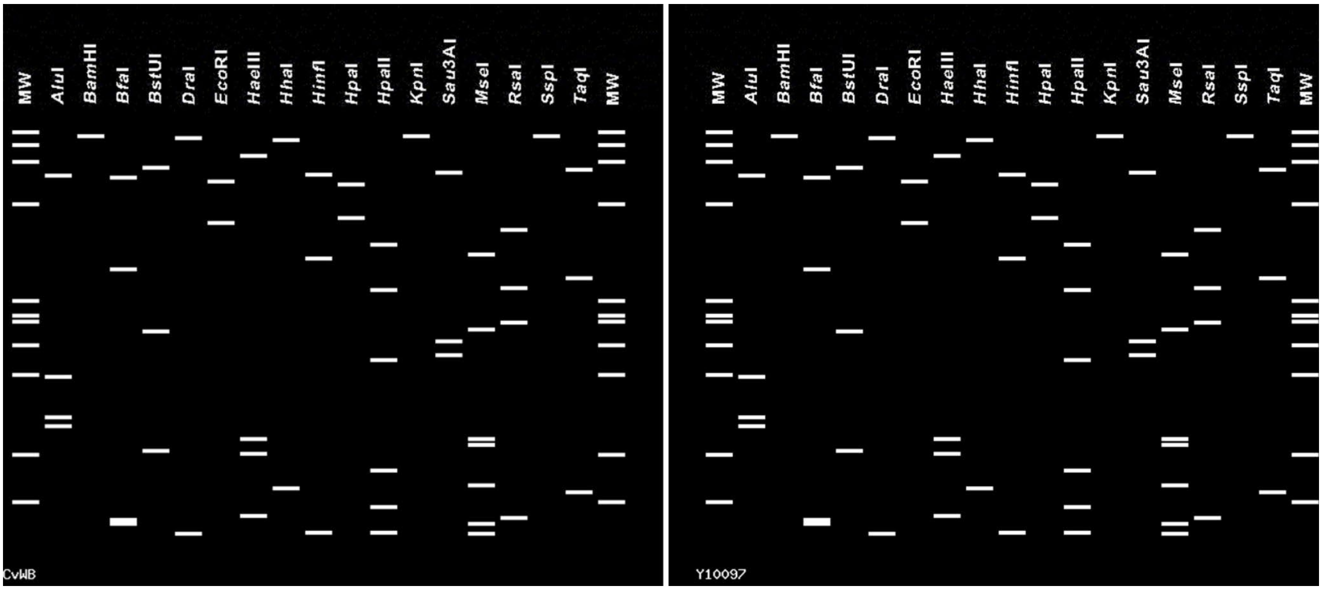

Fig. 4 Virtual RFLP patterns derived from in silico digestions of phytoplasma partial 16 S rDNA sequence fragment from *Convolvulus virgatus* witches' broom phytoplasma, representative of 16SrII-D strains detected in the present study (left) and reference strain of 16SrII-D subgroup (GenBank accession number: Y10097) (right)

weed in the vegetable fields is great mallow (*M. sylvestris*). The association of phytoplasmas from 16SrV (rubus stunt phytoplasma) (Jarausch et al. 2001), 16SrIX (Casati et al. 2016) and 16SrXII (Quaglino et al. 2013) groups with great mallow has been demonstrated. Furthermore, a phytoplasma from the 16SrII group was transmitted to great mallow with dodder from infected tomato (Hemmati et al. 2016). Natural infection of great mallow with a 16SrII phytoplasma strain is being reported here for the first time. Another naturally phytoplasma-infected plant was *P. angulata* (cutleaf groundcherry) collected in and around sesame fields. Cutleaf groundcherry is an herbaceous annual plant that grows throughout the temperate regions of the world. The 16SrI-D subgroup phytoplasma strains have been previously detected in *P. angulata* in China (Wang et al. 2010); however, to our knowledge, this is the first report of the 16SrII-D subgroup phytoplasma infection of *P. angulata*. In the tomato fields, 16SrII-D phytoplasma strains were detected in several symptomatic white goosefoot (*C. album*) plants. Heretofore, phytoplasma strains belonging to16SrII-D subgroup (Taloh et al. 2020), 16SrIII, and 16SrXII groups (Safarova et al. 2011) have been found in white goosefoot.

Weeds and wild plants can serve as sources of the phytoplasma inoculum and may be alternative hosts of the insect vectors. Since weeds often grow abundantly in or around fields even in the absence of crops, their importance as phytoplasma reservoir cannot be overestimated (Mall et al. 2010). The interaction of secondary hosts and insect vectors could be of most significant importance in the epidemiology of phytoplasma diseases. In nature, most of the phytoplasma diseases are easily spread by leafhoppers. In Pakistan, a '*Ca*. P. australasia' strain associated with infected tomato plants has been transmitted by *Orosius albicinctus* leafhopper (Akhtar et al. 2018). In Iran, transmission of this phytoplasma to sesame with *Circulifer haematoceps* and *O. albicinctus* have been reported (Salehi et al. 2017). Moreover, in Iran, *O. albicinctus* has been previously reported as a vector of 16SrII-D and 16SrII-M phytoplasmas associated with cucumber and squash phyllody diseases (Salehi et al. 2015b). *O. albicinctus* was found as a prevalent leafhopper species carrying phytoplasmas of 16SrII group in Hormozgan province (unpublished data). The leafhoppers such as *O. albicinctus* are known as polyphagous insects that feed on various plant hosts including main crops and weeds, and therefore have the potential to inoculate a wide range of plant species. Available data reveal that a given leafhopper species may transmit more than one phytoplasma, while a given phytoplasma may be transmitted by more than one vector species (Weintraub and Beanland 2006).

In conclusion, results of this work showed that the 16SrII-D subgroup phytoplasma strains are the most prevalent phytoplasmas in the investigated fields in Hormozgan province in southern Iran. Furthermore, seven weed plants, including *C. virgatus*, *P. angulata*, *M. sylvestris*, *C. album*, *T. apollinea*, *E. cicutarium*, and *T. officinale* were shown to be hosts of the 16SrII group phytoplasma strains. Among these, *C. virgatus* and *T. apollinea* are perennial herbs and might have more significance because remain as a source of phytoplasma for a longer period of time. As a result, control of these weeds in and around the crop fields could significantly reduce the pathogen inoculum and decrease the disease incidence.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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