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Abstract

During 2015–18, surveys were conducted in the main eggplant growing areas of Iran and in all areas phytoplasma-type symptoms were observed. A

total of 350 symptomatic eggplant plants were collected and tested for the phytoplasma presence on 16S rDNA. Diversity of the detected phytoplasmas was verified by molecular analyses, dodder and graft transmission on experimental test plants. Phytoplasmas were detected in all symptomatic samples and, by using nucleotide sequence comparisons and virtual restriction fragment length polymorphism analyses of 16S rDNA, six subgroups including 16SrII-D and -V, 16SrIX-C and -I, 16SrVI-A and 16SrXII-A and molecular variants related to 16SrII-D, 16SrVI-A, 16SrIX-C subgroups were identified. Based on symptomatology in dodder and graft inoculated eggplant and periwinkle plants, the phytoplasmas enclosed in the identified subgroups were differentiable. Collectively, based on the results of the present study and considering the reported presence of phytoplasmas belonging to the same ribosomal subgroups in other crops, eggplant fields play an important role in the epidemiology of other diseases associated with these phytoplasmas in Iran.

Keywords

Solanum melongena
16SrII-D
16SrII-V
16SrVI-A
16SrXII-A
16SrIX-C
16SrIX-I

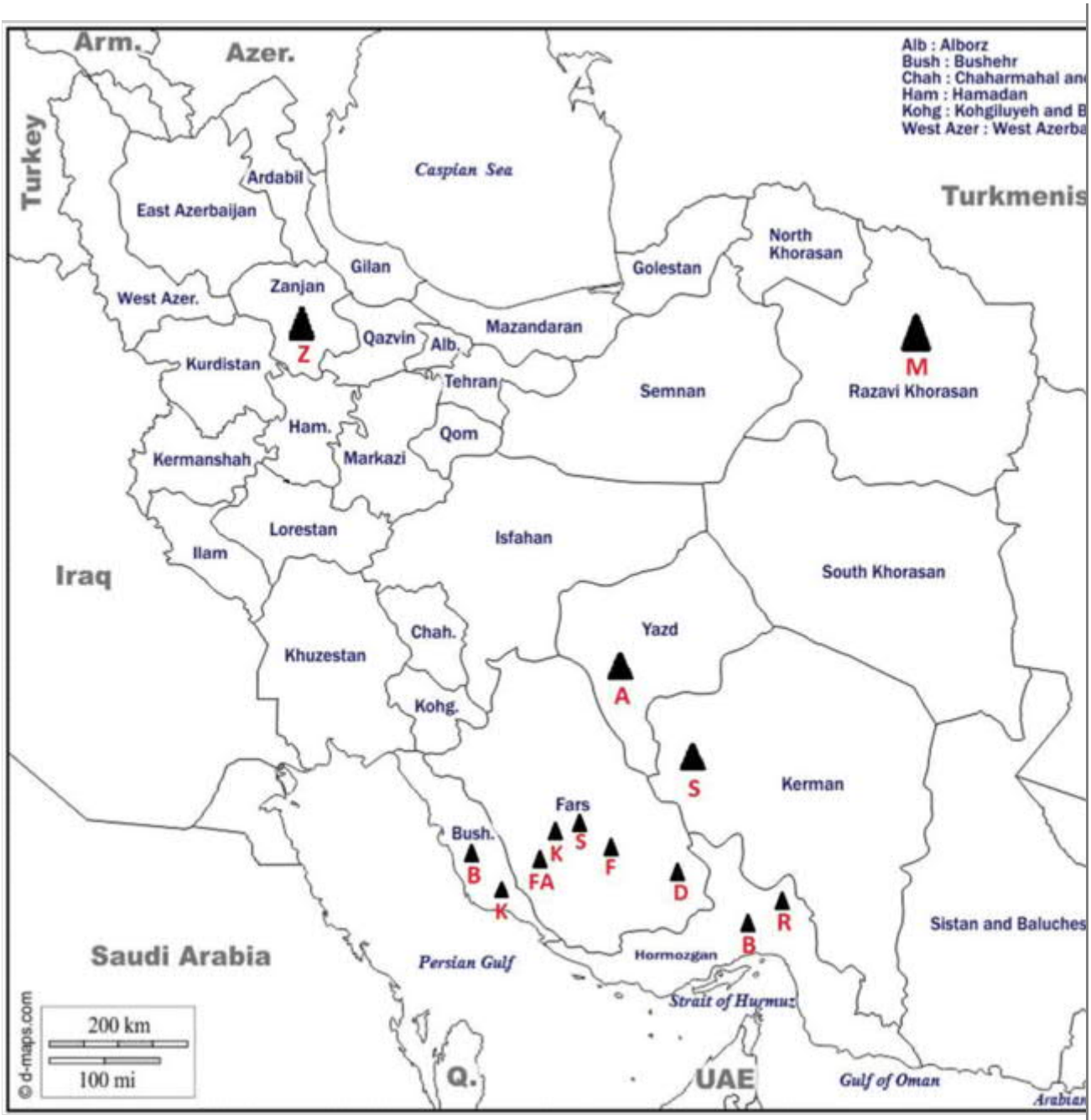
Phytoplasmas are associated with different destructive plant diseases worldwide (Bertaccini et al., 2014) and are transmitted mainly by leafhoppers, however they could be disseminated also by propagation materials and in several cases by seeds (Satta et al., 2019). Phytoplasma presence is associated with symptoms of yellowing, discoloration, witches' broom, dwarfing, virescence, and phyllody. More than 1,000 plant species from different plant families are reported as affected by phytoplasmas (Bertaccini & Duduk, 2009; Lee et al., 2000) and among them, vegetables growing in the major production areas worldwide, are infected by phytoplasmas ~~classified in~~ belonging to numerous ribosomal groups (Kumari et al., 2019). In particular, eggplant (*Solanum melongena* L.) was reported as infected with strains belonging to 16SrI in Japan, Bangladesh and India (Kelly et al., 2009; Kumar et al., 2012; Lee et al., 1998; Okuda et al., 1997), 16SrII in Oman, Egypt and India (Al-

Subhi et al., 2011; Omar & Foissac, 2012; Yadav et al., 2016), 16SrIII in Brazil (Amaral-Mello et al., 2011; Barros et al., 1998), 16SrVI in India, Turkey and Bangladesh (Azadvar & Baranwal, 2012; Sertkaya et al., 2007; Siddique et al., 2001), and 16SrXII in Romania and Southern Russia (Ember et al., 2011). Eggplant, with harvested areas of 5,312 ha, yield of 5,419 kg/ha and a production of 5,510 tons (FAOSTAT, 2018), is widely cultivated in Iran where the average production ranks the country as fifth in global production. Formerly the association of a 16SrIX-C phytoplasma with eggplant phyllody in Roodan (Hormozgan province of Iran) was reported (Tohidi et al., 2015). The present work reports genetic diversity of phytoplasmas associated with eggplant phyllody disease in several cultivation areas of Iran.

During 2015–2018, sampling of eggplant phyllody was carried out in the major eggplant growing areas of Fars (Khafr, Fassa, Firooz Abad, Sarvestan, Darab), Yazd (Abarkooh), Zanjan (Zanjan), Kerman (Sirjan), Khorasan Razavi (Mashhad), Bushehr (Bushehr, Kangan, Dashtestan) and Hormozgan (Roodan, Bandar Abbas) provinces of Iran (Fig. 1). In each area, five eggplant fields were randomly selected, and sampling was carried out at five points in 1000 m^2 field within a 1 m^2 by moving on a diagonal transect across each field. The percentage of eggplant phyllody disease incidence was calculated by number of plants with symptoms out of the total number of plants ~~observed~~ present within a 1 m^2 multiplied by 100. From each field, five eggplant phyllody affected plants were potted, transferred to greenhouse for disease transmission and molecular analyses.

Fig. 1

Map of Iran showing the sampling locations of the eggplant phyllody. In Fars area, K: Khafr, F: Fassa, FA: Firooz Abad, S: Sarvestan, D: Darab; in Yazd area, A: Abarkooh, in Zanjan, Z: Zanjan; in Kerman area, S: Sirjan, in Khorasan Razavi area, M: Mashhad, in Bushehr area, B: Bushehr, K: Kangan, D: Dashtestan and in Hormozgan area, R: Roodan, B: Bandar Abbas


Arm.
Azer.
Turkey
Caspian Sea
Turkmenis
Alb : Alborz
Bush : Bushehr
Chah : Chaharmahal an
Ham : Hamadan
Kohg : Kohgiluyeh and B
West Azer : West Azerba
Ardabil
East Azerbaijan
Gilan
Zanjan
West Azer.
Golestan
North Khorasan
Mazandaran
Qazvin
Alb.
Z
Kurdistan
Tehran
Semnan
M
Razavi Khorasan
Ham.
Qom
Kermanshah
Markazi
Lorestan
Ilam
Isfahan
Iraq
South Khorasan
Chah.
Yazd
Khuzestan
A
Kohg.
S
Kerman
Fars
Bush.
K
S
B
FA
F
D
R
K
B
Sistan and Baluches
Saudi Arabia
Persian Gulf
Hormozgan
Strait of Hurmuz
200 km
100 mi
© d-maps.com
Q.
UAE
Gulf of Oman
Arabian

After phytoplasma identification from potted eggplants, one representative of each phytoplasma subgroup identified was dodder transmitted from two infected eggplants to 10 seed-grown 3-month-old periwinkle plants (Salehi, Esmailzadeh Hosseini, Salehi, & Bertaccini, 2016e) under insect-proof conditions. For graft transmission, small axillary shoots from a symptomatic eggplant (representative of an identified subgroup) were used as scions and side grafted on five 12-week-old seed grown eggplant plants. Each rootstock received two scions. Grafted areas were wrapped with parafilm and plants were covered with plastic bags for a week to maintain humidity. Healthy seed grown eggplant and periwinkle plants (five plants per each trial) were left as healthy controls. Presence of phytoplasmas in dodder and graft inoculated plants was confirmed by nested PCR assay.

Total DNA was extracted from 0.2 g of midrib tissue of eggplant phyllody infected, and dodder and graft inoculated plants using the procedure described by Zhang et al. (1998). Total DNA extracted from symptomless seed-grown eggplant was used as negative control. Positive control was a symptomatic periwinkle plant infected with Fars alfalfa witches' broom phytoplasmas (16SrII-C subgroup) (Salehi et al., 2011). Total DNA samples were tested for phytoplasma presence using primer pair P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995) followed by R16F2n/R16R2 (Gundersen & Lee, 1996). The molecular weight of the PCR products was estimated by comparison with 100 bp DNA ladder (Fermentas, Vilnius, Lithuania).

The R16F2n/R16R2 primed PCR products of 54 samples from the surveyed areas (one sample per ~~each~~ area for which three sequences were screened) were ligated in pTZ57R/T vector and cloned into *Escherichia coli* DH5a cells using InsT / A clone^{M} PCR Product Cloning Kit (Fermentas, Vilnius, Lithuania) according to manufacturer instructions. The presence of the correct size insert was confirmed by restriction endonuclease analysis using *Eco*R1 and *Pst*1 enzymes. Three plasmid DNAs from recombinant colonies were purified using GF-1 PCR Clean-Up Kit (Vivantis, Malysia, HQ) and sequenced. Sequencing was performed by Macrogen ~~(South Korea)~~ on both strands by using M13F/M13R primers (BioNeer, DNA sequencing service, South Korea). The phytoplasma 16Sr DNA partial sequences obtained (1,250 bp) were used in Blastn analyses. Virtual RFLP was performed by *i*PhyClassifier (Zhao et al., 2009) to determine the ribosomal subgroup affiliation of the detected phytoplasmas. Partial 16S rDNA sequences of eggplant phyllody phytoplasma strains from Fars [Khafr, Fassa

(Nowbandegan, Zahedshahr), Firooz Abad (Jaydasht), Sarvesta, Darab], Yazd (Abarkooh), Zanjan (Zanjan), Kerman (Sirjan), Khorasan Razavi (Mashhad) Bushehr [Bushehr (Bushehr1), Kangan, Dashtestan (Borazjan1, Borazjan2, Borazjan3, Bondarooz)] and Hormozgan (Roodan, Bandar Abbas) obtained from the present study were aligned and phylogenetic trees and sequence homologies were generated using MEGA 6 software (Tamura et al., 2013). *Acholeplasma laidlawii* was used as out-group to root the trees. Bootstrapping was performed 1,000 times to estimate the stability and support for the tree branches.

The occurrence of eggplant phyllody was observed in all surveyed areas. The main disease symptoms were little leaf, internode shortening, flower virescence, phyllody, big bud, proliferation and sterility, witches' broom and stunting (Fig. 2). The highest disease percentage observed was 11% in Zahedshahr.

Fig. 2

Symptoms of eggplant virescence and phyllody in plants from Zanjan (left) and Fasa (right)

The disease ~~latent~~latency period varied between 6 weeks, in eggplants graft inoculated with 16SrII-A and -V subgroup strains, to 11 weeks in periwinkle plants dodder inoculated with 16SrIX-C strains. After dodder and graft inoculation of eggplant and periwinkle plants, at early stages of infection, there was no significant difference in symptoms among the diverse phytoplasma subgroups, except for 16SrVI-A strain, and the main symptoms were virescence, phyllody and moderate yellowing. At the late stage of infection, phytoplasma subgroups were differentiable from each other for the specific presence of virescence and phyllody (16SrII-A and –V), severe little leaf, internode shortening and stunting (16SrIX-C and –I), plant wilt and death (16SrVI-A), witches' broom and rosettes (16SrXII-A). However, in both eggplant and periwinkle plants 16SrII-A and –V were not differentiable on symptoms from each other, but resulted differentiable from those associated with the presence of phytoplasmas classified in the other ribosomal subgroups (Table 1 and Figs. 3 and 4).

Table 1

Ribosomal group/subgroup affiliation of eggplant phyllody disease associated phytoplasmas in Iran and their distribution

Ribosomal group	Ribosomal subgroup*	Location	GenBank accession numbers
16SrII	16SrII-D	Khafr	MT248286
		Bandar Abbas	MT240535
	16SrII-D (97%)	Bondarooz	MT248287
	16SrII-D (97%)	Borazjan3	MT248288
	16SrII-V	Syrjan	MT248285
16SrVI	16SrVI-A	Abarkooh	MG760570
		Zanjan	MT240537
	16SrVI-A (90%)	Mashhad	MT240536
16SrIX	16SrIX-C	Borazjan1	MT248280
		Roodan	MT248278
		Darab	MT248276
		Zahedshahr	MT248275

*, identity percentage is reported only for the strains in which it was lower than 100% to the sequence of the representative strain of each subgroup

Ribosomal group	Ribosomal subgroup*	Location	GenBank accession numbers
	16SrIX-C (91%)	Borazjan2	MT248284
	16SrIX-C (97%)	Sarvestan	MT248282
	16SrIX-C (97%)	Jaydasht	MT248281
	16SrIX-C (97%)	Nowbandegan	MT248283
	16SrIX-I	Bushehr1	MT248274
16SrXII	16SrXII-A	Kangan	MT248273

*, identity percentage is reported only for the strains in which it was lower than 100% to the sequence of the representative strain of each subgroup

Fig. 3

Eggplants 3-month-old graft inoculated with different eggplant phyllody phytoplasma strains four months after the grafting; a: 16SrIX-I; b: 16SrVI-A; c: 16SrIX-C; d: 16SrII-A and 16SrII-V; e: 16SrXII-A phytoplasmas. Left in d and e are healthy seed grown eggplants

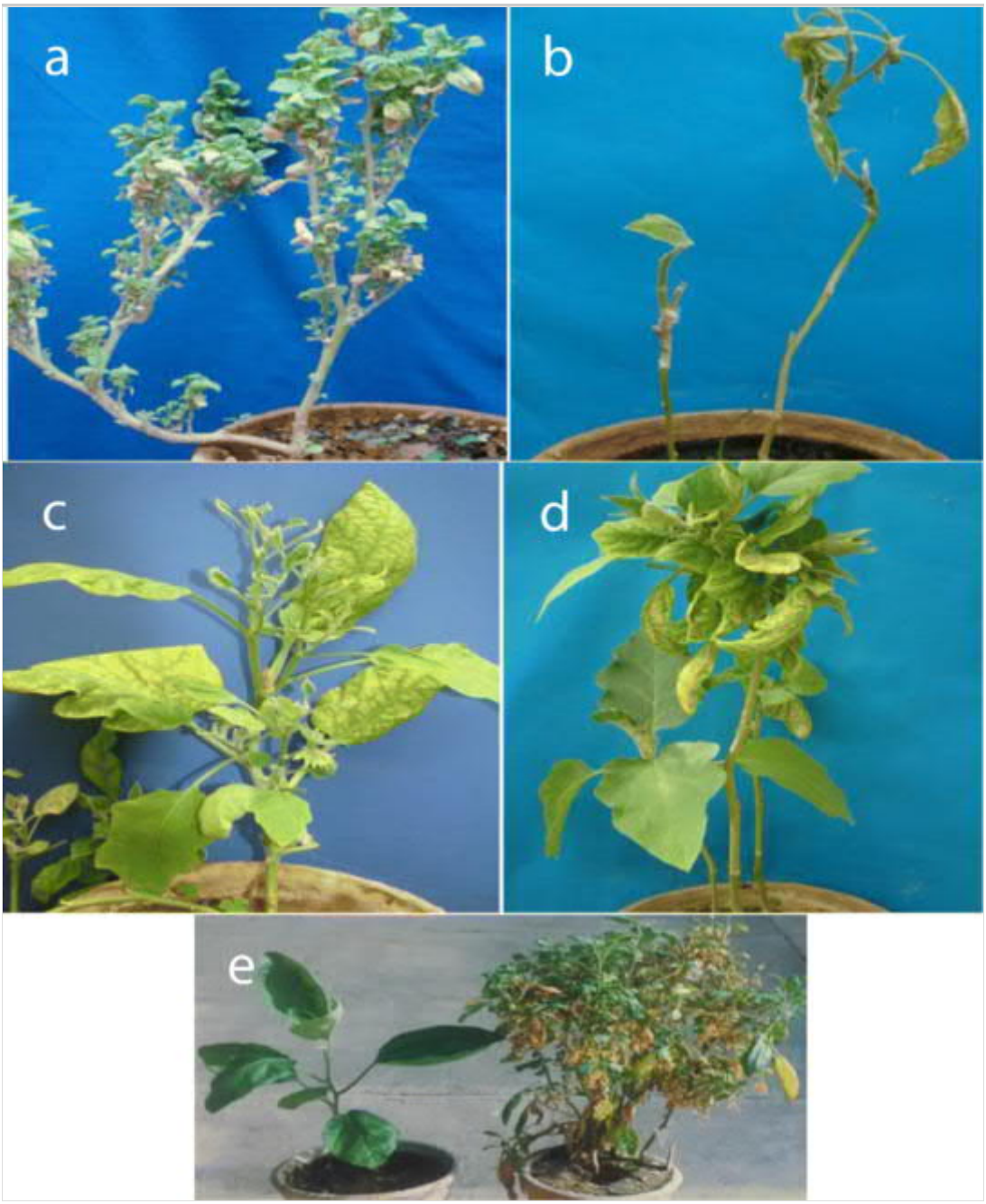


Fig. 4

Periwinkle plants 2-month-old dodder inoculated with different eggplant phyllody phytoplasma strains, four months after the beginning of the transmission trials. a: 16SrIX-C and 16SrIX-I phytoplasmas; b: 16SrVI-A phytoplasma; c: 16SrII-A and 16SrII-V phytoplasmas; d: 16SrXII-A phytoplasma; e: healthy seed grown periwinkle plant

DNA fragments of approximately 1,800 and 1,250 bp were amplified in direct and nested PCR, respectively from all the symptomatic eggplant plants, but no amplification was obtained from the asymptomatic plants. BLASTn search showed that eggplant phyllody strains were differentiable according to the diverse localities (Table 1) and the phylogenetic tree confirmed that the Iranian eggplant phyllody strains cluster with phytoplasmas enclosed in the diverse ribosomal groups listed above (Fig. 5). Since the sequences from eggplant phyllody phytoplasma samples collected in each province were identical to each other, only one representative of each province was submitted to GenBank (Table 1). Results of virtual RFLP analyses of eggplant phyllody strains showed the presence of six phytoplasma subgroups including

16SrII-V and -D, 16SrIX-C and -I, 16SrVI-A and 16SrXII-A, and of some that are variants of 16SrII-D, 16SrVI-A, 16SrIX-C (Table 1 and Fig. 6).

Fig. 5

Phylogenetic tree constructed by the Neighbor-Joining method using partial 16S rRNA gene sequences (1,250 bp) and ~~A~~.*Acholeplasma laidlaw*ii as the outgroup; 'Ca. P.': '*Candidatus* Phytoplasma'; numbers at the nodes are bootstrap (confidence) values based on 1,000 repetitions; GenBank accession numbers for sequences are given in parentheses following the phytoplasma names, Iranian eggplant phytoplasmas are in bold

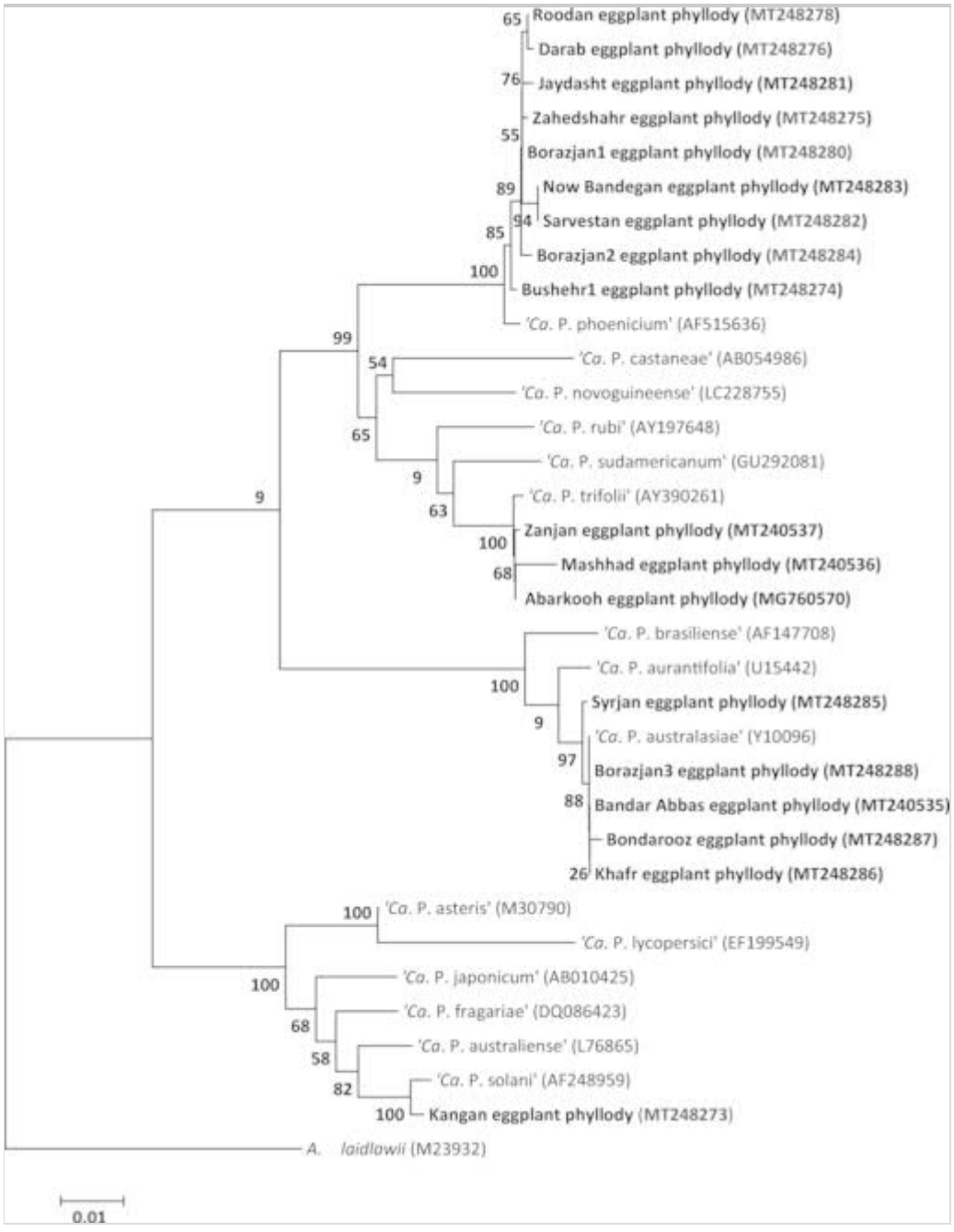
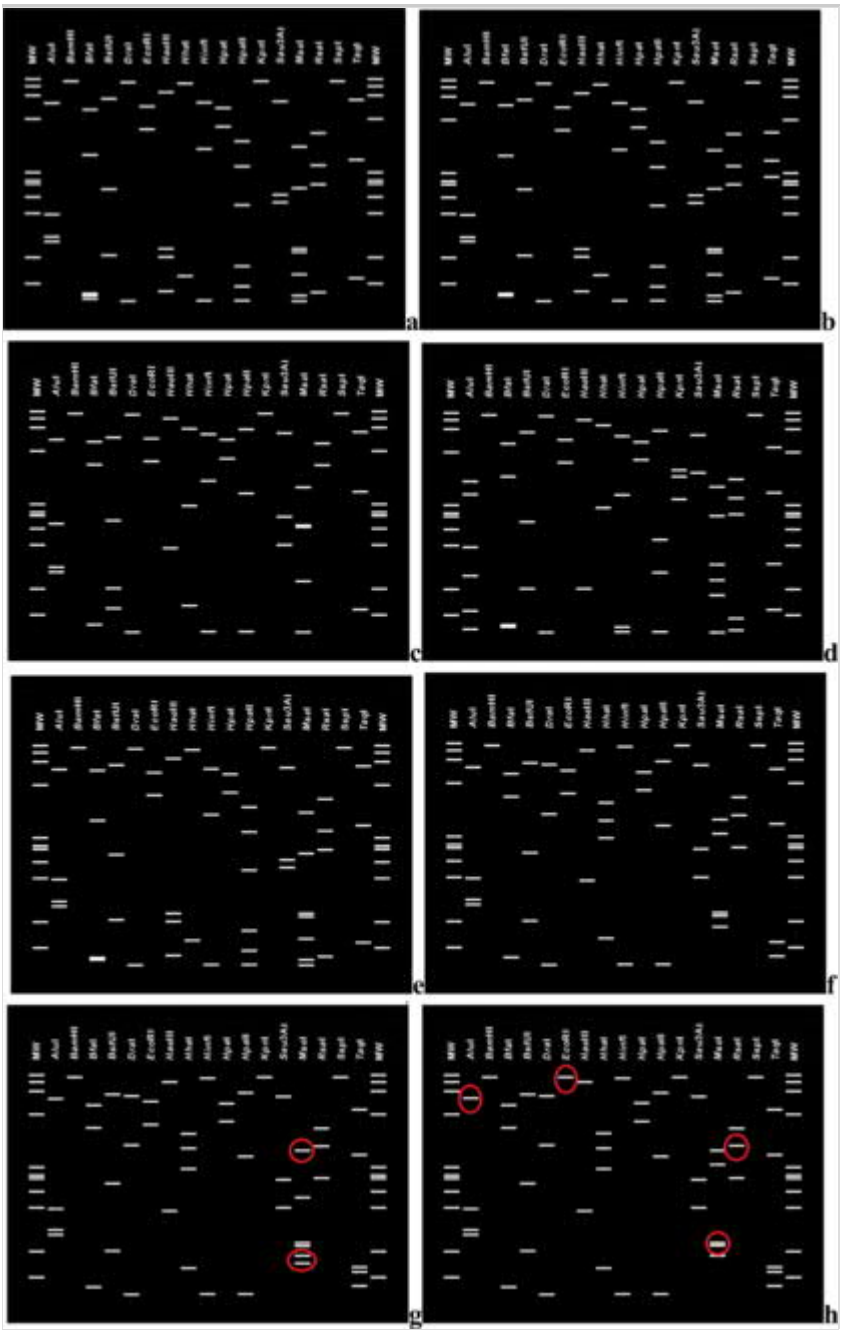


Fig. 6

Virtual RFLP pictures generated with *i*PhyClassifier from ~~in silico~~*in silico* digestion of the R16F2n/R16R2 fragments of the diverse eggplant phytoplasma strains from Iran. In a) v~~irtual RFLP profiles of~~ 16SrII-D strains Khafr (GenBank accession number, AC: MT248286), Bandar Abbas (AC: MT240535) and Bondarooz (AC: MT248287), Borazjan3 (AC: MT248288). In b) 16SrII-V (AC: MT248285) profiles. In c) strains 16SrVI-A, Yazd (AC: MG760570), Zanjan (AC: MT240537) and Mashhad (AC: MT240536). In d) strain 16SrXII-A, Kangan (AC: MT248273). In e) strains 16SrIX-C Borazjan1 (AC: MT248280); Roodan (AC: MT248278), Darab (AC: MT248276); Zahedshahr (AC: MT248275), and Borazjan2 (AC: MT248284). In f) strain 16SrIX-I, Bushehr1 (AC: MT248274). In g) strains related to 16SrIX-C (~~0.~~97%), Sarvestan (AC: MT248282); Nowbandegan (AC: MT248283). In h) strain related to 16SrIX-C (~~0.~~97%), Jaydasht (AC: MT248281). In g) and h) the red circles indicate enzymes differentiation from the 16SrIX-C profiles of the strains



In the present study 16SrII-V and -D phytoplasma subgroups were detected; the 16SrII group encloses 22 subgroups (from 16SrII-A to 16SrII-V) and two '*Candidatus* Phytoplasma' species ('*Ca*. P. aurantifolia' and '*Ca*. P. australasia'). The 16SrII-V subgroup is here reported for the first time in Iran in Syrjan (Kerman province) after its description in *Praxelis clematidea* phyllody disease in tropical and subtropical regions of China (Yang et al., 2017).

In Iran the presence of destructive phytoplasma diseases adjacent to eggplant fields may indicate a role in the epidemiology of eggplant phyllody for alfalfa witches' broom (Esmailzadeh Hosseini, Khodakaramian, Salehi, Fani, Bolok Yazdi, et al., 2015a, b, c, Esmailzadeh Hosseini, Khodakaramian, et al., 2016a,

b, c; Salehi et al. 2011~~b~~), tomato witches' broom (Salehi et al., 2014), parsley phyllody (Salehi, Esmailzadeh Hosseini, Salehi, & Bertaccini, 2016f), squash phyllody (Salehi et al., 2015), garden beet witches' broom (Mirzaie et al., 2007), sesame phyllody (Salehi, Esmailzadeh Hosseini, Salehi, & Bertaccini, 2016d), carrot witches' broom (Salehi, Esmailzadeh Hosseini, Salehi, & Bertaccini, 2016e), pot marigold phyllody (Esmailzadeh Hosseini, Salehi, et al., 2016) and pomegranate little leaf (Salehi, Esmailzadeh Hosseini, Rasoulpour, et al., 2016a). Moreover, the 16SrVI-A phytoplasma strains identified in alfalfa witches' broom, *Sophora alopecuroides* yellowing (Esmaeilzadeh-Hosseini et al., 2020; Esmailzadeh Hosseini, Khodakaramian, et al., 2016b), cabbage yellows (Salehi et al., 2007), witches' broom and yellowing in jujube plants (Babaei et al., 2020) and tomato big bud (Davoodi et al., 2019; Salehi, Salehi, & Masoumi, 2016b) were adjacent to eggplant fields in Abarkooh, Zanjan and Mashhad where the occurrence of eggplant phyllody was here reported. The phytoplasmas in the 16SrIX–C subgroup also were important in eggplant growing areas and were previously detected in Iran associated with sesame phyllody (Salehi, Esmailzadeh Hosseini, Salehi, & Bertaccini, 2016e), almond witches' broom (Salehi et al., 2006) and grapevine yellows (Salehi, Salehi, Taghavi, & Izadpanah, 2016c) diseases. The 16SrXII-A phytoplasma strains were associated with alfalfa witches' broom, *S. alopecuroides* yellowing (Esmaeilzadeh-Hosseini et al., 2020, Esmailzadeh Hosseini, Khodakaramian, et al., 2016b), *Vitis vinifera* yellows (Salehi, Salehi, Taghavi, & Izadpanah, 2016c) and decline (Ghayeb Zamharir et al., 2017), field bindweed witches' broom (Salehi et al., 2020), tomato witches' broom (Salehi & Esmailzadeh Hosseini, 2016).

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Two phytoplasma insect vectors, *Circulifer haematoceps* and *Orosious albicinctus* are found inside the eggplant fields and on many weeds, trees and shrubs in eggplant marginal fields. *C. haematoceps* was reported as vector of eggplant big bud phytoplasma in Iran (Salehi & Izadpanah, 1995). These two insect species in Iran are vectoring several of the phytoplasmas identified in eggplant (~~c;~~ Esmailzadeh Hosseini et al., 2007, 2011, 2017; Mirzaie et al., 2007; Salehi et al., 2015; Salehi, Esmailzadeh Hosseini, Salehi, & Bertaccini, 2016d).

The presence of phytoplasmas associated with eggplant phyllody in other crops and of *C. haematoceps* and *O. albicinctus* vectors of different phytoplasma subgroups in Iran provide indication that the eggplant fields may play an important role in the epidemiology of other diseases associated with

these phytoplasmas. Collectively, based on the results of the present study and considering the reported presence of phytoplasmas belonging to the same ribosomal subgroups in other crops, eggplant fields contribute to the maintenance and spreading of diseases associated with these phytoplasmas in Iran.

Declarations

Conflict of interest All authors affirm that 1) there exist no actual or potential conflict of interests to disclose, 2) the manuscript is original and has not been published previously (partly or in full), and is not under review for publication elsewhere, 3) all the necessary local, national and international standards, regulations and conventions, including normal scientific ethical practices, have been duly followed and respected. Additionally, all authors have endorsed the final version of the manuscript before submission.

Research involving human participants and/or animals The authors certify that no special permits were required for the fieldwork investigations. Investigations did not involve any species endangered or protected in Iran.

Informed consent All the authors declare that the principles of ethical and professional conduct were duly followed during the execution of this research. The research was funded by Agricultural Research, Education and Extension Organization (AREEO), Iran.

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