

Rapid Communication

Characterization of phytoplasmas related to 'Candidatus Phytoplasma asteris' subgroup *rpl-L* in Iran

Fereshteh Vali-Sichani, Masoud Bahar, Leila Zirak*

Division of Plant Pathology, Department of Plant Protection, College of Agriculture, Isfahan University of Technology, Isfahan 8415683111, Iran

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Abstract: In two of Iran's central provinces, several herbaceous plants showing phytoplasma disease symptoms were collected to detect 'Candidatus Phytoplasma asteris'-related phytoplasmas. Confirmation of an association of phytoplasmas with diseased plants was done using polymerase chain reaction (PCR) assays having the phytoplasma universal primer pairs P1/P7 followed by R16F2n/R16R2 in nested PCR. Then, for detection of 'Ca. P. asteris', DNA samples were subjected to amplification of *rp* and *tuf* genes using specific primer pairs rp(I)F1A/rp(I)R1A and fTufAy/rTufAy, respectively. Restriction fragment length polymorphism or RFLP analyses of *rp* gene fragments using *Tsp509I* restriction enzyme as well as sequence analyses indicated that 'Ca. P. asteris'-related phytoplasmas associated with carrot, niger seed and scallion plants in these regions, belong to the *rpl-L* subgroup. This research is the first report of carrot, niger seed, and scallion infection with phytoplasmas belonging to the *rpl-L* subgroup.

Key words: 'Candidatus Phytoplasma asteris', carrot, Iran, niger seed, *rpl-L*, scallion

Introduction

'Candidatus Phytoplasma asteris', the widespread phytoplasma species which is classified in the aster yellows group, infects a large number of annual and perennial plants worldwide. Based on restriction fragment length polymorphism (RFLP) and phylogenetic analyses of 16S rRNA, *rp*, *tuf*, *secY* and other genes, the aster yellows group has been differentiated into several distinct subgroups (Lee *et al.* 2004). Previously, phytoplasmas related to 'Ca. P. asteris' have been reported as the causal agent of several plant diseases in Iran (Babaie *et al.* 2007; Zirak *et al.* 2010; Hosseini *et al.* 2011), however, subgroup affiliations of these phytoplasmas is still unknown. In this study, we found that some of 'Ca. P. asteris'-related isolates, in the centre of Iran, belong to *rpl-L* subgroup. There has been an extensive spread of 'Ca. P. asteris' around the world, except for in Mexico and India, but the subgroup *rpl-L* phytoplasmas have not been reported anywhere up to now. This research is the first report of phytoplasmas belonging to *rpl-L* subgroup associated with carrot (*Daucus carota* L.), niger seed [*Guizotia abyssinica* (L.f.) Cass.], and scallion (*Allium fistulosum* L.) plants worldwide.

Materials and Methods

Several herbaceous plants including oilseeds, ornamentals, vegetables, and weeds, showing symptoms of phytoplasma diseases, were collected from Isfahan and Yazd provinces of central Iran.

An extraction of the total DNA from leaf midribs was done using the method originally described by Murray and Thompson (1980). For general detection of phytoplasmas, polymerase chain reaction (PCR) amplifications were performed using universal phytoplasma primer pair P1/P7, which amplify 1784 bp 16S rRNA, spacer region and 23S rRNA genes fragment (Deng and Hiruki 1991; Schneider *et al.* 1995), followed by primer pair R16F2n/R16R2 which amplify 1239 bp fragment in nested PCR (Lee *et al.* 1993). Also to amplify the *rp* operon, aster yellows group specific primer pair rp(I)F1A/rp(I)R1A was used to amplify a 1,212 bp fragment (Martini *et al.* 2007). Moreover, amplification of 940 bp fragment using the *tuf* gene specific primer pair fTufAy/rTufAy (Macone *et al.* 2000) confirmed the presence of 'Ca. P. asteris'-related isolates among the collected samples. The total volume of 20 µl of PCR mixtures contained 20 ng DNA, 0.2 mM each of dNTPs (Cinnagen, Iran), 1U of *Taq* DNA polymerase (Cinnagen), 0.5 µl of each of the primer pairs (20 pmol/µl), 2 mM MgCl₂ and 1X polymerase buffer. Finally, the reaction mixtures were electrophoresed in 1.2% (w/v) agarose gel prepared with Tris-Borate-EDTA (TBE) buffer, and stained with 5 µg/ml ethidium bromide. DNA bands were visualized with a UV transilluminator.

Then, the 1,212 bp *rp* gene fragments were subjected to single digestion using *AluI*, *MseI* and *Tsp509I* restriction enzymes according to the manufacturer's instructions (Fermentas, Lithuania). The restricted fragments

*Corresponding address:
lzirak@alumni.iut.ac.ir

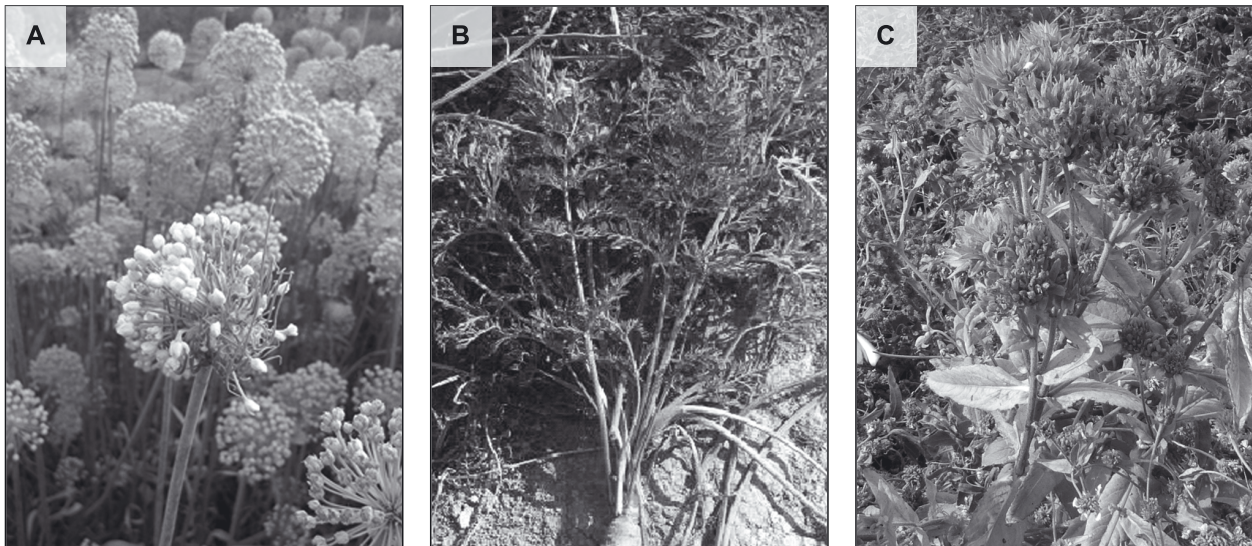


Fig. 1. Symptoms of diseases caused by subgroup *rpl-L* phytoplasmas on (A) scallion, (B) carrot and (C) niger seed, in the central regions of Iran

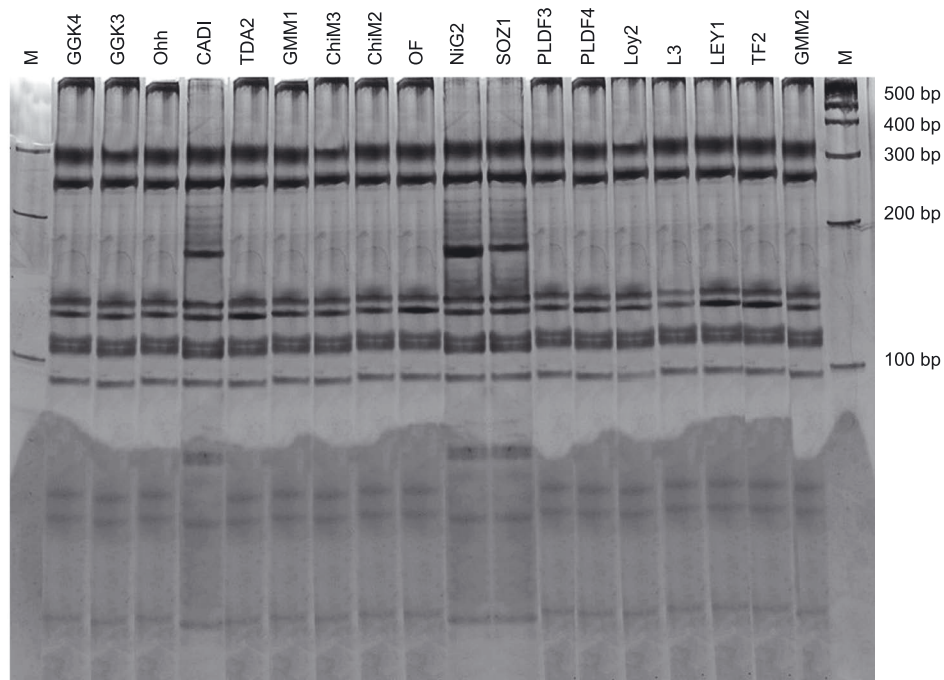


Fig. 2. RFLP analyses of 1212 bp *rp* gene sequences from Iranian '*Ca. P. asteris*'-related phytoplasmas using *Tsp509I* restriction enzyme. Isolates CADI, NiG2, and SOZ1 are related to subgroup *rpl-L* and others are related to subgroup *rpl-B*. Fragments were separated with electrophoresis through 12% polyacrylamide gel

were separated with electrophoresis through 12% polyacrylamide gel and stained with silver nitrate (Fig. 2).

Finally, three 1,212 bp *rp* gene fragments from infected carrot (isolate CADI), niger seed (isolate NiG2), and scallion (isolate SOZ1) plants were selected for *rp* gene sequence analyses. Also selected for sequence analyses was a 1784 bp 16S/23S rRNA gene fragment from the niger seed plant (isolate NiG2). All fragments were inserted into a PTZ57R/T vector using the InsTAClone[®] PCR Cloning kit, according to the manufacturer's instructions (Fermentas) and ligated into competent cells of *Escherichia coli* MC1061. The *rp* gene sequences were submitted in GenBank under accessions HM626105 (isolate NiG2),

HM626109 (isolate SOZ1), and HQ286477 (isolate CADI). Also, the rRNA sequence from isolate NiG2 was submitted in GenBank under accession JQ015289. For phylogenetic analyses, a tree was constructed by maximum parsimony analyses of *rp* gene sequences from three Iranian aster yellows phytoplasmas, and *rp* gene sequences of 21 aster yellows phytoplasmas (Fig. 3). A nearly full length 16S/23S rRNA sequence of isolate NiG2 and 30 sequences submitted in GenBank were used for construction of another tree (Fig. 4). Phylogenetic trees were constructed using MEGA 5.05 software, and bootstrapping was done in 1,000 replicates.

Results

After the general detection of phytoplasmas in collected plants, DNA samples which amplified expected fragments using universal primer pairs were subjected to amplification of *rp* and *tuf* genes to detect the probable '*Ca. P. asteris*'-related isolates. Amplification was done of the 1,212 bp *rp* genes using aster yellows specific primer pairs *rp*(I)F1A/*rp*(I)R1A from DNA samples of some plants. Also, amplification of 940 bp *tuf* gene fragments confirmed that the isolates are related to '*Ca. P. asteris*'.

All of the 1,212 bp *rp*(I)F1A/*rp*(I)R1A-amplified fragments were subjected to single digestion with *AluI*, *MseI* and *Tsp509I* restriction endonucleases for aster yellows subgroup differentiations according to a previous study (Lee *et al.* 2004). Using two *AluI* and *MseI* enzymes, no polymorphism appeared among the different isolates (data not shown). However, using the *Tsp509I* enzyme, isolates CADI, NiG2, and SOZ1 which were associated with symptomatic carrot, niger seed, and scallion, respectively, shared nearly the same patterns and were different from all other examined isolates (Fig. 2). Our previous work indicated that isolates which shared *Tsp509I* patterns were different from isolates CADI, NiG2, and SOZ1 belonging to subgroup *rpl*-B (Vali Sichani *et al.* 2014). Since there were two different types of *Tsp509I* RFLP patterns, it is probable that isolates CADI, NiG2, and SOZ1

belong to a subgroup different from subgroup *rpl*-B. In other words, a comparison between *Tsp509I* RFLP patterns of these three isolates with the isolates described by Lee *et al.* (2004) revealed that these three isolated could be related to subgroup *rpl*-L.

In the field, there were infected carrot shrubs showing typical phytoplasma disease symptoms include proliferation, leaf reddening and yellowing. Scallion shrubs showed flower malformation symptoms. Phyllody symptoms were observed in infected niger seed plants (Fig. 1). To characterise the phytoplasmas associated with carrot, niger seed, and scallion plants, *rp* gene fragments were sequenced. The blast search indicated that the Iranian carrot phytoplasma strain CADI, Iranian onion (scallion) phytoplasma strain SOZ1, and Iranian niger seed phyllody phytoplasma strain NiG2 shared a 99% similarity with maize bushy stunt phytoplasma (AY264858) – the member of subgroup *rpl*-L. However, a blast search of 16S/23S rRNA sequence showed that Iranian niger seed phyllody phytoplasma strain NiG2 shared a 99% identity with periwinkle virescence phytoplasma (DQ381535) and maize bushy stunt phytoplasma (AF487779) – the members of subgroup 16SrI-B.

The tree constructed with *rp* sequences of three Iranian aster yellows isolates and 21 aster yellows *rp* sequences which were previously used for *rpl* subgroups studied by Lee *et al.* (2004) showed that isolates CADI, SOZ1,

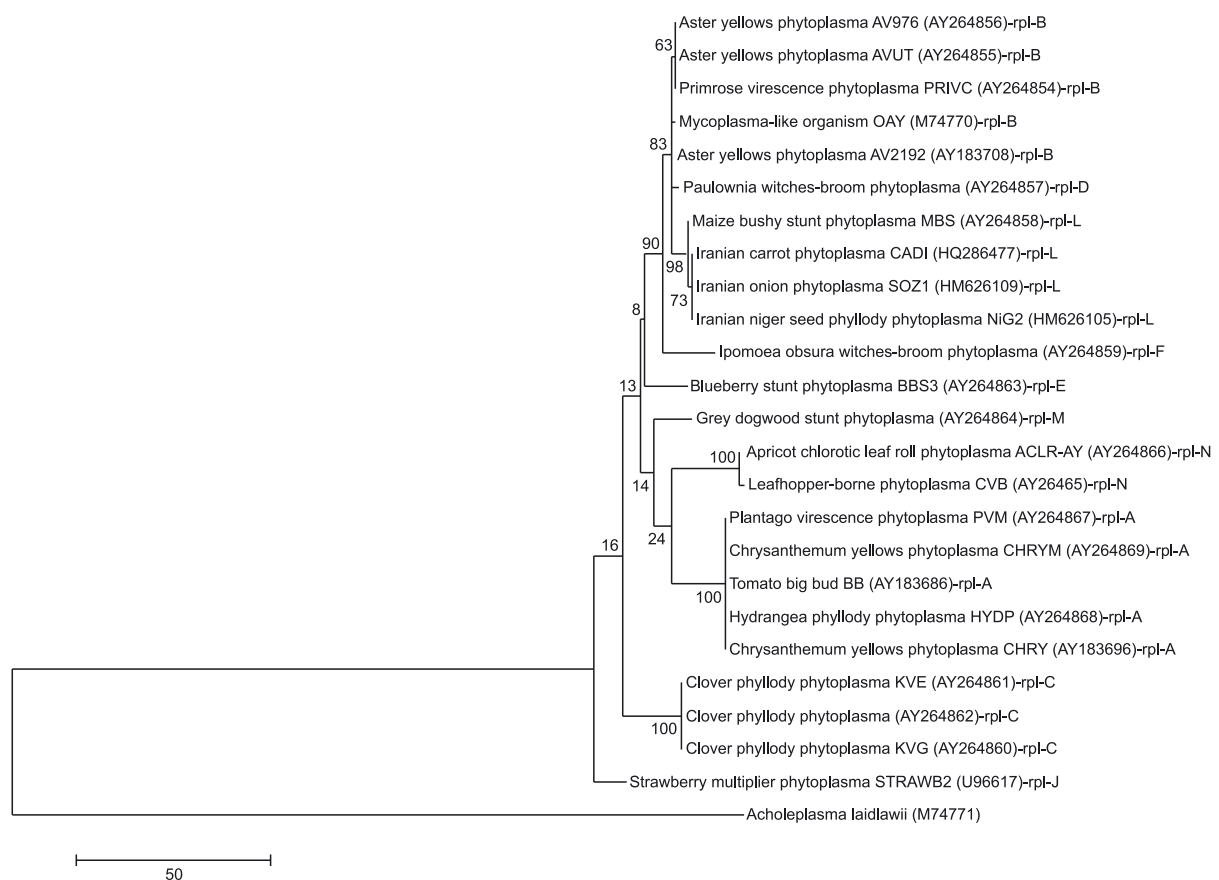


Fig. 3. A phylogenetic tree constructed by maximum parsimony analyses of 1.2 kb *rp* gene sequences from three Iranian '*Ca. P. asteris*'-related isolates CADI, NiG2, SOZ1, and 21 aster yellows phytoplasmas submitted in GenBank. *Acholeplasma laidlawii* is the outgroup to root the tree. Bootstrapping was done in 1,000 replicates. Phylogenetic analyses were performed using MEGA 5.05 software

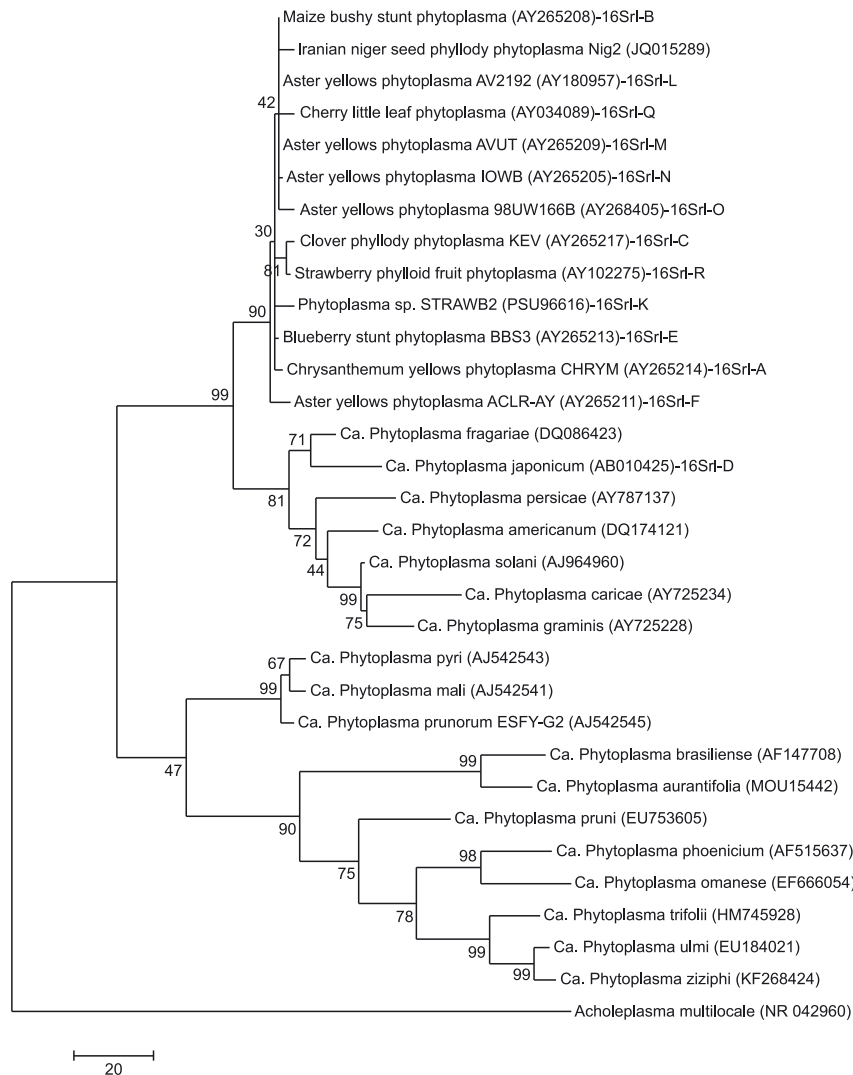


Fig. 4. A phylogenetic tree constructed by maximum parsimony analyses of the near full length 16S/23S rRNA gene from Iranian niger seed phyllody phytoplasma strain NiG2 and 30 phytoplasmas submitted in GenBank. *Achleplasma multilocale* is the outgroup to root the tree. Bootstrapping was done in 1,000 replicates. Phylogenetic analyses were performed using MEGA 5.05 software

and NiG2 were clustered with maize bushy stunt phytoplasma as a member of subgroup *rpI-L* (Fig. 3). Whereas, the phylogenetic tree constructed using 16S/23S rRNA gene sequences from isolate NiG2 and 30 phytoplasmas submitted in GenBank showed that, as expected, isolate NiG2 was clustered near the aster yellows phytoplasmas belonging to subgroup 16SrI-B (Fig. 4).

Discussion

In Iran, '*Ca. Phytoplasma asteris*' is a widespread phytoplasma species. According to previous studies, all of aster yellows phytoplasma isolates in Iran belong to the 16SrI-B or *rpI-B* subgroups (Babaie *et al.* 2007; Vali Sichani *et al.* 2014). But in this study, three isolates from carrot, niger seed, and scallion plants shared an RFLP pattern similar to maize bushy stunt phytoplasma, a member of subgroup *rpI-L* (Lee *et al.* 2004). Sequence analyses of *rp* operon as well as RFLP analyses confirmed that Iranian carrot phytoplasma strain CADI, Iranian onion (scallion) phytoplasma strain SOZ1, and Iranian niger seed phyllody

phytoplasma strain NiG2 could be introduced as new members of subgroup *rpI-L*. Yet, rRNA sequence analyses indicated that Iranian niger seed phyllody phytoplasma strain NiG2 has the highest similarity with maize bushy stunt phytoplasma. Based on 16S rRNA sequence analyses, maize bushy stunt phytoplasma was classified in subgroup 16SrI-B (Harrison *et al.* 1996), but *rp* gene sequence analyses indicated that it was a divergent lineage from other members of 16SrI-B and was clustered as a member of subgroup *rpI-L* (Lee *et al.* 2004). Comparison between *rp* gene nucleotide numbers of isolates CADI, NiG2, SOZ1, and maize bushy stunt phytoplasma revealed a difference in only one nucleotide. Phytoplasmas belonging to subgroup *rpI-L* have a limited distribution around the world and they were only reported from Mexico associated with maize bushy stunt disease (Lee *et al.* 2004), and India associated with Coorg black pepper yellows disease (Adkar-Purushothama *et al.* 2011). This research is the first report of scallion phytoplasma infection and the first record of subgroup *rpI-L* phytoplasma's association with carrot, niger seed, and scallion plants worldwide.

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