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Characterization and vector identification of phytoplasmas associated with cucumber and squash phyllody in Iran

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Abstract

Phytoplasmas associated with cucumber phyllody (CuP) and squash phyllody (SqP) in Yazd province of Iran were characterized by molecular analyses and biological studies. *Orosius albicinctus* leafhoppers testing positive for phytoplasma presence by polymerase chain reaction (PCR) successfully transmitted CuP and SqP phytoplasmas to healthy cucumber and squash plants. The phytoplasmas were also transmitted by *O. albicinctus* from cucumber and squash to periwinkle, alfalfa, cucumber, carrot, sesame, sunflower, pot marigold, eggplant, squash, tomato and parsley. Both phytoplasmas induced similar symptoms in the post-inoculated plants. Restriction fragment length polymorphism (RFLP) analysis of the 16S rDNA nested PCR products identified the CuP, SqP and *O. albicinctus* phytoplasmas as members of the 16SrII group. Sequence identity and phylogenetic analysis confirmed the placement of these phytoplasmas in the same clade of other phytoplasmas belonging to 16SrII group. Virtual RFLP analyses on 16S rDNA sequences allowed the affiliation of SqP phytoplasma to subgroup 16SrII-D, while the CuP phytoplasma was identified as representative of a new subgroup 16SrII-M. This is the first report on molecular characterization of the CuP and SqP phyllody phytoplasmas, including subgroup affiliation, identification of their leafhopper vector and determination of their plant host range in Iran.

Key words: cucumber, squash, phyllody, 16SrII phytoplasma, characterization, leafhopper, *Orosius albicinctus*, Iran.

Introduction

Phytoplasmas are cell-wall less bacteria that have very small genome sizes and are amongst the smallest self-replicating living organisms (Bertaccini *et al*., 2014). They infect numerous important food, fibre, fodder and timber crops, causing significant crop losses, and are transmitted by sap-feeding insects. Phytoplasma diseases of the Cucurbitaceae family have been reported in *Cucumis sativus* L., *Cucurbita maxima* Duchesne, *Cucurbita mixta* Pangalo, *Cucurbita pepo* L., *Lagenaria leucantha* Rusby, *Lagenaria siceraria* (Molina) Standley (McCoy *et al*., 1989; Seemüller *et al*., 1998), *Cucurbita moschata* Duchesne (Montano *et al*., 2006), *Luffa cylindrica* L. (McCoy *et al*., 1989; Lee *et al*., 1993; Gundersen *et al*., 1994; Montano *et al*., 2007a), *Momordica charantia* L. (McCoy *et al*., 1989; Montano *et al*., 2000), *Sechium edule* (Jacquin) Swartz (McCoy *et al*., 1989; Montano *et al*., 2000; Villalobos *et al*., 2002), and *Sicana odorifera* (Vellozo) Naudin (Montano *et al*., 2007b). Phytoplasmas associated with diseases in cucurbitaceae plants were molecularly characterized in *C. pepo* from Italy (Seemüller *et al*., 1998) and in *S. edule* from Costa Rica (Villalobos *et al*., 2002) where 16SrI group (‘*Ca*. P. asteris’) was detected. In *C. pepo* from Australia and Egypt (Davis *et al*., 1997; Omar and Foissac, 2012) and in *S. edule*, *M. charantia*, *S. odorifera*, *C. moschata*, and *L. cylindrica*, from Brazil, 16SrII group phytoplasmas were identified (Montano *et al*., 2000; 2006; 2007a; 2007b). In *L. cylindrica*, 16SrVIII group phytoplasmas were detected in Taiwan (Lee *et al*., 1993; Gundersen *et al*., 1994).

Iran is among the top ten producers of cucumber after China with field cultivation of 8,700 ha and a year production of 1,811,630 tonnes (FAOSTAT, 2013). During field surveys in 2004, cucumber (*C. sativus*) and squash (*C. pepo*) plants showing phyllody symptoms were observed in Abarkooh and Yazd areas (Yazd province, Iran). The present work reports biological and molecular characterization of phytoplasmas associated with cucumber (CuP) and squash phyllody (SqP), and the transmission experiments to identify insect vector(s) of both SqP and CuP.

Materials and methods

Disease incidence

In cucumber cultivations of Chahgeer five fields per crop were selected randomly and sampling was carried out randomly at five points in 1,000 m^2 fields within a 1 m^2 on a diagonal transect across each of the five fields. The percentage of CuP disease incidence was calculated by counting number of plants with symptoms out of total number of plants observed using the formula given below.

$$\% \text{ disease incidence} = \frac{\text{No. of symptomatic plants}}{\text{No. of plants observed}} \times 100$$

Source of phytoplasmas

Cucumber and squash plants with typical symptoms of phyllody were selected in fields located in Chahgeer location in Abarkooh area (140 Km west of Yazd), transferred to a greenhouse located in Zarghan (Fars prov-

ince, Iran) and used as sources for biological and molecular studies of the associated phytoplasmas. Leaf midribs (0.3 g) from symptomatic cucumber and squash plants collected in the fields and from the experimentally post-inoculated plants were subjected to total DNA extraction. A periwinkle [*Catharanthus roseus* (L.) G. Don] plant infected with a '*Ca.* P. aurantifolia' (Salehi *et al*., 2002) was used as positive control. Healthy *C. sativus*, *C. pepo*, *C. roseus*, *Medicago sativa* L. and *Solanum lycopersicum* L. grown from seeds collected in Zarghan fields were used as negative controls.

DNA extraction and PCR detection of phytoplasmas

Total DNA was extracted from plant (Zhang *et al*., 1998) and insect samples following the protocol of Doyle and Doyle (1990). The DNA quality and concentration was estimated by spectrophotometer and agarose gel electrophoresis (Sambrook *et al*., 1989). For PCR, 100 ng of total DNA extract was used. The universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) was used to amplify the 16S rRNA operon comprising the 16S rRNA gene, 16S-23S rRNA genes spacer region and the 5' end of the 23S rRNA gene. The amplification products were diluted 1: 29 with sterile deionized water and 1 µL was amplified in a nested PCR with the primer pair R16F2n/R2 (Gundersen and Lee, 1996). The PCR reaction was performed in 50 µL reaction mixtures containing 0.4 µM of each primer, 0.2 mM of each dNTP, 1.25 U Taq DNA polymerase (CinnaGen, Iran) and 5 µL 1X Taq polymerase buffer. The reaction cycled 35 times in a Bio-Rad (USA) thermal cycler with the following parameters: denaturing for 1 min at 94 °C (2 min of initial denaturation), annealing for 2 min at 55 °C and primer extension for 3 min at 72 °C (10 min of final extension). PCR conditions for the nested PCR were the same except that the annealing temperature was 58 °C. Following PCR, 5 µL of each PCR product were electrophoresed in a 1% (w/v) agarose gel containing 0.3 µg/mL ethidium bromide in 0.5 X TBE buffer (22.5 mM Tris-borate, 1 mM EDTA, pH 8.0) to verify amplification of target DNA.

Vector identification, PCR examination and phytoplasma transmission

Insects were collected weekly three times during fruit setting period from cucumber and squash fields affected by phyllody in Chahgeer (Abarkooh, Yazd province) with an insect sweep net, and sorted out by their gross morphology. Twenty specimens of each leafhopper species were subjected to nested PCR using P1/P7 and R16F2n/R16R2 primer pairs. *Orosius albicinctus* Distant (Rhynchota Cicadellidae) adult specimens, collected in the same plots that the adult insects of the species tested positive in PCR assay, were placed in three cages (20 specimens per cage). Each cage contained five young healthy cucumber or squash plants to test the *O. albicinctus* transmission ability immediately after field collection. The inoculation access time was 4 weeks, after which plants were sprayed with Metasystox-R to kill the adults and nymphs of *O. albicinctus*.

Host range studies

Followed taxonomical identification, non-inoculative colonies of *O. albicinctus* were developed by transferring single fertilized females to a healthy sugar beet plants for egg deposition and subsequent hatching. Non-inoculative colonies were frequently monitored for SqP and CuP phytoplasma presence by nested PCR. Highly inoculative *O. albicinctus* colonies were developed by transferring adult *O. albicinctus* from non-inoculative colonies to infected cucumber and squash plants, and the resulting young adults used to inoculate cucumber, squash, periwinkle, sunflower, sesame, alfalfa, carrot, sugar beet, arugula, parsley, rapeseed, onion, pot marigold, eggplant and tomato plants (table 1). The inoculation test for each plant species consisted of caging twenty five inoculative leafhoppers of each species on five plants in a pot. Fifteen plants (in 3 pots) for each species were inoculated. The inoculation feeding time on each plant species was three weeks. After the acquisition access period (AAP), plants were sprayed with insecticide and transferred to a separate insect-proof greenhouse for the monitoring of disease symptom appearance and PCR testing. Twenty five non-inoculative *O. albicinctus* were fed on five plants of each plant species used as negative controls. Cucumber, squash and test plants used in host range studies were grown from seed in a greenhouse sprayed with insecticide every two weeks. Six months post-inoculation, plants were tested for phytoplasma presence detection by nested-PCR assays.

RFLP analyses

Products from R16F2n/R2 nested PCR were digested with restriction endonucleases *Alu*I, *Hha*I, *Hin*fI, *Hpa*II, *Mse*I, *Rsa*I and *Taq*I (Fermentas, Vilnius, Lithuania). RFLP profiles were analyzed on 2% agarose electrophoresis gels followed by staining with ethidium bromide and visualization under a UV transilluminator. The 16S rDNA virtual RFLP patterns of CuP and SqP phytoplasmas were analyzed and compared to that of other phytoplasmas using *i*PhyClassifier (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 (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I (*Sau*3AI), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I].

Cloning and sequencing analyses

P1/P7 primed PCR products of the CuP and SqP phytoplasmas were ligated onto a pTZ57R/T vector and cloned into *Escherichia coli* (strain DH5α) cells using InsT/A clone PCR product Cloning Kit (Fermentas, Vilnius, Lithuania). Presence of the correct insert was confirmed after digestion with *Eco*RI and *Pst*I restriction endonucleases enzymes. Plasmid DNA from cultures of recombinant colonies was purified (High Pure Isolation Kit, Roche, Germany). Sequencing on both strands was performed by Macrogen (South Korea) using forward and reverse M13 primers. Internal primers were designed and used by the sequencing company. Nested PCR products of R16F2n/R2 from a phytoplasma detected in leafhoppers or the full lengths of the 16S rDNA from CuP and SqP phytoplasmas were sequenced and used for

Table 1. Results of plant species inoculation with insect vector (*Orosius albicinctus*) with CuP and SqP phytoplasmas.

Plant species Common name/ Family	Vector inoculation CuP	Vector inoculation SqP	Main disease symptoms	Disease latency (days)	PCR assay
Allium cepa L. Onion/ Amaryllidaceae	0/15[a]	0/15	NS	0	-
Beta vulgaris L. Sugar beet/ Chenopodiaceae	0/15	0/15	NS	0	-
Brassica rapa subsp. *rapa* L. Rapeseed/ Brassicaceae	0/15	0/15	NS	0	-
Calendula officinalis L. Pot marigold/ Asteraceae	6/15	10/15	FP, FV, SL, IS, ST, Y	49	+
Catharanthus roseus (L.) G. Don. Periwinkle/ Apocynaceae	11/15	9/15	FP, FV, SL, IS, ST, WB, Y	28	+
Cucurbita pepo L. Squash/ Cucurbitaceae	7/15	5/15	FP, FV, PS, Y, ST	29	+
Cucumis sativus L. Cucumber/ Cucurbitaceae	9/15	8/15	FP, FV, PS, Y, ST	27	+
Daucus carota L. Carrot/ Apiaceae	4/15	5/15	SL, IS, ST,Y	42	+
Eruca sativa Miller Arugula/ Brassicaceae	0/15	0/15	NS	0	-
Helianthus annuus L. Sunflower/ Asteraceae	5/15	2/15	FP, FV, SL, IS, PS, ST	39	+
Solanum lycopersicum L. Tomato/ Solanaceae	4/7	7/15	BB, IS, PC, PS, SL, ST, Y	30	+
Medicago sativa L. Alfalfa/ Fabaceae	6/15	9/15	FP, FV, IS, PC, PS, SL, ST, WB, Y	21	+
Petroselinum crispum Miller Parsley/ Apiaceae	7/15	3/15	PC, IS, SL, ST, WB, Y	46	+
Sesamum indicum L. Sesame/ Pedaliaceae	6/15	8/15	FP, FV, PS, SL, ST, WB, Y	34	+
Solanum melongena L. Eggplant/ Solanaceae	6/15	7/15	FP, FV, IS, PC, SL, ST, Y	24	+

[a] number of infected plants/number of inoculated plants.

BB: big bud; FP: floral proliferation; FV: floral virescence and phyllody; IS: internode shortening; NS: no symptoms; PC: proliferation of crown buds; PS: proliferation of stem buds; SL: small leaves; ST: stunting; WB: witches' broom; Y: foliar yellowing; - : no reaction; + : positive reaction.

further analyses. The sequences were compared with 16S rDNA sequences of phytoplasmas in GenBank using Blast from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Sequence identity and phylogenetic analyses

Full-length 16S rDNA sequences of thirty phytoplasmas including CuP and SqP were aligned using Clustal W. A phylogenetic tree was constructed using the neighbor joining method of MEGA5 software (Tamura *et al*., 2011). *Acholeplasma laidlawii* was used as outgroup to root the tree. Bootstrapping was performed 100 times to estimate the stability and support for the branches. The 16S rDNA sequence identity between strains was evaluated after alignments generated by using homology matrix distance option of DNAMAN version 4·02 (Lynon Corporation).

Results

Disease symptoms and incidence

Characteristic symptoms of CuP and SqP phyllody included proliferation of short spindly shoots along the stem (figures 1A and 1C); reduced size of leaves, shortening of internodes (figures 1A, 1C and 2A), fruit cracking (figure 1B), virescence and phyllody (figures 1D and 2B), floral proliferation, sterility, witches' broom, branch malformation (figures 2A and 2B) and failure to fruit especially during early infection. An infection rate of up to 60% was observed in the cucumber fields in Chahgeer.

Vector identification

Eight leafhopper genera including *Circulifer haematoceps* (Mulsant et Rey), *Neoaliturus fenestratus* (Herrich-Schaffer), *Empoasca decepiens* (Paoli), *Orosius*

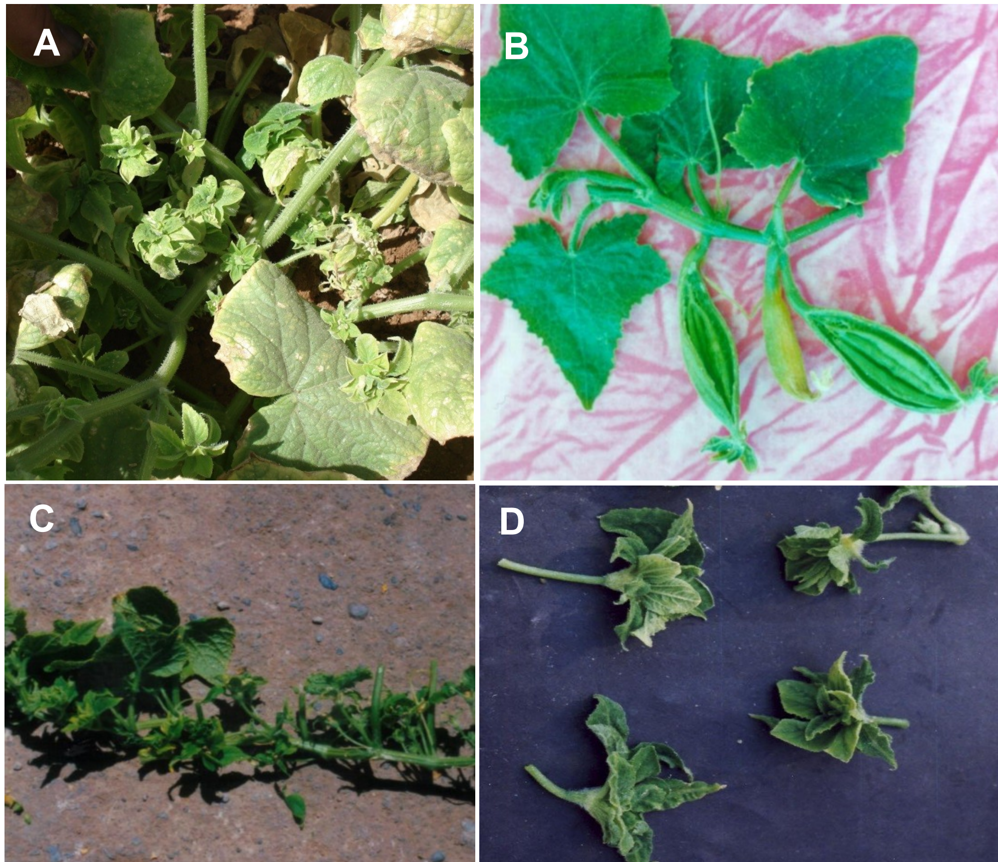


Figure 1. Symptoms of cucumber phyllody in Abarkooh and Yazd (Yazd province) as a result of phytoplasma presence. **A**: small leaves, internode shortening and flower virescence, phyllody and witches' broom; **B**: cracking of cucumber fruits; **C**: severe proliferation along the stem; **D**: flower virescence and phyllody.
(In colour at www.bulletinofinsectology.org)

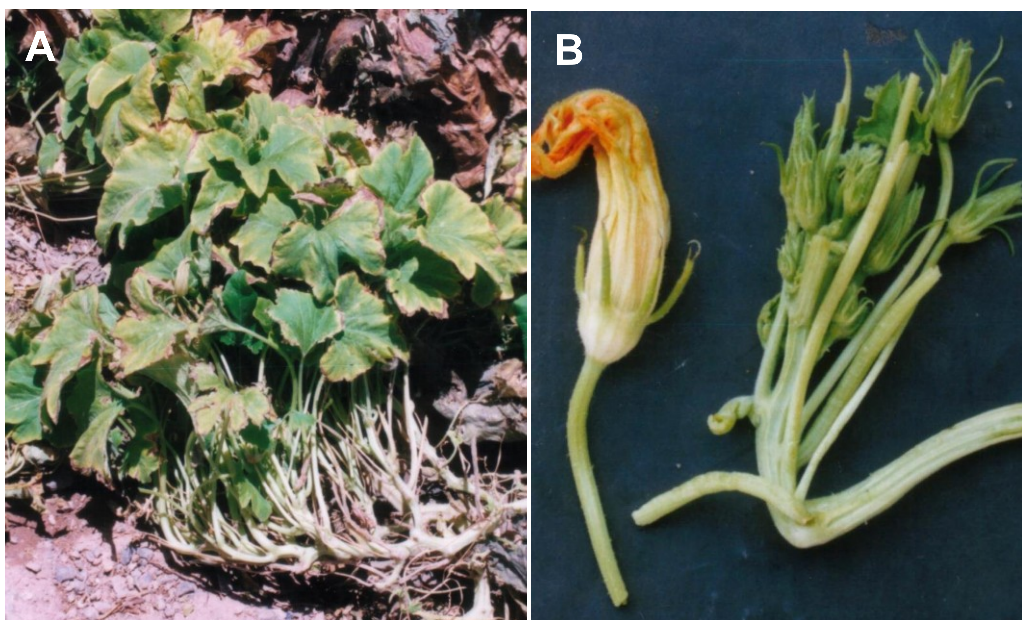


Figure 2. Symptoms of squash phyllody in Abarkooh (Yazd province). **A**: severe stem proliferation, chlorosis, little leaf, internode shortening and witches' broom; **B**: proliferation of abnormal flowers with virescence and phyllody symptoms (right) compared to a healthy flower (left).
(In colour at www.bulletinofinsectology.org)

albicinctus Distant, *Psammotettix striatus* L., *Austrogallia sinuata* Mulsant et Rey, *Eupteryx zelleri* (Kirschbaum) and *Zygina* sp. were collected in phyllody-affected cucumber and squash fields. Only *C. haematoveps* and *O. albicinctus* were reared on cucumber and squash plants under greenhouse conditions, while the other species were collected from different weeds in cucumber and squash fields.

Twenty samples of each species were tested for phytoplasma presence. Twenty one specimens of *O. albicinctus* collected from cucumber (11 out of 20 specimens) and squash (10 out of 20 specimens) were positive in nested PCR assays (data not shown). Other leafhopper species as well as *O. albicinctus* from non-inoculative colonies tested negative in PCR.

PCR positive *O. albicinctus* specimens were further evaluated as vectors using groups of twenty leafhoppers collected in the field and directly caged on healthy cucumber and squash plants. This leafhopper was able to transmit the CuP and SqP phytoplasmas to healthy squash and cucumber plants. Seven out of 15 squash and nine out of 15 cucumber plants exposed to this leafhopper developed symptoms of SqP and CuP diseases.

Phytoplasma host range

The host range of cucumber and squash phyllody phytoplasmas was examined following the inoculation of test plants by *O. albicinctus* reared on the infected *C. sativus* and *C. pepo* (table 1). Among test plants used, at least two out of 15 plants of alfalfa, carrot, cucumber, sesame, sunflower, pot marigold, eggplant, squash, tomato, periwinkle, and parsley developed the phytoplasma symptoms from both original host plant species. In contrast, *Eruca sativa*, rape, sugar beet and onion plants expressed no phytoplasma symptoms up to six months post-inoculation. No symptoms were observed when non-inoculative leafhoppers were exposed to the test plants. The phytoplasma presence in the symptomatic test plants was verified by PCR analysis (table 1). The RFLP patterns of the R16F2n/R2 amplicon in the infected test plants were identical to those of *C. sativus* and *C. pepo* phyllody agents (data not shown). No amplicons were obtained in PCR from asymptomatic plants inoculated by either the healthy or the inoculative leafhoppers.

The results showed that both squash and cucumber phyllody phytoplasmas express the same symptoms in the test plants (table 1). The minimum disease latency period in alfalfa, eggplant, carrot, cucumber, tomato, periwinkle, sesame, sunflower, squash, parsley, and pot marigold was 21, 24, 42, 27, 30, 28, 34, 39, 29, 46, 49 days, respectively (table 1).

Phytoplasma detection and identification

The RFLP patterns from the R16F2n/R2 amplicons of CuP and SqP phytoplasmas, and a phytoplasma amplified from collected *O. albicinctus* were 100% identical (figure 3) and similar to those previously published for members of the peanut witches' broom (16SrII phytoplasma group) (Lee *et al*., 1998). P1/P7 amplicons were amplified from *C. pepo* (18 out of 20 samples) and *C. sativus* (10 out of 20 samples) plants showing the phyllody symptoms in the field (data not shown). R16F2n/R2 PCR products were obtained for all the forty samples (data not shown); as well as in 11 (out of 20) and 10 (out of 20) samples of *O. albicinctus* leafhoppers collected, respectively, from infected cucumber and squash fields.

The 1.8 kbp DNA fragments amplified from one selected sample of *C. sativus* and one of *C. pepo* phyllody were cloned, sequenced and sequences were submitted to the GenBank data base under accession numbers KR822804 and KR822805, respectively. Sequence analysis showed that the 16S rDNA nucleotide sequences of phytoplasmas from cucumber and squash plants showed 99% identity to each other. Two representative R16F2n/R2 amplicons obtained from *O. albicinctus* collected from infected squash and cucumber fields, were also sequenced and they were shown to be 100% identical to the corresponding R16F2n/R2 sequences of SqP and CuP phytoplasmas. Blast searching showed that the phytoplasmas associated with CuP, SqP and *O. albicinctus* shared the highest sequence identity to phytoplasmas enclosed in the clade containing phytoplasmas of 16SrII group.

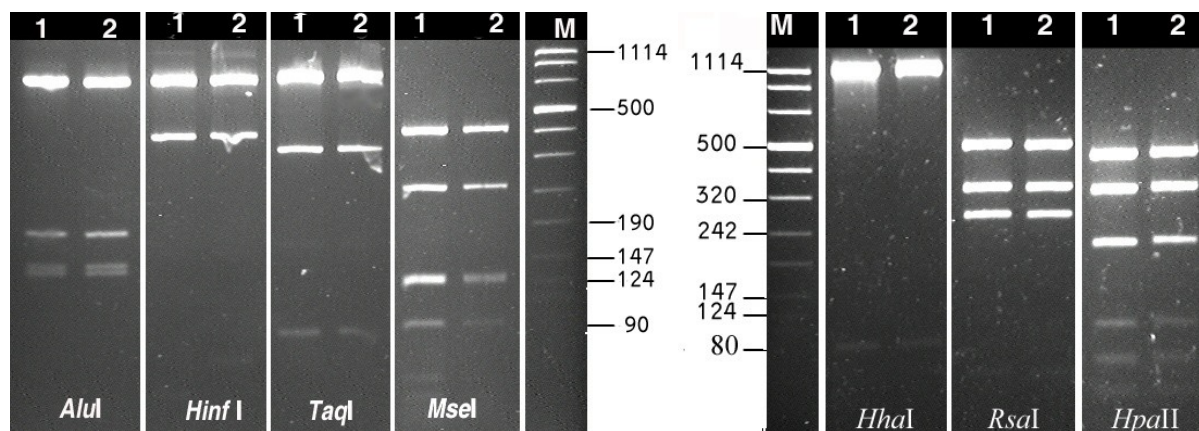
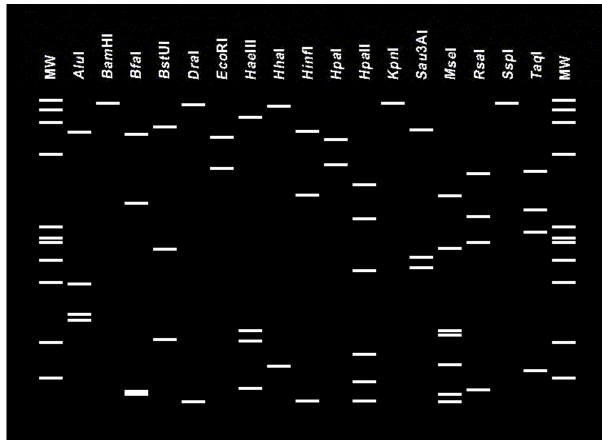
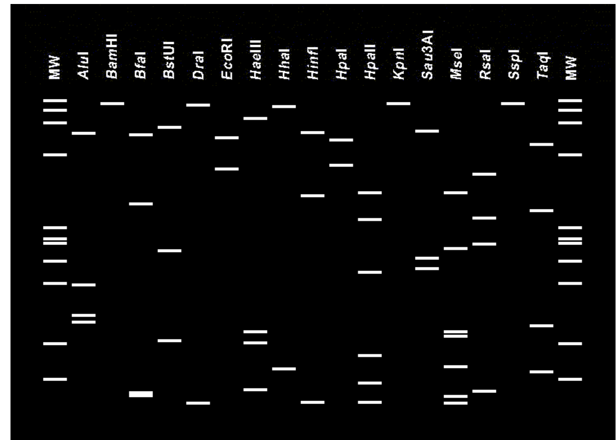


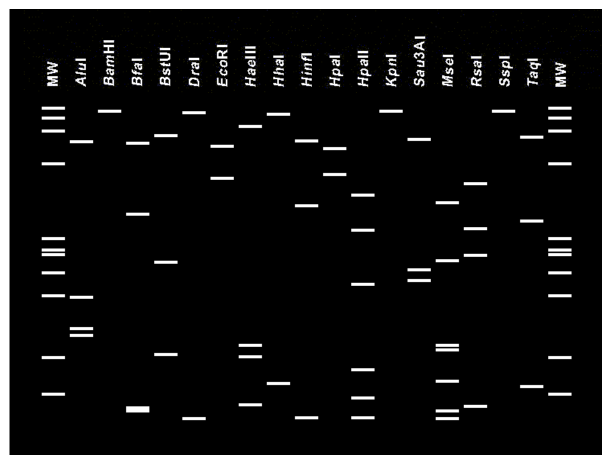
Figure 3. RFLP profiles of 16S rDNA amplicons obtained in nested PCR primed by P1/P7 and R16F2n/R16R2 from cucumber and squash phyllody phytoplasmas (lanes 1 and 2 respectively). Lane M, DNA molecular weight marker VIII from Roche Life Science. DNA products were digested with the enzymes listed at the bottom of the figures.



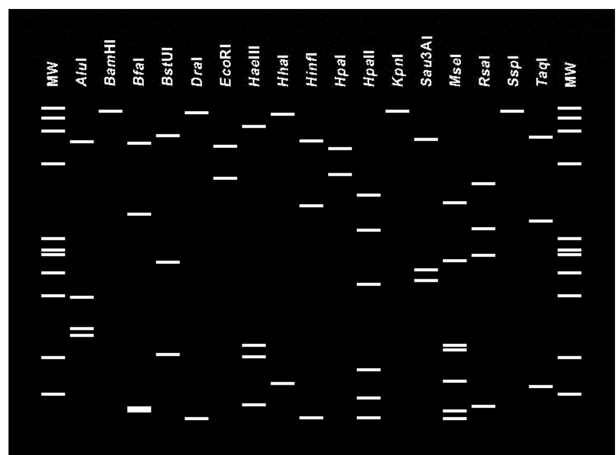
Peanut witches' broom
(16SrII-A, GenBank accession number, L33765)



Cucumber phyllody
(16SrII-M, GenBank accession number, KR822804)



'*Ca*. P. australasia'
(16SrII-D, GenBank accession number, JQ868448)



Squash phyllody
(16SrII-D, GenBank accession number, KR822805)

Figure 4. Virtual RFLP patterns of 1.2 kb 16S rDNA fragments of representative strains of subgroups 16SrII-A and 16SrII-D compared with profiles of cucumber and squash phyllody phytoplasmas using online iPhyClassifier program (Zhao *et al*., 2009).

Virtual RFLP analyses

The R16F2n/R16R2 amplified regions from the 16S rDNA sequences of 12 phytoplasmas, representatives of 12 16SrII subgroups (A-L) and of phytoplasmas associated with cucumber and squash phyllody were each digested *in silico* with 17 restriction enzymes (data not shown). This analysis showed that SqP phytoplasma is identical to '*Ca*. P. australasia', representative of 16SrII-D subgroup. Based on *Taq*I enzyme, CuP and SqP phytoplasmas were differentiable: SqP had three sites for *Taq*I but CuP had one more site for this enzyme. For the 16 other restriction enzymes CuP and SqP were not differentiable. Regarding number of *Taq*I sites CuP phytoplasma was similar to peanut witches' broom, representative of 16SrII-A subgroup but based on positions of *Taq*I, it was differentiable from it (figure 4). Collectively based on the number and position of *Taq*I restriction enzyme, among reported subgroups of 16SrII group CuP is an unique phytoplasma that could be affiliated to a new subgroup 16SrII-M.

Phylogenetic analysis

The phylogenetic tree generated by the analysis of nearly full length 16S rDNA sequences of different 16Sr groups, and those of the squash and cucumber phyllody phytoplasmas is shown in figure 5. Squash and cucumber phyllody phytoplasmas are in the same clade with phytoplasmas belonging group 16SrII supported by high bootstrap values; however they are in two different branches that supports the finding that they belong to separate subgroups. The closest phytoplasmas to the squash phyllody were those related to '*Ca.* P. australasia' (GenBank numbers Y10097), representatives of 16SrII-D subgroup. The homology percentage among 16S rDNA sequences of selected 16SrII phytoplasmas was determined and the results are presented in table 2. Both strains from Iran had greatest homology (99.3%) with '*Ca.* P. australasia' strains that are members of 16SrII-D subgroup and homology of 89.1% with '*Ca*. P. asteris' (GenBank number M30790) that is affiliated to group 16SrI-B.

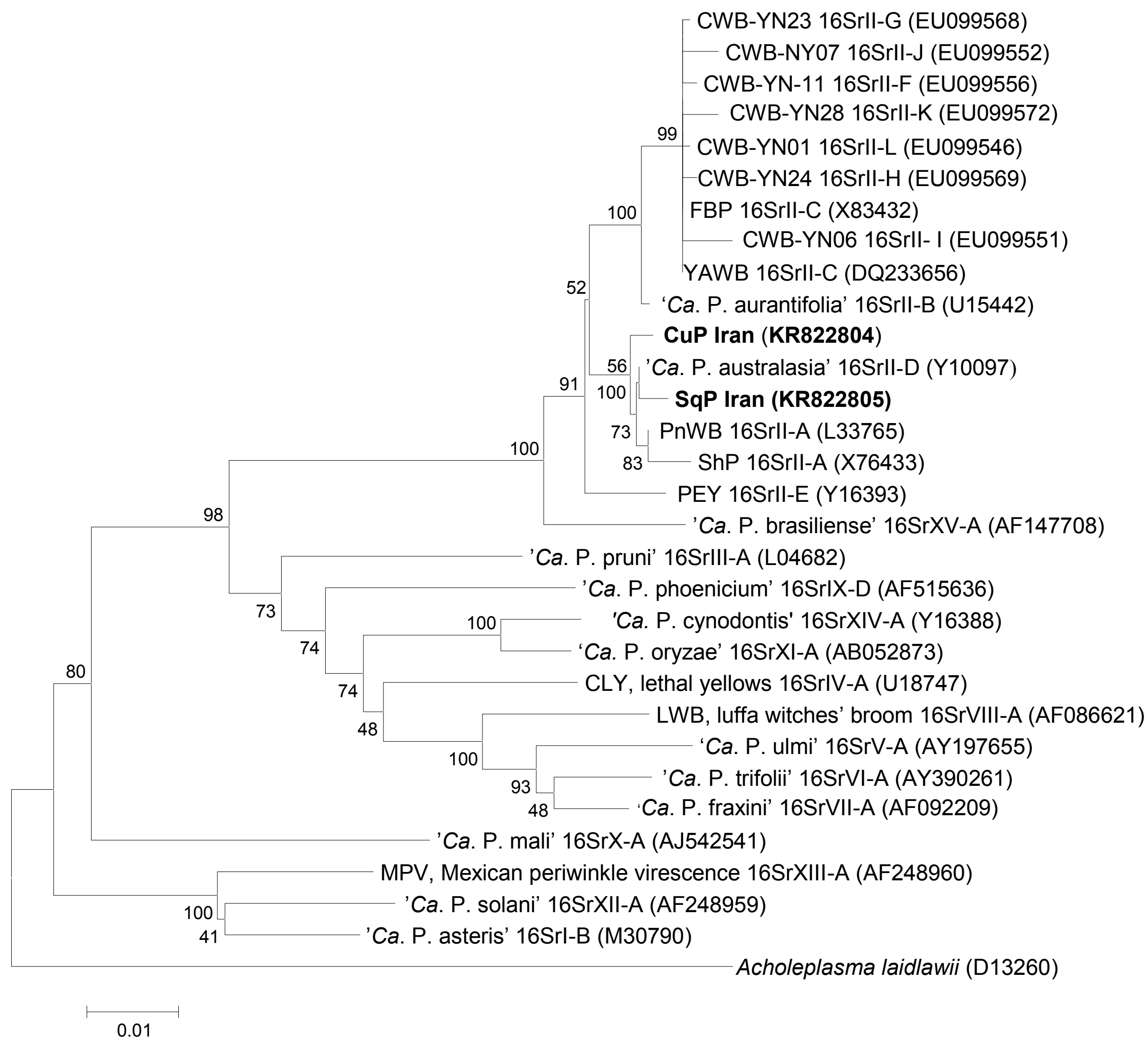


Figure 5. Phylogenetic tree constructed by the Neighbor-Joining method of 16S rRNA gene sequences from 30 phytoplasmas and *A. laidlawii*, as outgroup. The cucumber and squash phyllody phytoplasmas are bolded. Numbers at the nodes are bootstrap values based on 100 repetitions. CLY: coconut lethal yellowing; CuP: cucumber phyllody; SqP: squash phyllody; CWB: cactus witches' broom; FBP: faba bean phyllody; LWB: luffa witches' broom; MPV: Mexican periwinkle virescence; PEY: *Pichris echioides* yellows; PnWB: peanut witches' broom; ShP: sunhemp phyllody; YAWB: Yazd alfalfa witches' broom; '*Ca*. P.': '*Candidatus* Phytoplasma'. GenBank accession numbers for sequences are given in parentheses followed by the phytoplasma grouping. Bar, 1 nucleotide substitution per 100 nucleotides.

Discussion

Phyllody disease is an emerging threat to cucurbitaceous and other susceptible plants in Yazd province showing high percentages of infected cucumber plants that frequently did not bear fruits, and induce yield losses that in cases of early infection reaches 100%. Symptoms of cucumber phyllody was also observed in Iranian provinces of Fars (Salehi *et al*., 2005), Sistan-Baluchistan, Hormozgan and Bushehr, but phytoplasmas were not identified. A 16SrII-related phytoplasma has been reported from Kerman (Iran) province (Tazehkand *et al.*, 2010).

Direct and nested PCR assays using phytoplasma universal primers confirmed phytoplasma presence in symptomatic cucumber and squash samples and experimentally inoculated plants. RFLP analyses showed that phytoplasmas associated with CuP and SqP were 100% identical and belong to 16SrII ribosomal group. Virtual RFLP analyses indicated that SqP phytoplasma belongs to16SrII-D subgroup while CuP phytoplasma may be representative of a new subgroup 16SrII-M. Phylogenetic analysis using 16S rDNA full-length sequence confirmed the clustering of CuP and SqP phytoplasmas within the 16SrII group in the same clade with 16SrII-A and 16SrII-D subgroup phytoplasmas, respectively.

The fact that CuP and SqP phytoplasmas were transmitted by the same vector species (*O. albicintctus*), and that each of the CuP and SqP phytoplasmas was reciprocally transmitted to squash and cucumber, respectively, and both phytoplasmas generated the same symptoms in common experimental host plants indicated that

Table 2. Pairwise homology (%) among cucumber and squash phyllody phytoplasmas and selected phytoplasmas in group 16SrII using 16S rDNA sequences.

	'*Ca*. P. asteris'	'*Ca*. P. aurantifolia'	'*Ca*. P. australasia'	CoP	**CuP**	FBP	PnWB	PEY	**SqP**
'*Ca*. P. asteris'	100								
'*Ca*. P. aurantifolia'	90.2	100							
'*Ca*. P. australasia'	89.8	98.7	100						
CoP	90.0	99.5	98.7	100					
CuP	**89.1**	**98.0**	**99.3**	**98.0**	**100**				
FBP	89.3	99.1	98.2	99.1	**97.8**	100			
PnWB	89.3	98.2	99.3	98.1	**99.0**	98.5	100		
PEY	89.8	98.3	98.5	98.1	**97.9**	97.6	97.9	100	
SqP	**89.1**	**98.0**	**99.3**	**98.0**	**99.4**	**97.8**	**99.0**	**97.9**	**100**

'*Ca*. P. asteris' (16SrI, GenBank accession number, M30790); '*Ca.* P. aurantifolia' (16SrII-B, GenBank accession number, U15442); '*Ca.* P. australasia' (16SrII-D, GenBank accession number, JQ868448); CoP, cotton phyllody (16SrII-F, GenBank accession number, EF186827); CuP, cucumber phyllody (GenBank accession number, KR822804); FBP, faba bean phyllody (16SrII-C, GenBank accession number, X83432); PnWB, peanut witches' broom (16SrII-A, GenBank accession number, L33765); PEY, *Picris echoides* yellows (16SrII-E, GenBank accession number, Y16393); SqP, squash phyllody (GenBank accession number, KR822805). In bold data referred to CuP and SqP phytoplasmas.

CuP and SqP diseases may be associated with biologically undistinguishable phytoplasmas. Eight leafhopper genera including *C. haematoceps*, *N. fenestratus*, *E. decepiens*, *O. albicinctus*, *P. striatus*, *A. sinuata*, *E. zelleri* and *Zygina* sp. were collected from cucumber and squash fields in Chahgeer (Abarkooh, Yazd province). Among collected leafhoppers only *O. albicinctus* was PCR assay positive and in transmission experiments was proven for the first time as the natural vector of both CuP and SqP phytoplasmas. This leafhopper species was also collected from alfalfa, sesame and sugar beet fields in Chahgeer. *O. albicinctus* was previously reported as alfalfa witches' broom phytoplasma vector in Iran (Salehi *et al.*, 1995). *C. haematoceps* has been previously reported in Iran as the efficient vector of *Spiroplasma citri* (Salehi *et al*., 1993) and of some phytoplasma diseases, including sesame phyllody (Salehi and Izadpanah, 1992), rapeseed phyllody (Salehi *et al*., 2011) and cabbage yellows (Salehi *et al*., 2006). *P. striatus* has been also reported as vectors of a 16SrI-related phytoplasma in Iran (Salehi *et al*., 2005).

Association of a 16SrII-D subgroup phytoplasmas with *C. pepo* has been previously reported in Egypt (Omar and Foissac, 2012). CuP and SqP phytoplasmas were experimentally transmitted to alfalfa, cucumber, squash, carrot, eggplant, sunflower, pot marigold, parsley, sesame and tomato plants using *O. albicinctus*. Natural infection of these test plants with '*Ca.* P. aurantifolia' - related phytoplasmas (16SrII group) were reported from surveyed areas in Yazd province (Salehi *et al*., 2005; Esmailzadeh Hosseini *et al*., 2011). Phytoplasmas associated with alfalfa witches' broom (Salehi *et al*., 2014), sunflower (Salehi *et al*., 2015) and squash phyllody belong to subgroups 16SrII-C and -D respectively, while cucumber phyllody is a member of a new phytoplasma subgroup. The presence of different subgroups of group 16SrII in different plant species suggest a high level of genetic diversity and host susceptibility among the 16SrII phytoplasma strains affecting distinct Cucurbitaceae species in Chahgeer. Results indicate that 16SrII-related phytoplasmas are widespread in Yazd province. This is the first report of the molecular characterization of CuP and SqP phyllody phytoplasmas, and the identification of their common leafhopper vector and possible plant host range in Iran. The results of the present work show that cucumber and squash fields are sources of 16SrII phytoplasmas and *O. albicinctus* is a possible vector also for phytoplasma infection of other important crops in Yazd province.

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