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ORIGINAL ARTICLE

Occurrence and molecular characterization of a 16SrI-R subgroup phytoplasma associated with *Aquilegia vulgaris* phyllody disease

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Abstract

During 2016–2017 surveys, carried out for phytoplasma diseases in ornamental plants in Chaharmahal and Bakhtiari provinces, Iran, found symptoms of virescence, phyllody, reduced size of leaves and flowers in columbine (*Aquilegia vulgaris*). Total DNAs extracted from symptomatic and symptomless plants were tested for the presence of phytoplasma using P1/P7 and R16F2n/R16R2 primers in direct and nested PCR producing amplicons of about 1.8 and 1.2 kb, respectively, from all symptomatic *A. vulgaris* plants, but not from symptomless ones. The consensus sequence of the detected phytoplasma named *Aquilegia* phyllody (APh) was 100% identical with strains clustering to phytoplasmas enclosed in the 16SrI group as also confirmed by phylogenetic analyses. Both real and virtual restriction fragment length polymorphism analysis of R16F2n/R16R2 amplicons showed profiles that were identical to each other and indicated the affiliation of the APh phytoplasma to the 16SrI-R subgroup. This is the first report of a 16SrI-R phytoplasma associated with this *A. vulgaris* phyllody disease.

Keywords: columbine disease, phytoplasma, 16SrI-R subgroup

Introduction

Aquilegia vulgaris L. commonly known as columbine is an herbaceous plant belonging to the Ranunculaceae family growing in meadows and woodlands as a wild plant, and native to many areas of North America. However, numerous varieties are widely grown in floriculture worldwide or used as medicinal plants (Bastida *et al*. 2010; Noutsos *et al.* 2015). Phytoplasmas belonging to 16SrI-L and 16SrI-M subgroups were reported in columbine in Lithuania (Samuitiene *et al.* 2004). Columbine is mainly cultivated in Iran as an ornamental plant and the objective of this study was to identify the phytoplasmas associated with a recently detected *A. vulgaris* phyllody (APh) pathogen.

Materials and Methods

During 2016–2017 surveys for phytoplasma diseases of ornamental plants in Chaharmahal and Bakhtiari provinces, Iran, found flower phyllody and virescence in columbine plants in different floriculture fields in the Shahrekord area. Disease incidence was determined by sampling four ornamental plant fields in different regions. The number of symptomatic plants out of the total number of plants was calculated. Forty symptomatic plants and five symptomless plant were collected and subjected to molecular studies to verify the possible presence of phytoplasma and its identity. Total DNA was extracted from 0.3 g of midrib tissue of fresh leaves using the method described by Healey *et al.* (2014). Total DNA extracted

from healthy *A. vulgaris* plants grown from seeds in an insect proof greenhouse was used as a negative control. Total DNA of a periwinkle plant dodder inoculated with a phytoplasma strain associated with alfalfa (16SrXII-A) was used as a positive control (Esmailzadeh Hosseini *et al.* 2016). Assessment of extracted total DNA quality and quantity was estimated by spectophotermeter and agarose gel electrophoreses using standard techniques (Green and Sambrook 2012) and 1 µl (100 ng) of nucleic acid was used for each polymerase chain reaction (PCR).

The phytoplasma universal primer pair P1/P7 (Deng and Hiruki 1991; Schneider *et al.* 1995) was used for PCR assays. The amplified products diluted 1 : 30 in sterile deionized water were subjected to nested PCR using R16F2n/R16R2 primer pair (Gundersen and Lee 1996). The PCR mixtures containing 1 µl of diluted DNA and 0.4 µM of each primer were amplified using Emeraldamp PCR master mix (Takara Bio Inc., Japan). The reaction was cycled 35 times in a programmable thermocycler (Quanta Biotech, UK) for 30 s at 94°C for denaturation (2 min in the first cycle), 30 s at 55°C for annealing and 1 min at 72°C for primer extension (10 min in the final cycle). Nested PCR was performed under the same conditions except at 58°C for annealing temperature. Amplification results were verified by electrophoresis in an agarose gel 1% (w/v) in Tris-borate-EDTA (TBE) buffer and visualized with a UV imaging system (Isogene-Life Science, The Netherlands) after ethidium bromide staining.

The R16F2n/R16R2 amplified products of 20 *Aquilegia* phyllody samples exhibiting phyllody symptoms were digested separately with *Mse*I, *Hha*I, *Alu*I, *Hae*III, *Rsa*I, *Hpa*II, *Taq*I, *Kpn*I, *Tha*I and *Bfa*I restriction enzymes following the manufacturer's instructions (Thermo Scientific, Lithuania). The restriction products were examined in 8% polyacrylamide gel, stained and visualized as described above. The resulting RFLP patterns were compared with those previously published for 16S rDNA of other phytoplasmas (Lee *et al.* 1998; Jomantiene *et al.* 2011).

The R16mF2/R16mR1 (Gundersen and Lee 1996) primed PCR products (1.4 kb) from five samples of *A. vulgaris* phyllody phytoplasma (APh) were directly sequenced from both sides and the R16F2n/R16R2 trimmed fragment was assembled and compared with the sequences deposited in GeneBank database using BlastN and aligned with BioEdit tool. Amplified 16S rDNA consensus sequence of the APh phytoplasma was aligned with sequences of a number of phytoplasmas using ClustalW. Phylogenetic trees were constructed using the neighbor-joining method with MEGA7 software (Kumar *et al.* 2016). Bootstrapping was used 1,000 times to estimate the stability and support for the branches. *Acholeplasma laidlawii* were used as an outgroup to root the trees. The online tool *i*PhyClassifier (Zhao *et al.* 2009) was used for virtual restriction fragment length polymorphism (RFLP) analysis to determine the APh phytoplasma group affiliation. The sequences of the DNA fragments amplified with R16F2n/R16R2 primers pair were digested *in silico* with the 17 restriction enzymes.

Results

Infected *A. vulgaris* plants showed flower phyllody and virescence, stunting, small leaves, and a decrease in flower size (Fig. 1). The plants died 2–3 months

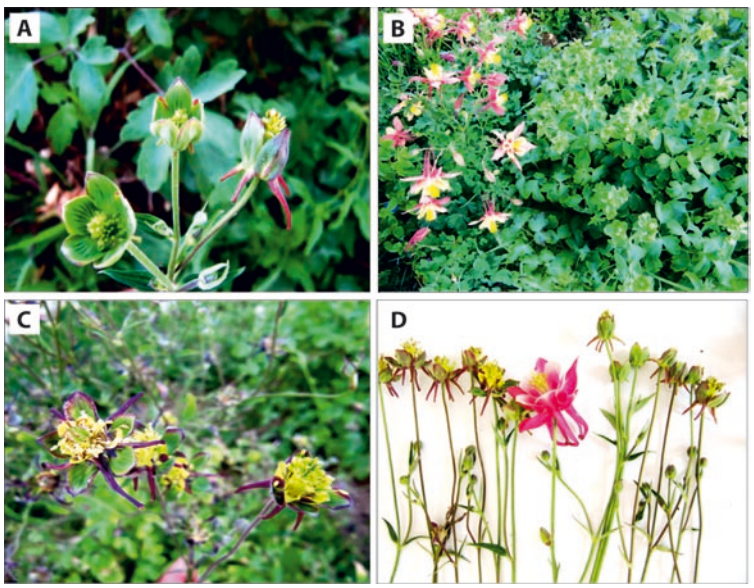

Fig. 1. Symptoms of *Aquilegia* phyllody disease from Chahrmahal and Bakhtiari provinces (Iran): A and B – flower virescence in different stages; C – phyllody and flower malformation; D – reduced flower size and color change during the progression of the disease in infected flowers (right and left) in comparison to an asymtomatic flower (center)

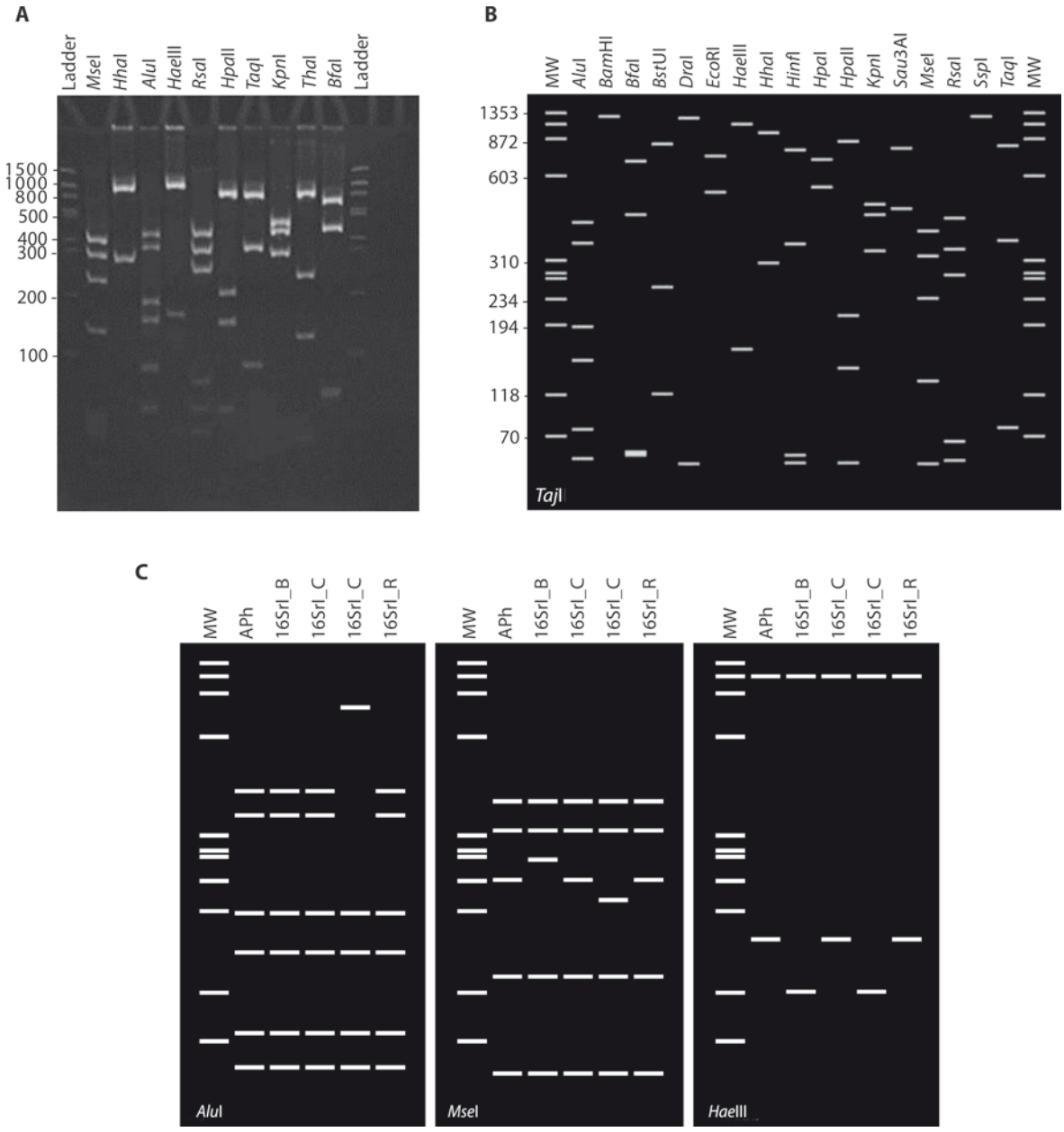

Fig. 2. A – polyacrylamide gel showing RFLP profiles of 16S rDNA amplified by nested PCR using P1/P7 followed by R16F2n/R16R2 from one representative *Aquilegia* phyllody phytoplasma (GenBank accession number MK307856) and the restriction profiles for other samples were the same. PCR products were digested by *Alu*I, *Hha*I, *Hpa*II, *Rsa*I, *Taq*I, *Kpn*I, *Hae*III, *Tha*I, *Bfa*I and *Mse*I. Ladder 100 bp DNA ladder (Bio Basic Inc., Canada); B – virtual RFLP generated with *i*PhyClassifier from *in silico* digestion of the R16F2n/R16R2 fragments of the *A. vulgaris* phyllody phytoplasma; C – virtual RFLP comparison between APh strain and phytoplasmas enclosed in 16SrI-C (clones rRNAa and rRNAb, respectively) and 16SrI-R using informative restriction enzyme that confirmed the subgroup affiliation. Phytoplasma subgroups are indicated in the picture, no name lane is APh; MW, ladder as in B, enzymes used are at the bottom of the picture

after the first appearance of disease symptoms. Average symptomatic plant percentage was 10% in different surveyed fields.

PCR amplicons of ~1.8 and ~1.2 kbp were obtained from all 40 symptomatic *A. vulgaris* plants from different fields and positive control using P1/P7 and R16F2n/R16R2 primer pairs in direct and nested PCR assays, respectively. No amplification was obtained from symptomless *Aquilegia* samples. An RFLP pattern of the 1.2 kb 16S rDNA amplicons of all symptomatic *A. vulgaris* samples from the surveyed areas were identical to each other and to members of the aster yellows phytoplasma group 16SrI (Fig. 2A).

The five ~1.4 kb DNA fragments amplified with R16mF2/R16mR1 from the APh samples were directly sequenced and the R16F2n/R16R2 trimmed fragments

showed 100% sequence identity when compared to each other. A 1.2 kb fragment from one representative sample of Shahrekord APh was submitted to GenBank database under the accession number MK307856 and the sequences for other samples were indistinguishable. BLAST search showed that this phytoplasma shares 99.4% identity with '*Candidatus* Phytoplasma asteris' (GenBank accession number M30790) (subgroup 16SrI-B). Results of RFLP analysis using the *i*PhyClassifier confirmed that virtual RFLP patterns with all 17 enzymes of the APh strain were identical (similarity coefficient 1.00) to the phytoplasma reference pattern of the cherry bunchy leaf phytoplasma from Lithuania (GenBank accession number HM067754), member of 16SrI-R subgroup (Fig. 2B). RFLP analyses with endonucleases *Alu*I, *Hae*III and *Mse*I from the examined plants further confirmed identical profiles for the *Aquilegia* samples (data not shown). Moreover, virtual RFLP analyses using as control phytoplasma strains belonging to 16SrI-B, the two sequences of the 16SrI-C subgroups (rRNA-A and rRNA-B GenBank accession numbers AF222065-66, respectively) and 16SrI-R indicated that these enzymes are useful to differentiate and identify this strain (Fig. 2C). Phylogenetic analysis confirmed that the APh phytoplasma clustered within the 16SrI phytoplasma with the strawberry phylloid fruit phytoplasma (GenBank accession number AY102275) and some other phytoplasmas classified in the 16SrI-R and 16SrI-C subgroups (Fig. 3).

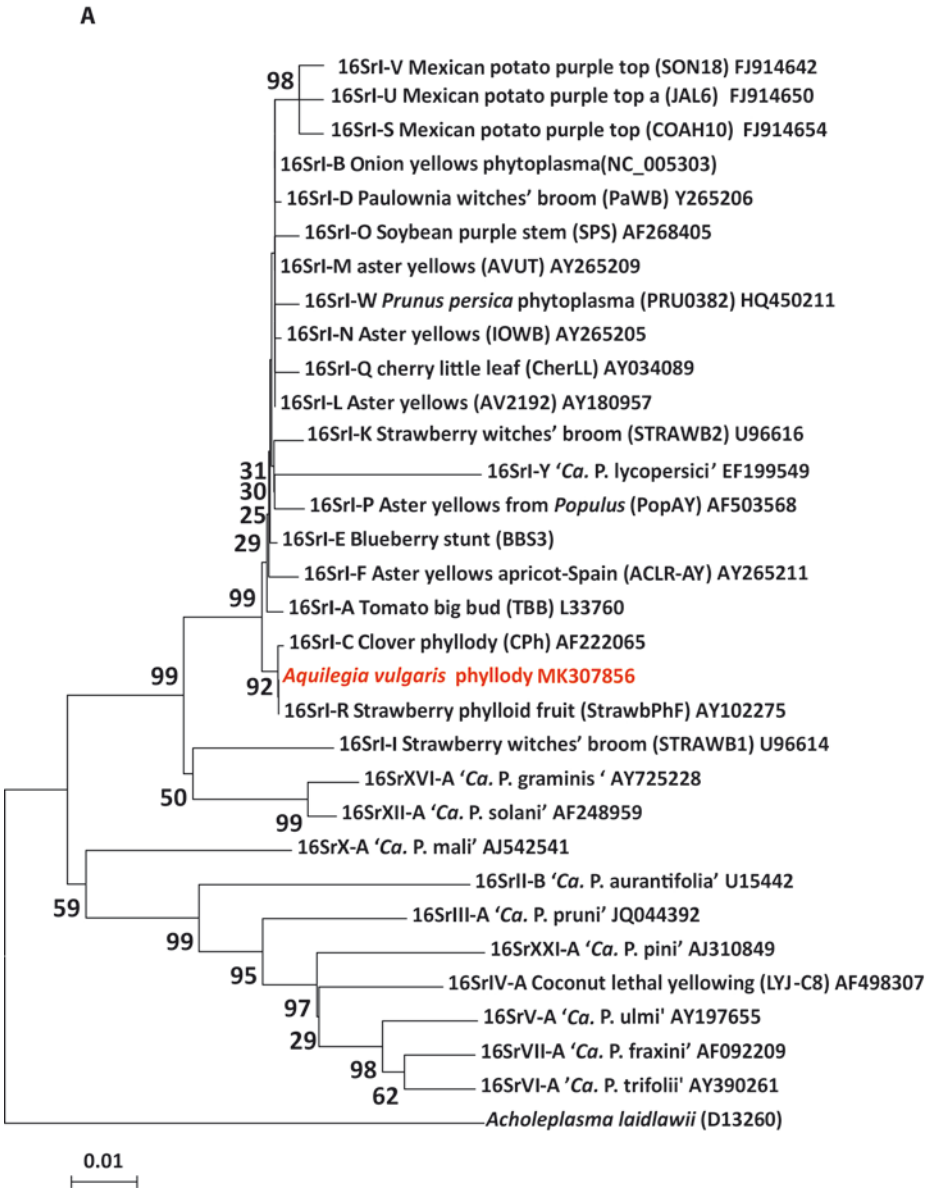

Fig. 3. A – phylogenetic tree constructed by the neighbor-joining method of 16S rRNA gene sequences from 32 phytoplasmas including *Aquilegia* phyllody phytoplasma (in red) and *A. laidlawii* as outgroup. Numbers at the nodes are bootstrap values based on 1,000 repetitions. Only values above 20 are shown. The ribosomal subgroup is on the left of the strain name and GenBank accession number is on the right

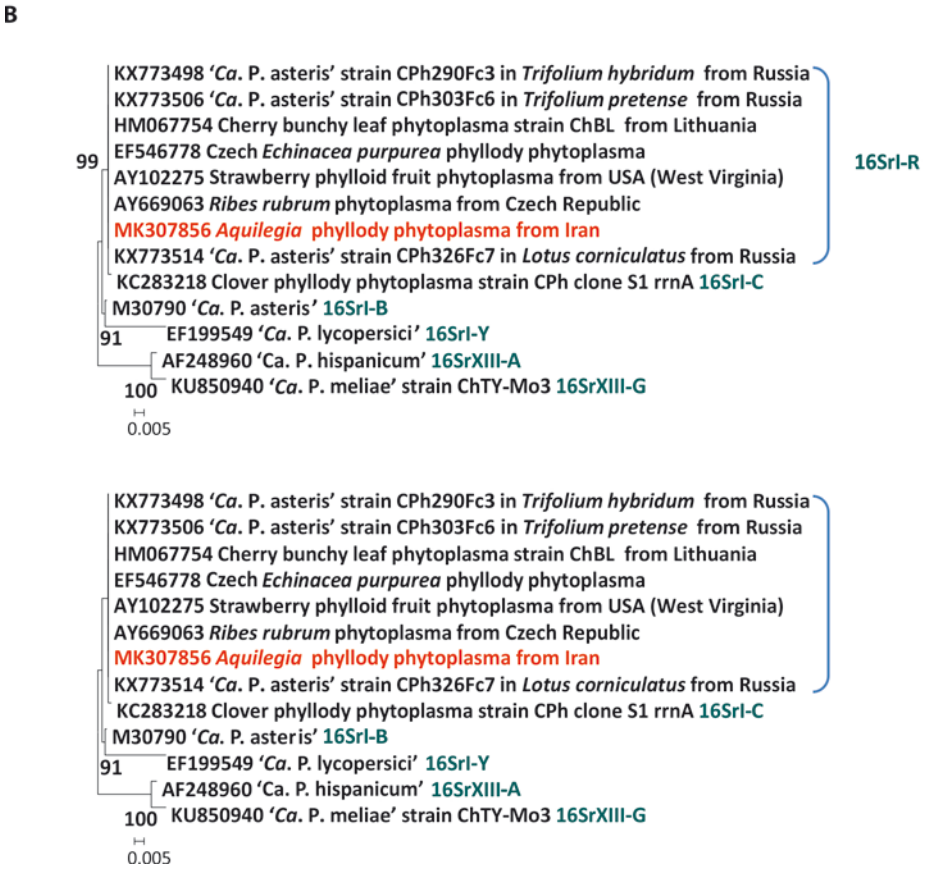

Fig. 3. B – phylogenetic analysis by maximum likelihood method based on the Tamura-Nei model (Tamura *et al.* 1993). Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 13 phytoplasma nucleotide sequences in group 16SrI. The sequences from 16SrXIII group were used as an outgroup. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016)

Discussion

Phytoplasma diseases associated with different phytoplasma groups and subgroups have been found in a variety of ornamental plants. These pathogens can seriously affect ornamentals by significantly impacting growth and flower quality, such as shape and color. Different phytoplasma groups have been reported from ornamental plants worldwide among which the 16SrI group members have the greatest distribution and host range (Kaminska 2008; Bellardi *et al.* 2018). Phytoplasmas belonging to 16SrI and 16SrIII have been reported to be associated with virescence, phyllody, proliferation, stunting and yellowing in such members of the Ranunculaceae family as *A. vulgaris, Delphinium* sp., and *Ranunculus* hybrid cv. Pauline (Samuitiene *et al.* 2004; Harju *et al.* 2008; Parrella *et al.* 2008).

The RFLP patterns of the APh phytoplasma are consistent with the affiliation of these phytoplasma strains to the subgroup 16SrI-R previously reported from strawberry and cherry in West Virginia (USA) and Lithuania, respectively (Jomantiene *et al.* 2002, 2011; Cieslinska *et al.* 2006). Interestingly, sequences from phytoplasmas of the same subgroup have also been found in infected *Lotus corniculatus* (GenBank accession number KX773514), *Trifolium pratense* (Accession number KX773506), and *Trifolium hybridum* (Accession number KX773498) from Russia (Girsova *et al.* 2017). Jomantiene *et al.* (2011) reported phytoplasmas associated with the 16SrI-C subgroup as being closely related to those of the newly established 16SrI-R subgroup.

In spite of several reports about the presence of aster yellows phytoplasmas (16SrI) in Iran (Babaie *et al.* 2007; Asghari Tazehkand *et al.* 2010; Rashidi *et al.* 2010; Esmailzadeh Hosseini *et al.* 2015a, b, c; Salehi *et al.* 2016, 2018) this is the first phytoplasma affiliated to the 16SrI-R subgroup in the country. This creates a greater awareness of the diversity of phytoplasmas in Iran. This in turn is helpful for planning the management of phytoplasma diseases that is primarily based

on controlling both the insect vectors and the main plant hosts that are phytoplasma reservoirs. Perennial ornamental plants such as *A. vulgaris* may play an important role as natural reservoirs of 16SrI-R phytoplasma that appear to be spreading more than is described in literature reports, in diverse areas of the world and on both herbaceous as well as shrubby and woody host species. The presence of this phytoplasma in cherry, strawberry and red currant (Jomantiene *et al.* 2002, 2011; Cieslinska *et al.* 2006; Přibylová *et al.* 2011) is an indication of its potentially dangerous presence in agricultural ecosystems. More research is needed for the verification of the presence of this phytoplasma in other plant hosts and for insect vector identification to avoid possible epidemic outbreaks for this emerging phytoplasma in Iran and in other parts of the world.

Conclusions

This study indicates the association of phytoplasmas in subgroup 16SrI-R with the phyllody disease of *A. vulgaris* in Iran. The identification of the phytoplasma makes it possible to verify its presence as an emerging disease in Iran and it is helpful for planning disease management considering its reported ability to infect fruit and small fruit crops in other parts of the world.

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