3 Biotech (2019) 9:65
https://doi.org/10.1007/s13205-019-1596-9

ORIGINAL ARTICLE

Association of a '*Candidatus* Phytoplasma aurantifolia'-related strain with apricot showing European stone fruit yellows symptoms in Iran

Rasoul Rasoulpour[1] · Mohammad Salehi[1] · Assunta Bertaccini[2]

Received: 8 August 2018 / Accepted: 25 January 2019 / Published online: 4 February 2019

Abstract
During 2012–2015 surveys in some orchards in Faraghe (Iran), a number of apricot trees showed symptoms resembling those associated with the phytoplasma disease known as European stone fruit yellows that are severe leaf roll, yellowing and die back. The presence of an infectious agent was confirmed by graft transmission experiments in which all the previously symptomless GF-677 (peach × almond) trees showed phytoplasma-type symptoms. The phytoplasma presence was confirmed by nested PCR assays using DNA extracted from samples from both field collected and graft-inoculated trees. The sequences of four nested PCR products from symptomatic apricot and experimentally graft-inoculated GF-677 trees were 100% identical to each other. RFLP and phylogenetic analyses carried out on these sequences allowed to cluster the strain infecting apricot trees in Iran with the16SrII-C subgroup phytoplasmas. This is the first report of a 16SrII-C phytoplasma associated with leaf roll and yellowing of the leaves in apricot trees.

Keywords Stone fruits · Apricot tree · Leaf roll · Yellowing · 16SrII phytoplasma

Introduction

Phytoplasmas are wall-less plant pathogenic bacteria associated with diseases of great economic importance (Lee et al. 2000; Bertaccini et al. 2014). The phytoplasma classification based on 16S rRNA gene allows to distinguish 43 '*Candidatus* Phytoplasma' species (Bertaccini and Lee 2018). RFLP analysis of 16S rRNA gene is also used for classification of phytoplasma strains in 33 16Sr groups and more than 200 subgroups (Zhao and Davis 2016). These techniques were used for rapid detection and identification of phytoplasmas in different crops throughout Iran. To date, phytoplasma strains belonging to 11 '*Candidatus* Phytoplasma' species have been identified in different plants including herbaceous and woody cultivated species (Salehi et al. 2005, 2006; Faghihi et al. 2010; Ghayeb Zamharir et al. 2017; Salehi and Esmailzadeh Hosseini 2017). Several phytoplasma diseases from different countries are known as a major threat for the production of stone fruits. '*Ca*. P. pruni', '*Ca*. P. phoenicium' and '*Ca*. P. prunorum' are among the most important phytoplasmas associated with significant losses to different *Prunus* species (Foissac and Wilson 2010). However, the European stone fruit yellows (ESFY, '*Ca*. P. prunorum') is the most important phytoplasma disease of *Prunus* species with a distribution apparently limited to the Europe and Asia Minor (Jarausch et al. 2001).

Apricot is one of the most important stone fruits produced in Iran, mainly cultivated in the northwest, central and south regions. In surveys performed during 2012–2015 in orchards in Faraghe (Abarkooh, Yazd province, Iran), in the center of Iran, a disease consisting of upward leaf roll and yellow symptoms on the whole crown was observed. Studies were, therefore, carried out to verify the presence and association of phytoplasmas using biological and molecular methods.

Materials and methods

Sample collection

Apricot trees with and without symptoms were selected for sampling in four locations of Faraghe (30 km West of

✉ Mohammad Salehi
salehi_abarkoohi@yahoo.com

[1] Plant Protection Research Department, Fars Agricultural and Natural Resources Research and Education Center, AREEO, Zarghan, Iran

[2] Department of Agricultural and Food Sciences, Alma Mater Studiorum-University of Bologna, Bologna, Italy

Abarkooh, Yazd province, Iran). Sixteen samples (four per location from four trees) were collected from chlorotic leaf roll-affected apricot trees (Felakei cultivar). Symptomless samples (2) were also included as negative controls.

Disease transmission

Two-year-old GF-677 (peach × almond) trees propagated by cuttings and nested PCR tested (Salehi et al. 2016) to verify the phytoplasma absence were used for the transmission of the apricot chlorotic leafroll disease agent. Each GF-677 tree was side grafted with three scions consisting of small branches with two to three leaves, from symptomatic apricot trees. Eight GF-677 trees (two for location) were grafted and four trees were left as control and maintained under insect-proof nets.

DNA extraction and polymerase chain reaction (PCR)

The midribs and petioles of symptomatic leaves (1 g) were used for total DNA extraction (Zhang et al. 1998) followed by a further purification using DNP™ high-yield DNA purification kit (CinnaGen, Iran). DNA from symptomless trees and a periwinkle plant infected with the lime witches' broom phytoplasma (Salehi et al. 2005) was used as negative and positive control, respectively. Direct PCR was carried out by the universal primer pair P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) followed by nested PCR using the primer pair R16F2n/R2 (Gundersen and Lee 1996).

Restriction fragment length polymorphism (RFLP) analysis

Each nested PCR product (8 µl, 500 ng DNA) was digested separately with the restriction enzymes *Hae*III, *Mse*I, and *Taq*I (Fermentas, Vilnius, Lithuania) as described (Rasoulpour et al. 2017). The PCR product from a periwinkle plant infected with Yazd alfalfa witches' broom phytoplasma (16SrII-C, GenBank Accession number DQ233656) (Salehi et al. 2015) was used as control. The resulting patterns were compared with those of the control and other phytoplasmas (Lee et al. 1998). Virtual RFLP analysis using *i*PhyClassifier was also used to confirm phytoplasma subgroup affiliation (Zhao et al. 2009).

Sequencing of PCR products and phylogenetic analysis

Amplified products with R16F2n/R2 primers from four affected apricot trees (one per location) and four experimentally inoculated GF-677 trees were cloned and sequenced as described (Rasoulpour et al. 2017). The 16S rDNA sequences (1250 bp) were used for search in the GenBank database at the National Center for Biotechnology Information (NCBI). Nucleotide sequences of phytoplasmas from different groups were then compared with a representative of the Iranian samples using MEGA7 (Kumar et al. 2016) and a phylogenetic tree was constructed.

Results and discussion

Disease symptomatology and transmission

In the selected orchards in Faraghe area, up to 12.5% of the apricot trees showed symptoms of chlorotic leafroll on one branch or on the whole crown with scattered die back of several branches (Fig. 1). The graft transmission from symptomatic apricot trees induced symptoms of little leaf, internode shortening, yellowing and stunting (Fig. 2) in all the inoculated GF-677 trees. The minimum time between inoculation and symptom expression was of 21 months and confirmed the transmissibility of the pathogen to stone fruits other than apricot indicating its possible relevance for further epidemic to this and other stone fruit species. All GF-677 trees were phytoplasma positive in nested PCR, while control plants were negative for phytoplasma presence and showed no symptoms.

PCR amplification and sequence analysis

In direct PCR, the expected length fragment was amplified only from the lime witches' broom affected periwinkle plant, while in nested PCR the expected 1250 bp fragments were obtained from 14 out of the 16 samples from symptomatic apricot, from all the GF-677 graft-inoculated trees and from the positive control. In direct and nested PCR assays, no bands were observed for two symptomatic trees and the negative controls. The RFLP patterns of all the PCR products were identical with the enzymes used (Fig. 3).

The sequences of four nested PCR products from symptomatic apricot and graft-inoculated GF-677 trees were 100% identical and one of them from Faraghe was submitted to the GenBank (accession number KU962122). BLASTn analysis of this fragment showed the maximum sequence identity with phytoplasmas in the peanut witches' broom (16SrII) group and in particular 100% identity with faba bean phyllody phytoplasma (GenBank accession number X83432), reference strain for the 16SrII-C subgroup and 98.9% identity with '*Ca*. P. aurantifolia' enclosed in the 16SrII-B subgroup sequences. The phylogenetic analysis (Fig. 4) allow the clustering of this phytoplasma with phytoplasmas in the 16SrII group closest to those enclosed in the 16SrII-C subgroup. Results of phylogenetic analysis and of virtual RFLP (Figs. 5 and 6) confirmed that this phytoplasma is clustering

Fig. 1 Symptoms observed in diseased apricot trees in Faraghe (Abarkooh, Yazd province, Iran): **a** severe leaf roll on a branch, **b** leafroll and dieback, **c** leaf roll and yellowing on one branch, **d** general yellowing on the whole tree

Fig. 2 Little leaf, internode shortening and yellowing in a GF-677 tree graft inoculated with the apricot chlorotic leafroll agent from Faraghe (Abarkooh, Yazd province, Iran) (**a**) in comparison with a healthy one (**b**)

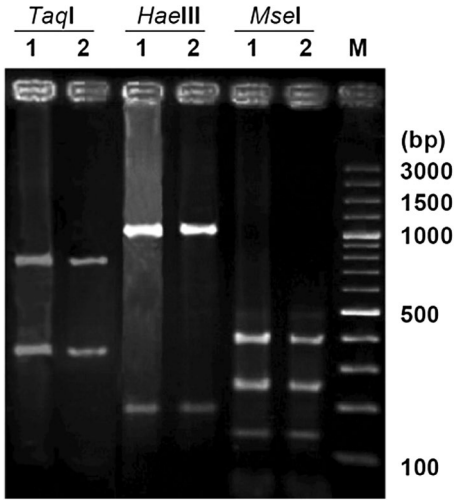

Fig. 3 RFLP profiles of nested PCR products using P1/P7 and R16F2n/R2 primer pairs from the phytoplasma detected in the apricot chlorotic leaf roll from Faraghe and Yazd alfalfa witches' broom phytoplasma (lanes 1 and 2, respectively). Lane M, 100-bp DNA molecular weight marker (Fermentas, Vilnius, Lithuania). DNA products were digested with enzymes listed at the top of the figure

with phytoplasmas in the 16SrII group and is classified in the 16SrII-C subgroup. The apricot chlorotic leafroll disease detected in Iran resulted, therefore, to be associated with the presence of phytoplasmas in this ribosomal subgroup.

The leaf roll, yellowing and die back symptoms observed in the apricot trees in Abarkooh are undistinguishable from those associated with the presence of '*Ca*. P. prunorum' in the same species in Europe (Necas and Krska 2005; Cieslinska 2011). Recently, a phytoplasma strain related to '*Ca*. P. prunorum' was found in association with a plum yellow leaf stunt disease in North of Iran (Allahverdi et al. 2014). However, in Iran the association of '*Ca*. P. aurantifolia'-related strains with diseases of other stone fruits including sweet cherry, plum and almond has also been described (Zirak et al. 2009a, b, 2010) but this is the first report of a '*Ca*. P. aurantifolia'-related strain in apricot. The presence of two symptomatic apricot trees that resulted PCR negative may be related to the low concentration or to the uneven distribution of the phytoplasma in the tissues since the severity and distribution of symptoms in the trees may be related to the disease progression steps.

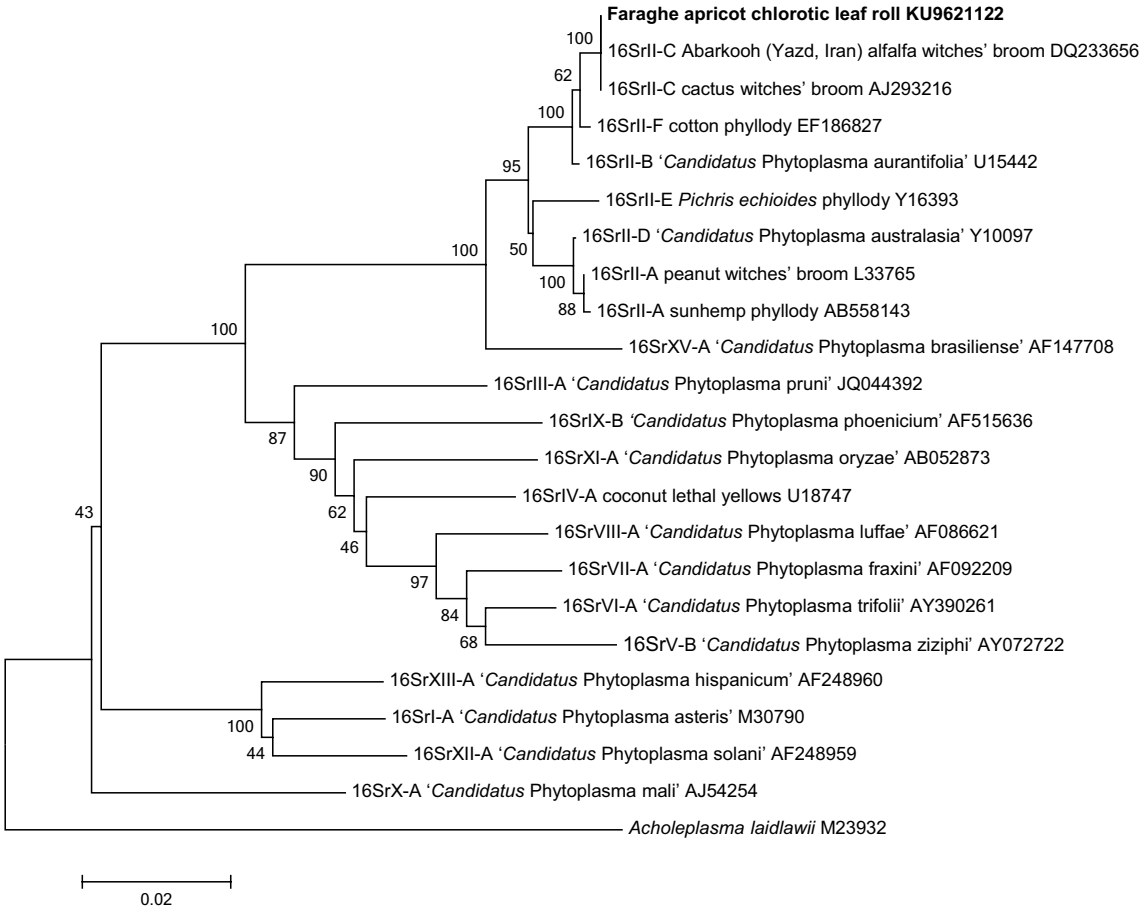

Fig. 4 Phylogenetic tree constructed by neighbor-joining analysis of 16S rRNA gene sequences from 21 phytoplasmas including Faraghe apricot chlorotic leaf roll phytoplasma (in bold). *Acholeplasma laidlawii* was used as out-group. GenBank accession numbers are indicated on the right. Numbers at the nodes are confidence values based on 1000 repetitions

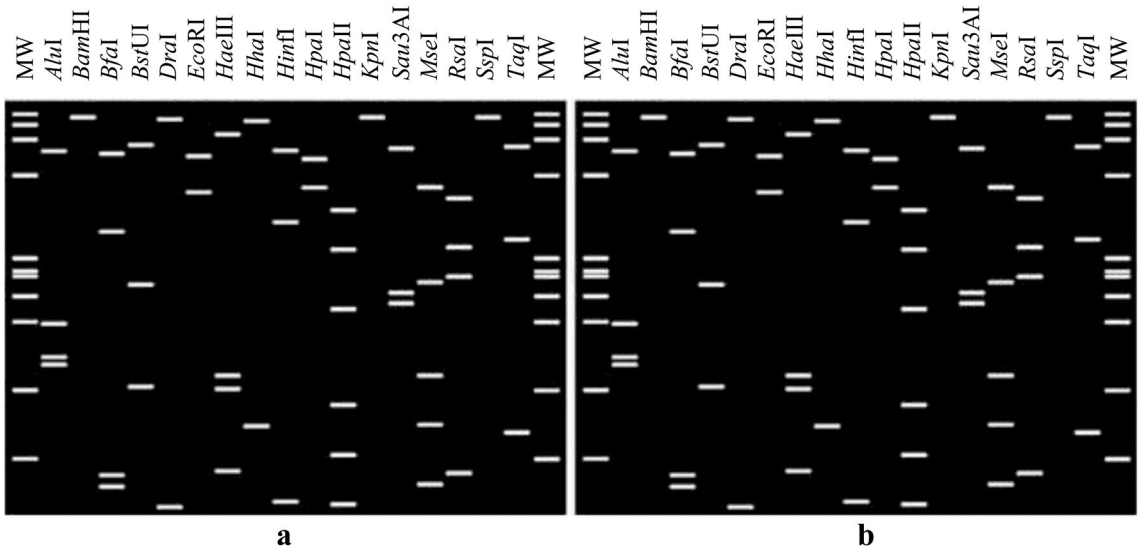

Fig. 5 Computer-simulated virtual RFLP patterns obtained from in silico digestions of 1.2 kb fragments of faba bean phyllody phytoplasma (16SrII-C, GenBank accession number X83432) (**a**), and apricot chlorotic leafroll phytoplasma (**b**) with 17 restriction enzymes using the online *i*PhyClassifier. Lane MW: phiX174 RFI DNA *Hae*III digest; fragment sizes (bp) from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72

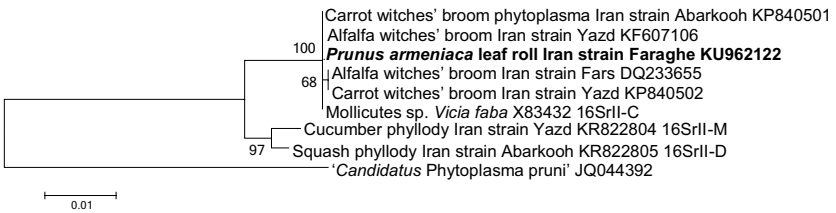

Fig. 6 Phylogenetic tree constructed by neighbor-joining analysis of 16S rRNA gene sequences from nine phytoplasmas including Faraghe apricot phytoplasma (in bold). '*Candidatus* Phytoplasma pruni' (16SrIII-A) was used as out-group. GenBank accession numbers are on the right followed by phytoplasma ribosomal subgroups when different from 16SrII-C

Platymetopius rostratus, *Phlepsius intricatus, Psammotettix alienus* (Cicadellidae) and *Laodelphax striatellus* and *Toya propinqua* (Delphacidae) were identified in an insect collection trial on grasses covering these apricot orchards (data not shown). Among these collected insect species *P. alienus* and *L. striatellus* are potential vectors of phytoplasmas (Cvrković et al. 2011; Orságová et al. 2011) and may spread the disease among the apricot trees in the orchards; however, this aspect requires further studies. Apricot propagation by grafting or import of asymptomatic infected apricot seedlings can represent further sources of inoculum. In the Abarkooh cultivation areas, the major phytoplasmas identified so far are mainly classified in the 16SrII-C subgroup (Fig. 6) and were detected in alfalfa (Esmailzadeh Hosseini et al. 2015), carrot (Salehi et al. 2016) and cucurbits (Salehi et al. 2015) growing as mixed cultivation with or around the apricot trees. Further researches are required to verify the apricot disease epidemiology and the relationship between the phytoplasmas detected in the herbaceous crops and apricot trees to design a reliable disease control strategy.

Acknowledgements This research was supported by a Grant from Fars Agricultural and Natural Resources Research and Education Center, AREEO, Zarghan, Iran.

Compliance with ethical standards

Conflict of interest There is no conflict of interest.

References

Allahverdi T, Rahimian H, Babaeizad V (2014) Association of '*Candidatus* Phytoplasma prunorum' with plum yellow leaf stunt in Iran. Iran J Plant Pathol 50:99

Bertaccini A, Lee I-M (2018) Phytoplasmas: un update. In: Rao GP, Bertaccini A, Fiore N, Liefting L (eds) Phytoplasmas: plant

pathogenic bacteria—I. Springer Nature Singapore Pte Ltd., Singapore, pp 1–30
Bertaccini A, Duduk B, Paltrinieri S, Contaldo N (2014) Phytoplasmas and phytoplasma diseases: a severe threat to agriculture. Am J Plant Sci 5:1763–1788
Cieslinska M (2011) European stone fruit yellows disease and its causal agent '*Candidatus* phytoplasma prunorum'. J Plant Prot Res 5:441–447
Cvrković A, Jović J, Mitrović M, Krstić O, Krnjalić S, Toševski I (2011) Potential new hemipteran vectors of "stolbur" phytoplasma in Serbian vineyards. Bull Insect 64(Suppl):S129–S130
Deng S, Hiruki C (1991) Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J Microbiol Methods 14:53–61
Esmailzadeh Hosseini SA, Khodakaramian G, Salehi M, Fani SR, BolokYazdi HR, Raoufi D, Jadidi O, Bertaccini A (2015) Status of alfalfa witches' broom phytoplasma disease in Iran. Phytopathog Mollicutes 5(1-Suppl):S65–S66
Faghihi MM, Siampour M, Zaeifi M, Bagheri AN, Salehi M, Samavi S (2010) First report of a phytoplasma associated with *Periploca aphylla* witches' broom in Iran. Plant Pathol 59:400
Foissac X, Wilson MR (2010) Current and possible future distributions of phytoplasma diseases and their vectors. In: Weintraub PG, Jones P (eds) Phytoplasmas: genomes plant hosts and vectors. CAB International, Oxford
Ghayeb Zamharir M, Paltrinieri S, Hajivand S, Taheri M, Bertaccini A (2017) Molecular identification of diverse '*Candidatus* Phytoplasma' species associated with grapevine decline in Iran. J Phytopathol 165:407–413
Gundersen DE, Lee I-M (1996) Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathol Medit 35:144–151
Jarausch W, Jarausch-Wertheim B, Danet J-L, Broquaire JM, Dosba F, Saillard C, Garnier M (2001) Detection and identification of European stone fruit yellows and other phytoplasmas in wild plants in the surroundings of apricot chlorotic leaf roll-affected orchards in southern France. Eur J Plant Pathol 107:209–217
Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874
Lee I-M, Gundersen-Rindal DE, Davis RE, Bartoszyk IM (1998) Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int J Syst Bacteriol 48:1153–1169
Lee I-M, Davis RE, Gundersen-Rindal DE (2000) Phytoplasma: phytopathogenic mollicutes. Annu Rev Microbiol 54:221–255
Necas T, Krska B (2005) Detection of phytoplasma ESFY in apricot trees using phloem and petioles. Plant Prot Sci 41:132–140
Orságová H, Březíková M, Schlesingerová G (2011) Presence of phytoplasmas in hemipterans in Czech vineyards. Bull Insect 64(Suppl):S119–S120
Rasoulpour R, Salehi M, Salehi E (2017) Detection and partial characterization of a 16SrIX-C phytoplasma associated with hemp (*Cannabis sativa* L.) witches' broom in Iran. J Plant Pathol 99:219–223
Salehi M, Esmailzadeh Hosseini SA (2017) First report of a 16SrXI group phytoplasma ('*Candidatus* phytoplasma oryzae') associated with *Cyperus* spp. white leaf disease in Iran. J Plant Pathol 99:809
Salehi M, Heydarnejad J, Izadpanah K (2005) Molecular characterization and grouping of 35 phytoplasmas from central and southern provinces of Iran. Iran J Plant Pathol 41:62–64
Salehi M, Izadpanah K, Heydarnejad J (2006) Characterization of a new almond witches' broom phytoplasma in Iran. J Phytopathol 154:386–391
Salehi M, Siampour M, Esmailzadeh Hosseini SA, Bertaccini A (2015) Characterization and vector identification of phytoplasmas associated with cucumber and squash phyllody in Iran. Bull Insectol 68:311–319
Salehi M, Esmailzadeh Hosseini SA, Salehi E, Bertaccini A (2016) Molecular and biological characterization of a 16SrII phytoplasma associated with carrot witches' broom in Iran. J Plant Pathol 98:83–90
Schneider B, Seemüller E, Smart CD, Kirkpatrick BC (1995) Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: Razin S, Tully JC (eds) Molecular and diagnostic procedures in mycoplasmology, vol 1. Academic, San Diego, pp 369–380
Zhang YP, Uyemoto JK, Kirkpatrick BC (1998) A small-scale procedure for extracting nucleic acids from woody plants infected with various phytoplasmas for PCR assay. J Virol Methods 71:45–50
Zhao Y, Davis RE (2016) Criteria for phytoplasma 16Sr group/subgroup delineation and the need of a platform for proper registration of new groups and subgroups. Int J Syst Evol Microbiol 66:2121–2123
Zhao Y, Wei W, Lee I-M, Shao J, Suo X, Davis RE (2009) Construction of an interactive online phytoplasma classification tool, *i*PhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). Int J Syst Evol Microbiol 59:2582–2593
Zirak L, Bahar M, Ahoonmanesh A (2009a) Characterization of phytoplasmas associated with almond diseases in Iran. J Phytopathol 157:736–741
Zirak L, Bahar M, Ahoonmanesh A (2009b) Molecular characterization of phytoplasmas related to peanut witches' broom and "stolbur" groups infecting plum in Iran. J Plant Pathol 91:713–716
Zirak L, Bahar M, Ahoonmanesh A (2010) Characterization of phytoplasmas related to '*Candidatus* Phytoplasma asteris' and peanut WB group associated with sweet cherry diseases in Iran. J Phytopathol 158:63–65