Characterization of phytoplasmas related to aster yellows group infecting annual plants in Iran, based on the studies of 16S rRNA and *rp* genes

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Abstract: Several annual field crops, vegetables, ornamentals, oilseed crops, and weeds showing phytoplasma diseases symptoms were collected to detect phytoplasmas related to '*Candidatus* Phytoplasma asteris'. The collecting was done in the central regions of Iran. For general detection of phytoplasmas, 16S rRNA gene fragments were amplified using phytoplasma universal primer pair P1/P7 in polymerase chain reaction (PCR) followed by primer pair R16F2n/R16R2 in nested PCR. Then, for finer detection of phytoplasmas related to '*Ca.* P. asteris', DNA samples were used to extend the *rp* and *tuf* gene fragments by PCR using aster yellows group specific primer pairs rp(I)F1A/rp(I)R1A and fTufAy/rTufAy, respectively. Restriction fragment lenght polymorphism (RFLP) analysis of *rp* gene fragments using digestion with *Alu*I, *Mse*I, and *Tsp*509I restriction enzymes indicated that aster yellows group related phytoplasmas in these Iranian regions, belong to *rp*I-B subgroups. Sequence analysis of partial 16S rRNA and *rp* genes from representative phytoplasma isolates confirmed the RFLP results. This research is the first report of annual plants infected with phytoplasmas related to subgroup *rp*I-B in Iran.

Key words: annual plants, *'Candidatus* Phytoplasma asteris', *rp*I-B

Introduction

Aster yellows phytoplasma group comprises a large number of worldwide-spread phytoplasmas. '*Candidatus* Phytoplasma asteris' is the only *Candidatus* species which was described as a representative of the aster yellows phytoplasma group, including aster yellows subgroups 16SrI-A, 16SrI-B, 16SrI-C,16SrI-D, 16SrI-E, 16SrI-F, 16SrI-H,16SrI-K,16SrI-L,16SrI-M,16SrI-O,16SrI-P,16SrI-Q, and 16SrI-R according to restriction fragment lenght polymorphism (RFLP) analyses of 16S rRNA gene. This taxon is known to infect a large number of monocotyledonous and dicotyledonous herbaceous plants and trees, in over forty plant families worldwide (Lee *et al*. 2004).

Because the 16S rRNA gene is highly conserved, analysis of it is an inadequate parameter to resolve taxonomic rank at the subgroup level. Therefore, for resolve phylogenetic relationships, the multiple genes, such as the *tuf* and *rp* genes which have various degrees of sequence conservation, have been employed as additional phylogenic parameters for differentiation and classification of closely related phytoplasmas. Based on the *rp* gene sequences, eleven *rp*I subgroups, including *rp*I-A, *rp*I-B, *rp*I-C, *rp*I-D, *rp*I-E, *rp*I-F, *rp*I-J, *rp*I-K, *rp*I-L, *rp*I-M and *rp*I-N, were characterized in the aster yellows group. Throughout the world, subgroups *rp*I-A and *rp*I-B are more prevalent than other *rp*I subgroups (Marcone *et al.* 2000; Lee *et al*. 2004; Cieślińska *et al*. 2011).

Recently in the central regions of Iran, we have observed various annual plants showing symptoms suggesting phytoplasma diseases. Previously, based on 16S rRNA gene studies, phytoplasmas related to '*Ca.* P. asteris' have been reported as the causal agents of several plant diseases in Iran (Salehi *et al.* 2005; Babaie *et al.* 2007; Asghari Tazekand *et al*. 2010; Rashidi *et al.* 2010; Zirak *et al*. 2010; Hosseini *et al*. 2011). Up till now, however, the *rp*I subgroup distributions have not been studied. Therefore, the aims of this study were the detection and characterization of aster yellows subgroups which infect different annual plants in the central regions of Iran, based on 16S rRNA and *rp* genes studies.

Materials and Methods

Plant materials

About 80 herbaceous annual plants including cabbage lettuce (*Lactuca sativa* L.), canola (*Brassica napu*s L.), celery (*Apium graveolens* L.), China aster (*Callistephus chinensis* L.), chrysanthemum (*Chrysanthemum* sp.), French marigold (*Tagetes patula* L.), gaillardia (*Gaillardia* × *grandiflora*), hemp (*Cannabis sativa* L.), marigold (*Calendula* sp.), onion (*Allium cepa* L.), prickly lettuce (*Lactuca scariola* L.), potato (*Solanum tuberosum* L.), sesame (*Sesamum indicum* L.), sunflower (*Helianthus annuu*s L.), tomato (*Lycopersicon esculentum* Mill.),

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and turnip (*Brassica rapa* var. L. *rapa*) showing several phytoplasma diseases symptoms, were collected from four provinces: Isfahan, ChaharMahal-O-Bakhtiari, Markazi, and Yazd located in the centre of Iran. The collecting was done during 2008 and 2009 autumn surveys (Fig. 1).

DNA extraction

Total genomic DNA was extracted from leaf tissues of infected plants and healthy plants using the Murray and Thompson method (1980). About 100 mg of leaf midribs were used for DNA extraction. Finally the nucleic acid precipitates were dissolved in 30 µl sterilized distilled water and stored at –20°C until use.

PCR analyses and primer pairs

As templates for polymerase chain reaction (PCR) amplifications, DNA extracted from infected and healthy plants was used. For general detection of phytoplasmas, PCR amplifications were performed using two sets of universal phytoplasma primer pairs. The primer pair P1/P7 was employed to amplify 1784 bp 16S rDNA fragment (Deng and Hiruki 1991; Schneider *et al.* 1995). Then, 30-fold diluted PCR products from the first amplifications were used in nested PCR using the R16F2n/R16R2 primer pair which amplified a 1239 bp fragment (Lee *et al.* 1993). Also, to amplify the *rp* operon which is encompassed with *rps19*, *rpl22* and *rps3* genes, the rp(I)F1A/rp(I)R1A primer pair, specific to aster yellows group, was used to amplify a 1212 bp fragment (Martini *et al.* 2007) (Fig. 2). Moreover, amplification of the 940 bp fragment using aster yellows *tuf* gene specific primer pair fTufAy/rTufAy approved the presence of the aster yellows group related phytoplasmas among the collected isolates (Marcone *et al.* 2000).

The total volume of 20 µl PCR mixtures contained 10–20 ng DNA, 0.2 mM of each of dNTPs (Cinnagen, Iran), 0.5 µl of each primer pair (20 pmol/µl), 1X polymerase buffer, 1.6 mM $MgCl_2$, and 1U of *Taq* DNA polymerase (Cinnagen). Finally reaction mixtures were analysed by electrophoresis in 1.2% (w/v) agarose gel using Tris-Borate EDTA (TBE) buffer, and stained with 5 µg/ml ethidium bromide. An ultra-violet (UV) transilluminator was used to visualise DNA bands.

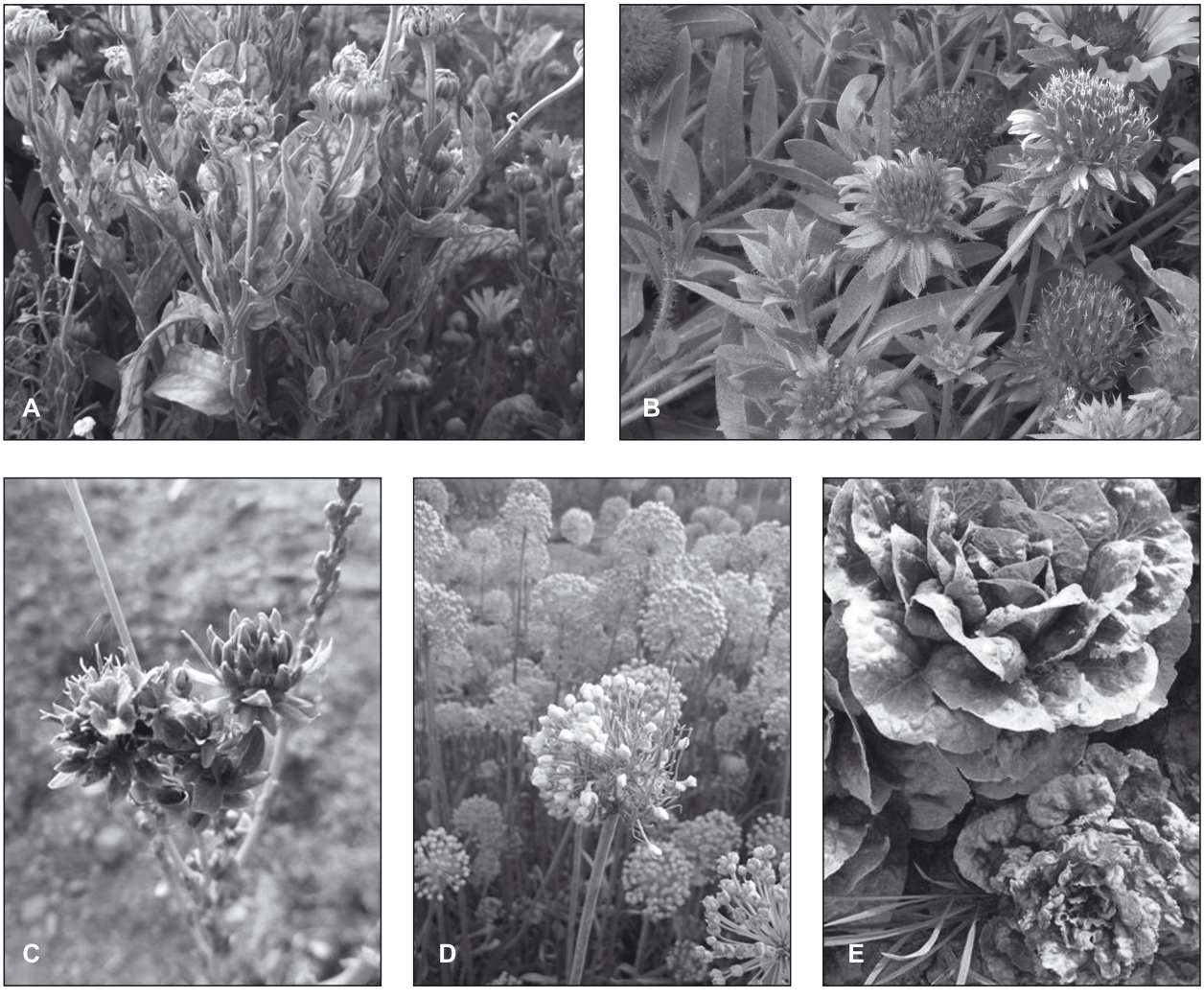

Fig. 1. Symptoms of diseases caused by '*Ca*. P. asteris' on annual plants in the central regions of Iran.
A – Gaillardia; B – French marigold; C – Prickly lettuce; D – Onion; E – Cabbage lettuce

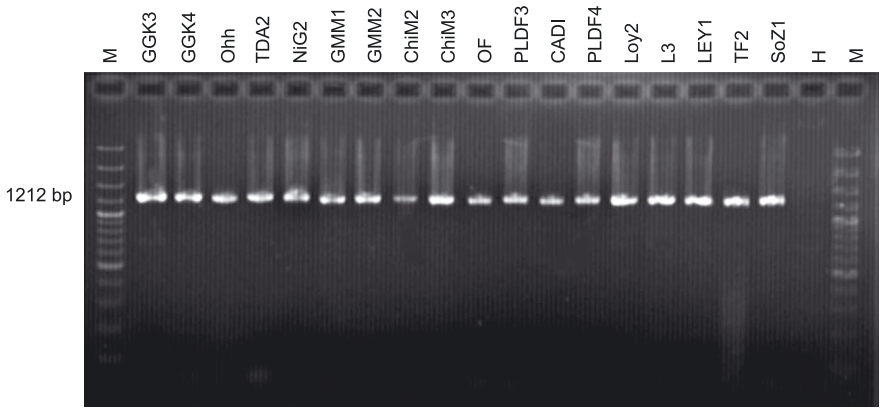

Fig. 2. 1212 bp fragment amplified using primer pair rp(I)F1A/rp(I)R1A in PCR assays. Line M is ladder 100 plus and line H is a healthy plant. DNA fragments were electrophoresed in 1.2% (w/v) agarose gel in Tris-Borate EDTA (TBE) buffer and stained with 5 μg/ml ethidium bromide

RFLP analyses

At first, the 1239 bp 16S rDNA fragments amplified using primer pair R16F2n/R16R2, were used in digestion analysis with *Cfo*I restriction enzyme according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania) (Fig. 3). Also, a R16F2n/R16R2 amplified sequence from an Iranian aster yellows group related isolate was used for subgroup affiliation studies using the website tool *i*PhyClassifier (Zhao *et al.* 2009). Furthermore, all isolates which amplified 1212 bp *rp* gene fragments using primer pair rp(I)F1A/rp(I)R1A were subjected to single digestion using *Alu*I, *Mse*I and *Tsp*509I restriction enzymes according to the manufacturer's instructions (Fermentas) (Fig. 4).

The *Cfo*I RFLP profiles were analysed by electrophoresis in 2% (w/v) agarose gel using TBE buffer, stained with 5 μg/ml ethidium bromide. The *Alu*I, *Mse*I and *Tsp*509I restriction profiles were separated with electrophoresis through 12% polyacrylamide gel for *Mse*I and *Tsp*509I enzymes and 5% polyacrylamide gel for *Alu*I enzyme and stained with silver nitrate.

Cloning and sequences analysis

Six 1212 bp fragments amplified using primer pair rp(I) F1A/rp(I)R1A from infected cabbage lettuce, China aster, gaillardia, onion, prickly lettuce, and tomato plants were selected to *rp* genes sequence analysis. An 1784 bp P1/P7 amplified fragment from cabbage lettuce plants was also selected for 16S rRNA sequence analysis. All fragments

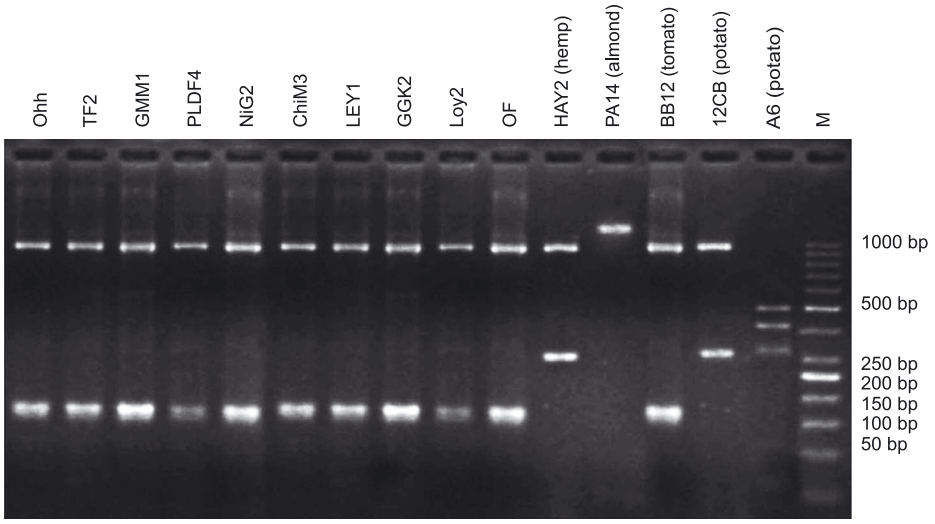

Fig. 3. RFLP analysis of 1239 bp 16S rDNA fragments amplified by primer pair R16F2n/R16R2 from phytoplasmas collected in central Iran using *Cfo*I restriction enzyme. Isolates 12CB and HAY2 are reference strains of 16SrXII group, isolate BB12 is reference strain of 16SrI group, isolate PA14 is reference strain of 16SrII group and isolate A6 belongs to 16SrVI group

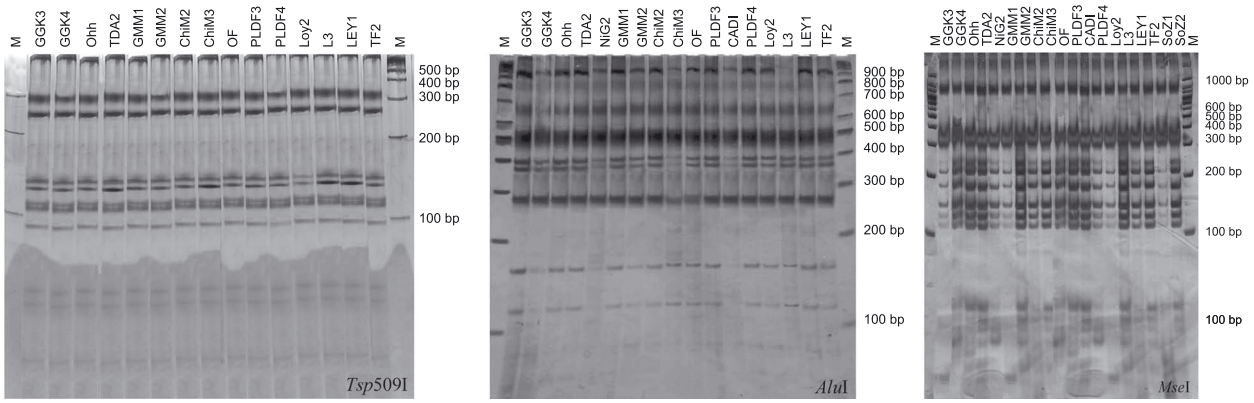

Fig. 4. RFLP analysis of 1212 bp *rp* genes fragments amplified by primer pair rp(I)F1A/rp(I)R1A from Iranian aster yellows phytoplasmas. *Mse*I and *Tsp*509I restriction fragments were separated with electrophoresis through 12% polyacrylamide gel and *Alu*I restriction fragments were separated with electrophoresis through 5% polyacrylamide gel

Table 1. Characteristics of aster yellows group related phytoplasmas and other phytoplasma isolates used in this study

Isolate	Province/Region	Host plant	Main symptom	Subgroup		Accession No.	
				rp	16Sr	*rp*	16S rRNA
PLDF4	Isfahan/Fereidan	Prickly lettuce	Phyllody	*rp*I-B	16SrI-B	HM626108	–
GMM1	Markazi/Mahalat	Gaillardia	Phyllody	*rp*I-B	16SrI-B	HQ286479	–
TF2	Markazi/Mahalat	Tomato	Little leaves	*rp*I-B	16SrI-B	HQ286478	–
ChiM3	Markazi/Mahalat	China aster	Yellowing and Stunting	*rp*I-B	16SrI-B	HQ286480	–
Ohh	Isfahan/Hasse	Onion	Flower malformation	*rp*I-B	16SrI-B	HM626107	–
LEY1	Isfahan/Ziar	Cabbage lettuce	Leaf deformation	*rp*I-B	16SrI-B	HM626106	JQ015290
BB12	Ardebil/Khalkhal	Tomato	Big bud	–	16SrI-B	–	GU797557
12CB	Hamedan/Hamedan	Potato	Purple top	–	16SrXII-A	–	EU661607
HAY2	Yazd/Abarkoh	Hemp	Witches'-broom	–	16SrXII-A	–	JF441274
PA14	Isfahan/Najafabad	Almond	Witches'-broom	–	16SrII-C	–	FJ184381
A6	Alborz/Karaj	Potato	Witches'-broom	–	16SrVI-D	–	FJ427295

were ligated into PTZ57R/T vector using the InsTAClone® PCR Cloning kit, following the instructions given by the manufacturer (Fermentas). An aliquot of the ligation mixture was used to transform competent *Escherichia coli* MC1061. Sequencing of the obtained clones was performed at Macrogen (Seoul, Korea). The accession numbers of partial *rp* and 16S rRNA genes are shown in table 1.

A phylogenetic tree was conducted by parsimony analysis of *rp* gene sequences of six Iranian aster yellows related phytoplasmas and 21 *rp* gene sequences submitted in GenBank (Fig. 5). Also, nearly the full length 16S rRNA sequence of an Iranian aster yellows related phytoplasma (cabbage lettuce phytoplasma strain LEY1) and 40 16S rRNA sequences submitted in GenBank, were used for constriction of another tree (Fig. 6). Phylogenetic trees were constructed using MEGA4 software and were subjected to bootstrap analyses in 1,000 replicates. The *Acholeplasma laidlawii* isolates were used as outgroups of both trees.

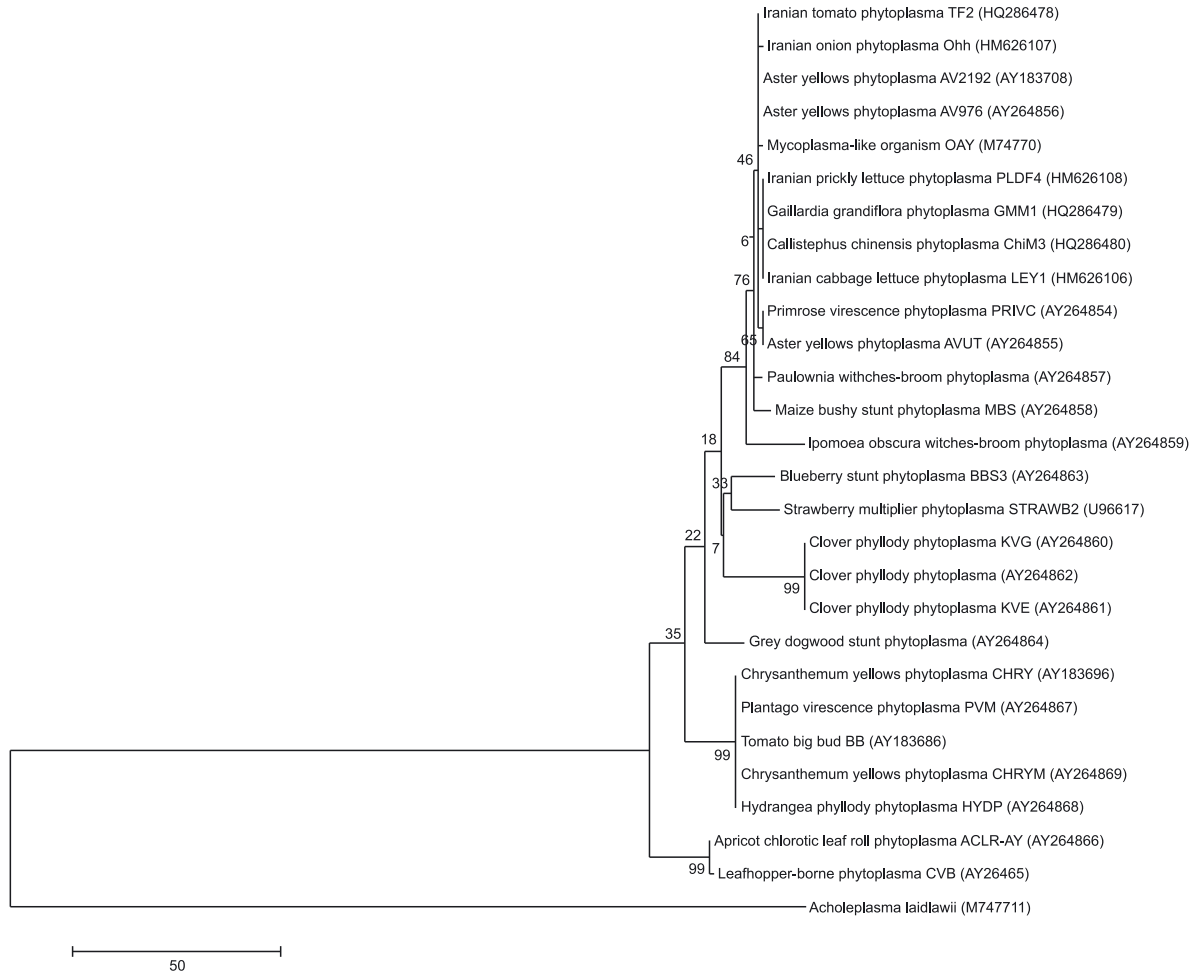

Fig. 5. Phylogenetic tree constructed by maximum parsimony analysis of partial 1.2 kb *rp* sequences (*rps19, rpl22* and *rps3* genes) from six Iranian aster yellows phytoplasmas and 21 phytoplasmas available in GenBank database. *Acholeplasma laidlawii* is outgroup to root the tree. Bootstrap analyses were done in 1,000 replicates. Phylogenetic analyses were performed using MEGA 5.05 software

Results

Detection of phytoplasmas

During the autumn of 2008 and 2009, about 80 annual plants including field crops, vegetables, ornamentals, oilseed crops, and weeds showing various symptoms of phytoplasma diseases were collected from Isfahan, ChaharMahal-O-Bakhtiari, Markazi, and Yazd provinces located in the central regions of Iran. Infected plants exhibited several typical symptoms of phytoplasma diseases (Fig. 1).

For general detection of phytoplasma infections, PCR and nested PCR amplifications using two phytoplasma universal primer pairs P1/P7 followed by R16F2n/R16R2 were performed. About 40 DNA samples amplified 1784 bp fragment in first PCR assays. However, many isolates in which there appeared phytoplasmal diseases symptoms, could not produce any band in the first PCR amplifications. Therefore, they were subjected for nested PCR amplifications using primer pair R16F2n/R16R2. The 1239 bp band was amplified from DNA of 65 symptomatic plants.

After a general detection of phytoplasmas using phytoplasma universal primer sets, all of the DNA samples which were positive in phytoplasma detection assays, were subjected to amplify *rp* and *tuf* genes to detect the probable aster yellows group related isolates. The 1212 bp *rp* genes and 940 bp *tuf* gene fragments were amplified using aster yellows specific primer pairs rp(I)F1A/rp(I)R1A (Fig. 2) and fTufAy/rTufAy, respectively, from DNA samples of only 20 symptomatic cabbage lettuce, China aster, gaillardia, onion, prickly lettuce, and tomato plants. The amplification results indicated that those isolates are related to '*Ca.* P. asteris'. Other DNAs which were amplified using primer pairs P1/P7 or R16F2n/R16R2 did not amplify any expected fragments using either rp(I)F1A/rp(I)R1A or fTufAy/rTufAy primer pairs. Therefore they were removed from further experiments. No band was obtained from negative controls using healthy plants.

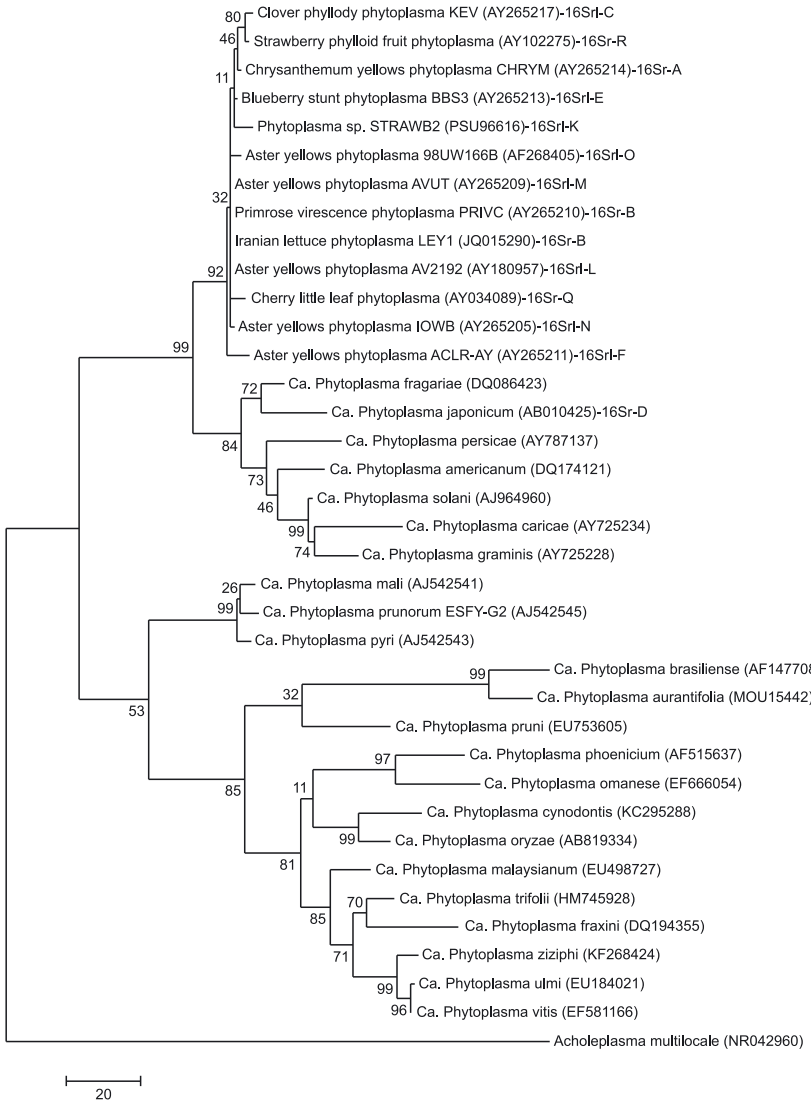

Fig. 6. Phylogenetic tree constructed by maximum parsimony analysis of near full length 16S rRNA gene sequence from Iranian cabbage lettuce phytoplasma strain LEY1 and 35 phytoplasma sequences downloaded from GenBank database. *Acholeplasma multilocale* is outgroup to root the tree. Bootstrap analyses were done in 1,000 replicates. Phylogenetic analyses were performed using MEGA 5.05 software

Real and virtual RFLP analyses

In addition to group specific primer pairs which were used for primary differentiation of aster yellows group related isolates from other phytoplasmas, *Cfo*I restriction enzyme was used for digestion of all 1239 bp R16F2n/R16R2 amplified fragments. A previously reported Iranian tomato big bud phytoplasma representative isolate for aster yellows group, potato purple top, and hemp witches'-broom phytoplasmas; the representatives of stolbur group, almond witches'-broom phytoplasma; the representative of peanut WB group and potato witches'-broom phytoplasma; and the representative of clover proliferation group, were used as reference strains in RFLP analysis. The RFLP analysis results indicated that *Cfo*I restriction enzyme could differentiate aster yellows group related isolates from other phytoplasma groups but there was no polymorphism between aster yellows group related isolates (Fig. 3). Therefore, the 16S rRNA sequence from a cabbage lettuce phytoplasma was used for subgroup affiliation studies using the virtual *i*PhyClassifier tool. The result indicated a 0.97 similarity with onion yellows phytoplasma (accession NC_005303), the reference strain of the 16SrI-B subgroup.

Next, 1212 bp *rp* genes fragments were subjected to single digestion with *Alu*I, *Mse*I, and *Tsp*509I restriction enzymes as previously described for aster yellows subgroup differentiations (Lee *et al.* 2004). The results indicated that Iranian aster yellows isolates shared patterns nearly the same as the subgroup *rp*I-B (Fig. 4).

Phylogenetic analyses

To identify and characterise the Iranian aster yellows related phytoplasma isolates infecting annual plants in central Iran, *rp* gene fragments amplified from six DNA samples of infected cabbage lettuce, China aster, gaillardia, onion, prickly lettuce, and tomato were sequenced.

The blast search results indicated that Iranian cabbage lettuce phytoplasma strain LEY1, Iranian prickly lettuce phytoplasma strain PLDF4, *C. chinensis* phytoplasma strain ChiM3, Iranian onion phytoplasma strain Ohh, Iranian tomato phytoplasma strain TF2, and *G. grandiflora* phytoplasma strain GMM1 share a 99% similarity with aster yellows phytoplasma strain AV2192 (accession AY183708), primrose virescence phytoplasma strain PRIVC (accession AY264854), and other phytoplasmas. All of them were previously described as members of the subgroup *rp*I-B by Lee *et al*. (2004).

Also, the 16S rRNA fragment of cabbage lettuce plant (strain LEY1) was sequenced as a representative of the *rp*I-B subgroup members. A Blast search of 16S rRNA sequences showed that this isolate shared a 99% identity with periwinkle virescence phytoplasma (accession DQ381535), a member of the 16SrI-B subgroup.

Two phylogenetic trees were constructed by parsimony analysis of *rp* and 16S rRNA gene sequences. The tree constructed with *rp* sequences of six Iranian aster yellows related isolates and 21 sequences related to *rp*I subgroups which were previously used for *rp*I subgroups studies by Lee *et al*. (2004), indicated that, as expected, isolates ChiM3, GMM1, LEY1, Ohh, PLDF4, and TF2 were clustered with *rp*I-B subgroup phytoplasmas (Fig. 5). However, the 16S rRNA gene sequence from isolate LEY1 and 40 phytoplasmas submitted in GenBank were used for another phylogenetic tree construction. Some of these phytoplasmas were collected from several regions in Iran. As expected, cabbage lettuce plant strain LEY1 was clustered near the aster yellows phytoplasmas, especially isolates related to subgroup 16SrI-B (Fig. 6).

Discussion

Because of 16S rRNA gene's importance in phytoplasma phylogenetic studies, in the present study, the 16S rRNA gene was also used for RFLP and phylogenetic analyses. In some previous studies, it was reported that *Cfo*I restriction enzyme is a suitable enzyme to differentiate aster yellows phytoplasmas from other taxonomically near phytoplasmas, such as the stolbur group (Girsova *et al.* 2008; Vali Sichani *et al.* 2011). However, after *Cfo*I enzyme digestion, no polymorphism was found among the tested Iranian aster yellows related phytoplasma isolates (Fig. 3). To verify the subgroup affiliations based on the 16S rRNA gene; the 16S rRNA sequence from isolate LEY1 was analysed with the *i*PhyClassifier virtual tool. Surveyed sequences shared a high similarity (coefficient 0.97) with the restriction pattern of the reference strain of the 16SrI-B subgroup, onion yellows phytoplasma OY-M (accession NC_005303).

Lee *et al*. (2004) performed RFLP analyses using restriction enzymes *Alu*I, *Mse*I, and *Tsp*509I to digest *rp* gene fragments for the *rp*I subgroup designations. A comparison of the *rp* RFLP patterns of ChiM3, GMM1, LEY1, Ohh, PLDF4, and TF2 isolates with isolates described by Lee *et al*. indicated that when using these three restriction enzymes, these phytoplasma isolates have a RFLP pattern similar to the restriction patterns of aster yellows phytoplasma strain AV2192, primrose virescence phytoplasma and other phyoplasmas representatives of the subgroup *rp*I-B (Lee *et al*. 2004) (Fig. 4).

Sequence analysis of *rp* operon as well as RFLP analyses clearly indicated that the Iranian aster yellows group related phytoplasma isolates ChiM3, GMM1, LEY1, Ohh, PLDF4, and TF2 are related to the subgroup *rp*I-B. Also, the results of 16S rRNA blast searches and virtual RFLP analyses confirmed that isolate LEY1 (representative of the *rp*I-B subgroup) has the highest similarity with the 16SrI-B subgroup phytoplasmas.

Several studies were accomplished on detection and characterization of phytoplasmas related to '*Ca.* P. asteris' in Iran (Salehi *et al.* 2005; Babaie *et al.* 2007; Asghari Tazekand *et al*. 2010; Rashidi *et al.* 2010; Zirak *et al.* 2010; Hosseini *et al.* 2011). However, all were done based on 16S rRNA gene studies, and up till now, there have not been any reports on *rp*I subgroup distributions in Iran. Finally, we recorded the presence of phyoplasmas related to subgroups *rp*I-B in Iran for the first time. In view of the fact that phytoplasmas related to subgroups *rp*I-B infect several annual plants in central Iran, it could be true that unknown insect vectors are involved in the development of diseases in these regions.

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References

Asghari Tazehkand S., Hosseini Pour A., Heydarnejad J., Massumi H., Azadvar M. 2010. Identification of phytoplasmas associated with cultivated and ornamental plants in Kerman province. Iran. J. Plant Pathol. 158 (11–12): 713–720.

Babaie G., Khatabi B., Bayat H., Rastgou M., Hosseini A., Salekdeh G.H. 2007. Detection and characterization of phytoplasma infecting ornamental and weed plants in Iran. J. Plant Pathol. 155 (6): 368–372.

Cieślińska M. 2011. Less common phytoplasma infecting stone fruit trees. J. Plant Prot. Res. 51 (4): 435–440.

Deng S., Hiruki C. 1991. Genetic relatedness between two nonculturable mycoplasma like organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology 81 (12): 1475–1479.

Girsova N., Bottner K.D., Mozhaeva K.A., Kastalyeva T.B., Owens R.A., Lee I.M. 2008. Molecular detection and identification of group 16SrI and 16SrXII phytoplasmas associated with diseased potatoes in Russia. Plant Dis. 92 (4): 654–661.

Hosseini P., Bahar M., Madani G., Zirak L. 2011. Molecular characterization of phytoplasmas associated with potato purple top disease in Iran. J. Phytopathol. 159 (4): 241–246.

Kunkel L.O. 1932. Celery yellows of California not identical with the aster yellows of New York. Contrib. Boyce Thompson Inst. 4: 405–414.

Lee I.M., Gundersen-Rindal D.E., Davis R.E., Bottner K.D., Marcone C., Seemüller E. 2004. *'Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. Int. J. Syst. Evol. Microbiol. 54 (4): 1037–1048.

Lee I.M., Hammond R.W., Davis R.E., Gundersen D.E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. Phytopathology 83 (8): 834–842.

Marcone C., Lee I.M., Davis R.E., Ragozzino A., Seemüller E. 2000. Classification of aster yellows group phytoplasma based combined analysis of rRNA and *tuf* gene sequences. Int. J. Syst. Evol. Microbiol. 50 (5): 1703–1713.

Martini M., Lee I.M., Bottner K.D., Zhao Y., Botti S., Bertaccini A., Harrison N.A., Carraro L., Marcone C., Khan A.J., Osler R. 2007. Ribosomal protein gene-based phylogeny for finer differentiation and classification of phytoplasmas. Int. J. Syst. Evol. Microbiol. 57 (9): 2037–2051.

Murray M.G., Thompson W.F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8 (19): 4321–4325.

Rashidi M., Ghosta Y., Bahar M. 2010. Molecular identification of a phytoplasma associated with Russian olive witches'-broom in Iran. Eur. J. Plant Pathol. 127 (2): 157–159.

Salehi M., Heydarnejad J., Izadpanah K. 2005. Molecular characterization and grouping of 35 phytoplasmas from central and southern provinces in Iran. Iran J. Plant Pathol. 41: 62–65.

Schneider B., Seemüller E., Smart C.D., Kirkpatrick B.C. 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas: Molecular and diagnostic procedures in mycoplasmology. p. 369–380. In: "Molecular and Diagnostic Procedures in Mycoplasmalogy". Vol. 1. (S. Razin, J.G. Tully, eds.). Academic Press Inc., San Diego, 451 pp.

Vali Sichani F., Bahar M., Zirak L. 2011. Characterization of stolbur (16SrXII) group phytoplasmas associated with *Cannabis sativa* witches'-broom disease in Iran. Plant Pathol. J. 10 (4): 161–167.

Zhao Y., Wei W., Lee I.M., Shao J., Suo X., Davis R.E. 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 (10): 2582–2593.

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 (1): 63–65.