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Research Article

Characterization of 16SrII group phytoplasmas associated with alfalfa (*Medicago sativa*) witches' broom disease in diverse areas of Iran

Seyyed Alireza Esmailzadeh Hosseini[1,2], Gholam Khodakaramian[1*], Mohammad Salehi[3] and Assunta Bertaccini[4]

1. Plant Protection Department, Bu-Ali Sina University, Hamedan, Iran.
2. Plant Protection Research Department, Yazd Agricultural and Natural Resources Research and Education Center, AREEO, Yazd, Iran.
3. Plant Protection Research Department, Fars Agricultural and Natural Resources Research and Education Center, AREEO, Zarghan, Iran.
4. Department of Agricultural Sciences, *Alma Mater Studiorum*, University of Bologna, Italy.

Abstract: Alfalfa witches' broom (AWB) is one of the most important alfalfa diseases in Iran. To characterize 16SrII group phytoplasmas associated with this disease, symptomatic and asymptomatic plants were collected during 2013-2015 and subjected to direct and nested polymerase chain reaction (PCR) using P1/P7, R16mF2/R16mR2 and R16F2n/R16R2. PCR amplicons of ~1.8, ~1.4 and ~1.25 kb respectively, were obtained only from all symptomatic plants. Restriction fragment length polymorphism (RFLP) analysis of R16F2n/R2 amplicons showed that the phytoplasma associated with AWB disease were members of 16SrII group subgroups 16SrII-D and -C. Blast analysis of these amplicon sequences and sequence homology of collected strains and strain sequences retrived from GenBank (AWB strains Chahgeer, Juyom and Bushehr) confirmed that AWB phytoplasmas collected from Bafg, Ardakan, Bahabad and Herat (Yazd province), Nikshahr (Sistan-Baluchestan), Bam, Zarand, Jiroft (Kerman province), Bushehr (Bushehr province), Tabas (South Khorasan province), Jowkar (Hamedan province) and Zardenjan (Esfahan province) cluster with phytoplasma strains enclosed in the 16SrII-D subgroup, while AWB strains from Chahgeer (Yazd province) and Juyom (Fars province) cluster with phytoplasma strains in the 16SrII-C subgroup. Based on these results the predominant strains of 16SrII phytoplasmas associated with AWB disease in Iran were classified in the 16SrII-D subgroup. In Ashkezar and Abarkouh in Yazd province entire alfalfa farm was infected with witches' broom disease. In 3 year alfalfa stands in Ashkezar alfalfa farms were plowed due to high incidence of the disease.

Keywords: Phytoplasma, 16SrII-C and -D subgroups, PCR, RFLP

Introduction

Forage and silage alfalfa *Medicago sativa* L. with production of about 21,500,000 tons per year and yield of 33076.9 kg/ha have an important role in Iran agriculture as the fifth producer in the world (FAOSTAT, 2013). AWB disease as one of the most important factors affecting alfalfa production was reported for the first time in the United States of America in 1925 (Haskell, 1926), and after that, reported to occur in many alfalfa growing areas in the world (Smarz *et al.*, 1981; Khadhair *et al.,* 1997; Peters *et al.,* 1999; Jomantiene *et al.,*

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* **Corresponding author**, e-mail: khodakaramian@yahoo.com
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2000; Marzachì *et al.,* 2000; Khan *et al.,* 2002; Conci *et al.,* 2005; Jones *et al.*, 2005; Getachew *et al.,* 2007; Li *et al.,* 2012; Starović *et al.,* 2012; Esmailzadeh Hosseini *et al.,* 2015a; 2015b; 2015c). In Iran, for the first time, AWB symptoms were observed in alfalfa farms in Sistan Baluchestan, Fars and Kerman provinces (Salehi and Izadpanah, 1993b). Recently, AWB phytoplasma disease was observed in all the major alfalfa growing areas on different cultivars of *M. sativa* especially in central and southern provinces of Iran (Salehi *et al.*, 1995; Raoofi and Salehi, 2012; Esmailzadeh Hosseini *et al.*, 2015a; 2015b; 2015dc). Phytoplasmas associated with AWB belong to diverse 16S rRNA group including 16SrI (Rashidi *et al.*, 2010), 16SrII (Salehi *et al.,* 2000; Esmailzadeh Hosseini *et al.,* 2015a; 2015b; 2015c), 16SrVI (Esmailzadeh Hosseini *et al.,* 2016a) and 16SrXII-A (Esmailzadeh Hosseini *et al.,* 2016 a,b). The insects *Orosius albicinctus* and *Neoaliturus haematoceps* were identified as AWB natural vectors in Iran (Salehi *et al.* 1995; Esmailzadeh Hosseini *et al.,* 2015a; 2015c).

The aim of the present work was to characterize phytoplasmas in 16SrII group associated with alfalfa witches' broom disease in Iran.

Materials and Methods

Plant sampling, infection percentage and disease severity

During 2013-2015, a survey (Fig. 1) was carried out in different alfalfa fields in Iran, and samples from *M. sativa* L. cultivars with typical witches' broom symptoms (Fig. 3) were collected and used for molecular studies. Infection percentage of AWB disease was determined by sampling within a 1 m^2 on a diagonal transect across 10 fields in each area. Number of infected plants over the total number of alfalfa plants was assigned as infection percentage (Esmailzadeh Hosseini *et al.* 2015b).

DNA extraction and nested PCR amplification

Total DNA was extracted from 0.2 g leaf midrib tissue of witches' broom affected alfalfa using Zhang *et al.* (1998) procedure and from alfalfa plants grown from seeds collected form asymptomatic plants maintained under insect proof cages and nested PCR negative to phytoplasma presence as negative control. A symptomatic periwinkle plant infected with lime witches' broom phytoplasma was used as positive control. Sterile distilled water was also added to each reaction as PCR mix negative control. DNA samples were tested for phytoplasma presence by direct PCR using P1/P7 primers (Deng and Hiruki, 1991; Schneider *et al.,* 1995) and nested PCR using R16F2n/R16R2 or R16mF2/R16mR2 (Gundersen and Lee, 1996) primer pairs. Primer pair P1/P7 amplifies a 1,800-bp fragment of the ribosomal operon which includes the 16SrRNA gene, the 16S-23S intergenic spacer region (SR) and a portion of the 5′ region of the 23S rRNA gene. R16mF2/R16mR2 and R16F2n/R16R2 primer pairs amplify 1,400 and 1,250 bp fragments respectively, in the 16S rRNA gene. PCR was performed in a 25 µl reaction volume containing 50 ng DNA, 0.4 mM of each primer, 0.25 mM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 units of Taq DNA polymerase (CinnaGen, Iran) in the buffer supplied by the manufacturer. Amplifications were carried out in a thermal cycler (Bio-Rad, USA) for 35 cycles as follows: 45 seconds denaturation at 94 °C (3 minutes for the first cycle), 45 seconds annealing at 58 °C and 2 minutes of extension at 72 °C. In the final cycle the extension step was extended to 10 minutes. PCR conditions for the nested PCR were the same except that the annealing temperature was raised to 55 °C. PCR products were separated in 1% agarose gels in 1X TBE buffer (108 g Tris-HCl, 55 g boric acid, 40 ml EDTA 0.5 M, pH 8.0 per 1 L). DNA bands were stained with ethidium bromide and visualized with a UV transilluminator (UVT- model 2020). The molecular weight of the PCR products was estimated by comparison with 100 bp DNA ladder (Fermentas, Vilnius, Lithuania).

Real and virtual restriction fragment length polymorphism (RFLP) analysis

RFLP analysis of nested PCR products (1,250 bp of 16S rRNA gene) was used for identification of the phytoplasma associated with AWB. The R16F2n/R16R2 amplified products were digested

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separately with each of the restriction enzymes, *Rsa*I, *Hae*III, *Taq*1, *Alu*I and *Mse*I according to the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). The restriction products were then separated by electrophoresis through a polyacrylamide gel, stained by ethidium bromide and visualized with a UV transilluminator. The resulting RFLP patterns were compared with those previously published for 16S rDNA of other phytoplasmas (Lee *et al.,* 1998).

Virtual RFLP analysis using *i*PhyClassifier (Zhao *et al.,* 2009) was used to determine subgroup affiliation of AWB phytoplasmas comparing the sequences of a representative strain of each area (Nikshahr, Bahabad, Bam, Bafq, Ardakan, Herat, Zardenjan, Jowkar, Zarand, Jiroft) to those of available phytoplasmas in 16SrII reported subgroups. Each aligned DNA 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, HhaI, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I (*Sau3A*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I.

Sequencing and phylogenetic analyses

The R16mF2/R16mR2 primed PCR products of 10 AWB strains from Nikshahr, Bahabad, Bam, Bafq, Ardakan, Herat, Zardenjan, Jowkar, Zarand and Jiroft were direclty sequenced in both directions using the amplification primers. The R16F2n/R16R2 1.25 kb fragments of 28 phytoplasmas including the AWB phytoplasmas described in this work and AWB sequences retrived from GenBank (AWB strains Chahgeer, Juyom and Bushehr) were separately aligned and phylogenetic trees were generated using MEGA6 software (Tamura *et al.,* 2013). *Acholeplasma laidlawii* was used as an outgroup to root the tree. Bootstrapping was performed 1,000 times to estimate the clade stability and support for the branches.

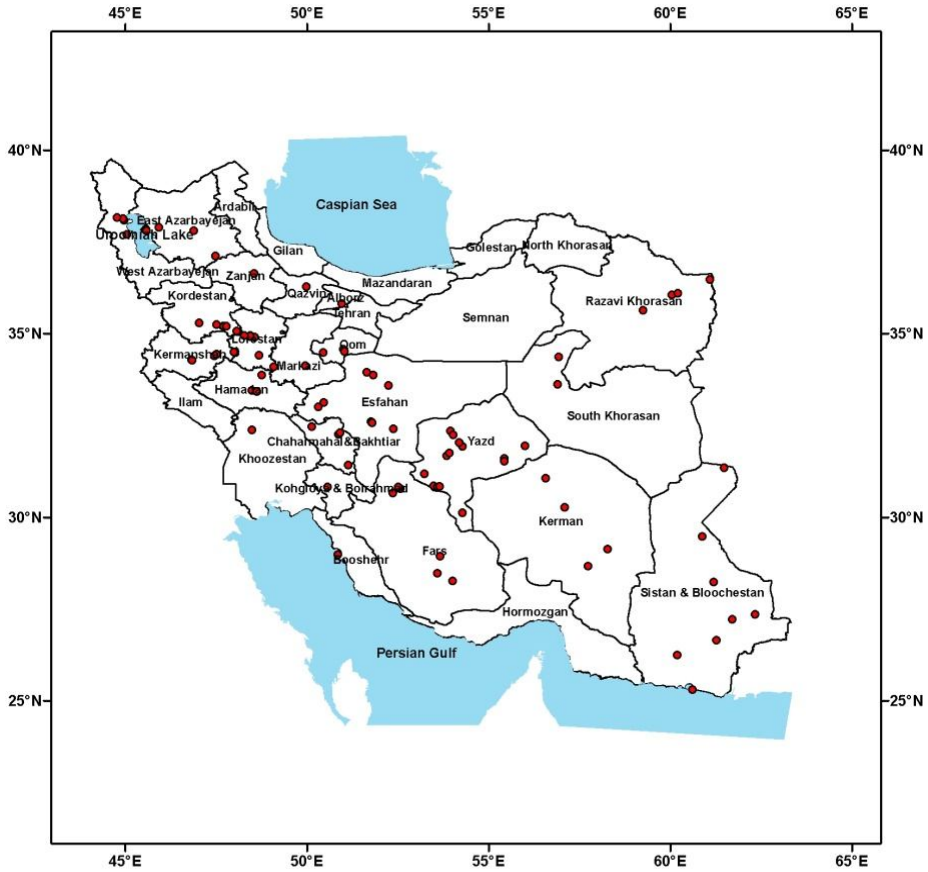

Figure 1 Alfalfa witches' broom (AWB) sampling areas in Iran alfalfa fields.

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Results

Infection percentage and disease severity

AWB symptoms were observed in alfalfa fields all over the country especially in central, southern and east of Iran with different incidence shown in Fig. 2 using ArcGIS 10.2. The affected plants showed little leaf, yellowing, formation of adventitious shoots, witches'broom, dwarfing, internode shortening, flower virescence, phyllody, proliferation and sterility, leaf yellowing, leaf curling, stunting, decline and death (Fig. 3). The highest incidence was found in Ashkezar and Abarkouh in Yazd province where entire field was infected. In Ashkezar the 3 years stand alfalfa farms were plowed due to the high incidence of disease.

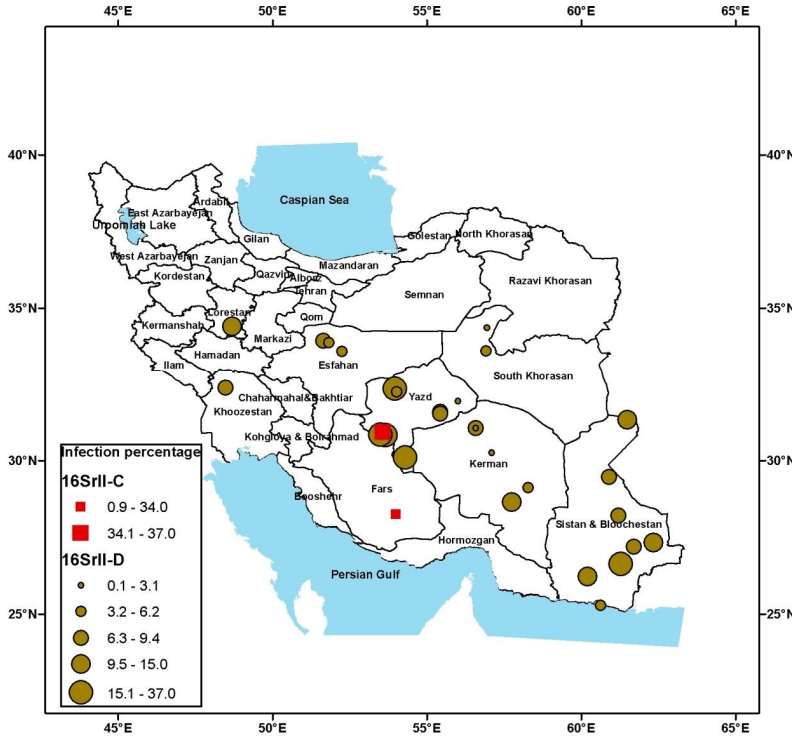

Figure 2 Disease incidence of phytoplasmas in 16SrII subgroups –C and –D in Iran alfalfa fields.

Figure 3 A view of Alfalfa witches' broom (AWB) disease symptoms observed in Chahgeer (Abarkouh, Yazd province).

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Polymerase chain reaction and RFLP analyses

From all 702 symptomatic alfalfa plants collected in the surveyed areas and positive control DNA fragments of the expetected sizes were amplified in direct and nested PCR using P1/P7, R16mF2/R16mR2 and R16F2n/R16R2 primer pairs. No amplification was observed in DNA samples from 43 symptomless plants or from negative controls (Fig. 4).

The R16F2n/R16R2 primed nested PCR products (1.25 kbp) from AWB strains collected in different areas were analyzed by digestion with five enzymes and RFLP patterns resulted identical to those of peanut witches' broom phytoplasmas (16SrII group) (Lee *et al.,* 1998) (Fig. 5). AWB strains from Bafg, Ardakan, Bahabad and Heart (Yazd province), Nikshahr (Sistan-Baluchestan), Bam, Zarand and Jiroft (Kerman province), Tabas (South Khorasan province), Jowkar (Hamedan province) and Zardenjan (Esfahan province) were selected for further molecular characterization.

Sequencing and phylogenetic analysis

Among AWB phytoplasma strains from the different areas, the sequences of the R16mF2/R16mR2 amplicons obtained from samples collected in Bafg, Ardakan, Bahabad and Herat (Yazd province), Nikshahr (Sistan-Baluchestan), Bam, Zarand, Jiroft (Kerman province), Tabas (South Khorasan province), Jowkar (Hamedan province) and Zardenjan (Esfahan province) locations were assembled to the fragments corresponding to the R16F2n/R16R2 amplicons and were deposited in GenBank as accession numbers: KT943967, KT943969, KT943970, KT943961, KT634120, KT943971, KT943968, KT943962, KT943964, KT943965 and KT943963 respectively. Blast analysis, sequence homology and phylogenetic analysis confirmed the clustering of these phytoplasmas with those enclosed in 16SrII group (Fig. 6).

Computer-simulated restriction analysis with 17 restriction endonucleases using *i*PhyClassifier (Zhao *et. al.,* 2009) indicated that virtual RFLP patterns of all the sequenced phytoplasma strains were identical (similarity coefficient 1.00) to the reference pattern of 16SrII-D phytoplasmas (GenBank acc. no. Y10097) (Fig. 7c).

Formerly two AWB strains identified in Chahgeer (Yazd province) and Juyom (Fars province) and deposited in GenBank under acc. ns. DQ233655 and DQ233655 respectively, were attributed to 16SrII-C subgroup (Fig. 7b) with a similarity coefficient of 0.99 to the representative of 16SrII-C, cactus witches' broom phytoplasma YNO1 (GenBank acc. no. AJ293216), while a further AWB strain from Bushehr province (GenBank acc. no. JN860711) was attributed to the 16SrII-D subgroup.

The determination of homology percentages among a number of 16S rDNA sequences showed that the majority of the studied AWB strains had higher homology percentages with phytoplasmas enclosed in 16SrII-D and –C subgroups.

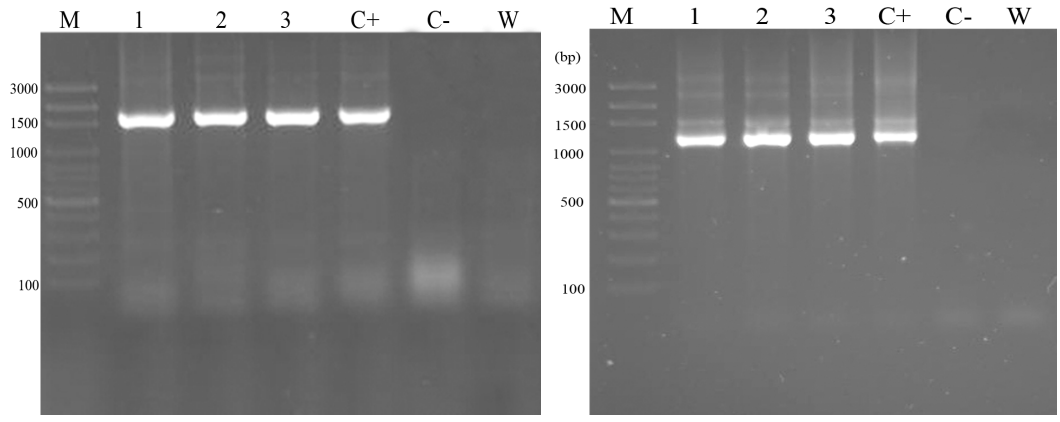

Figure 4 Electrophoresis of direct (left) and nested (right) PCR products using P1/P7 and R16F2n/R16R2 primer pair, respectively. Lanes 1-3, alfalfa witches' broom from Ardakan, Jiroft and Bam respectively. M: 100 bp DNA marker; W: water; C-: Healthy plant; C +: '*Ca.* P. aurantifolia'.

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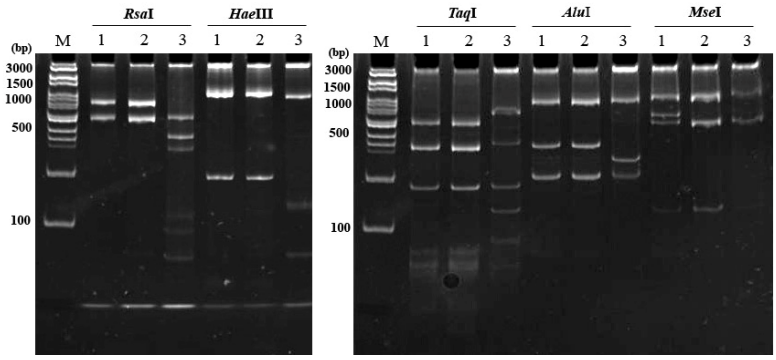

Figure 5 RFLP patterns in polyacrylamide gel electrophoresis of R16F2n/R16R2 nested PCR product of a 16SrII subgroup C from Abarkouh (lane 1), 16SrII-D from Esfahan (lane 2) and '*Ca.* P. aurantifolia' as control (lane 3) with *Rsa*I, *Hae*III, *Taq*1, *Alu*I and *Mse*I restriction enzymes. M: 100 bp DNA marker.

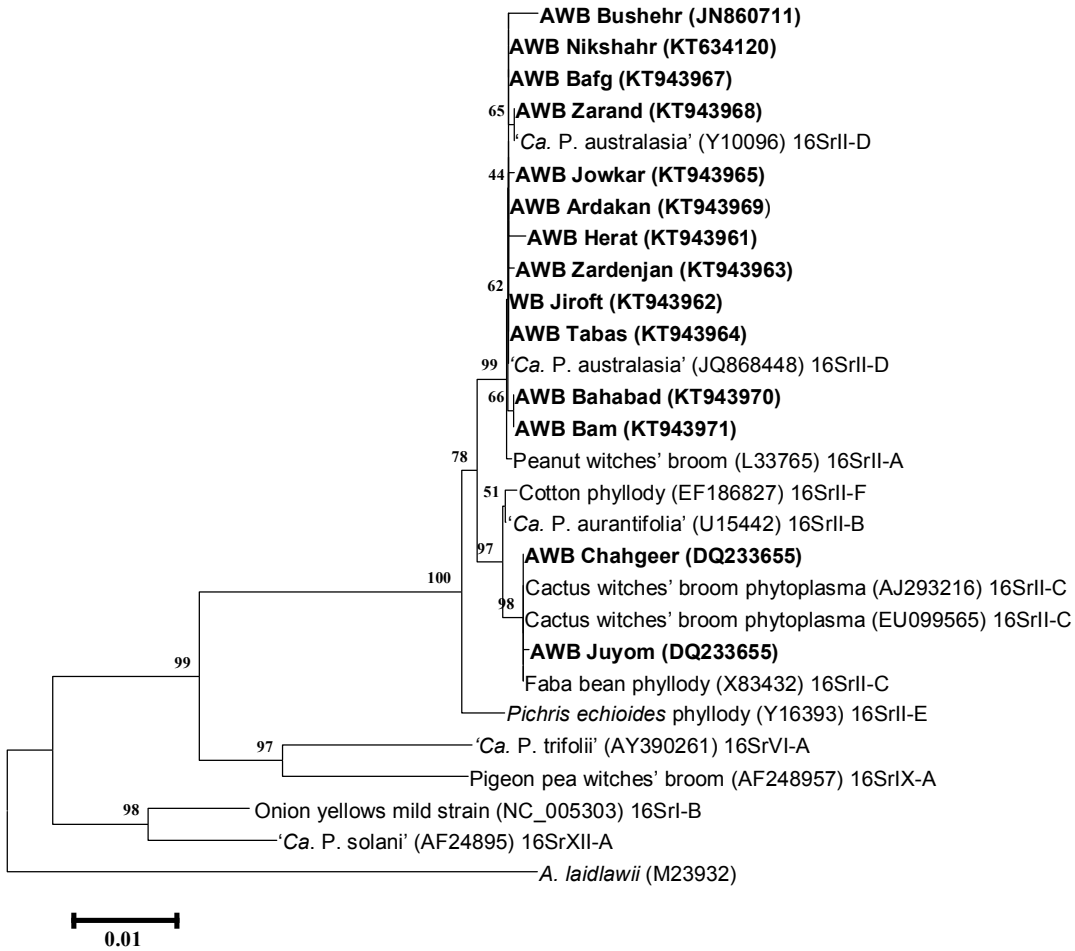

Figure 6 Neighbour-joining phylogenetic tree based on the 1,250 bp in the 16S rDNA sequence of the Alfalfa witches' broom (AWB) phytoplasma strains (in bold) and selected phytoplasmas from the GenBank. *Acholeplasma laidlawii* was used as an out group. GenBank accession numbers are shown in parentheses. Numbers on branches are bootstrap values of 1,000 replicates. '*Ca*. P.' stands for '*Candidatus* Phytoplasma' species, on the right of the branches are the ribosomal groups when available.

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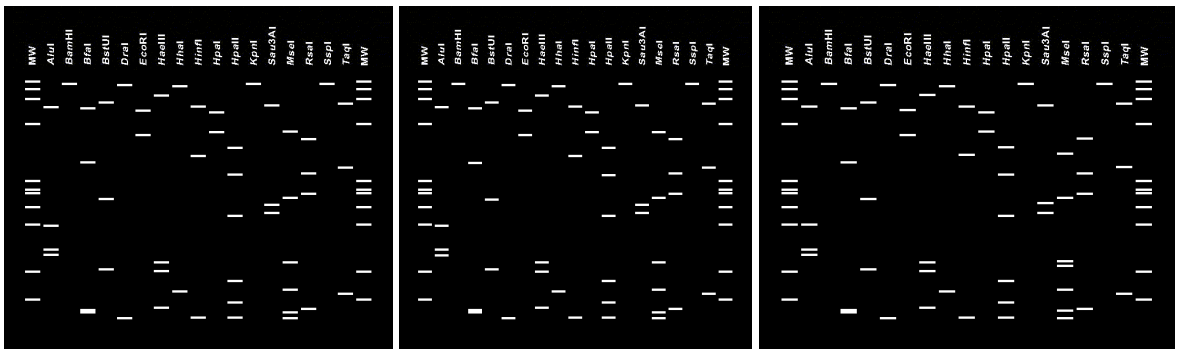

Figure 7 a: Virtual RFLP patterns of 1.2 kb 16S rDNA fragments of representative of 16SrII-C, cactus witches' broom phytoplasma YNO1 (GenBank acc. No. AJ293216); b: Alfalfa witches' broom (AWB) Chahgeer and Juyom strain; c: AWB strains collected from Bafg, Ardakan, Bahabad, Herat, Nikshahr, Bam, Zarand, Jiroft, Tabas, Jowkar and Zardenjan identical to the reference pattern of 16SrII-D reference phytoplasma (GenBank acc. No. Y10097) using online iphyclassifier program (Zhao *et al.,* 2009).

Discussion

During this study, AWB disease was observed all over the country with different incidence and severity. The majority of 16SrII-related AWB phytoplasmas detected in Fars, Yazd, Kerman, Esfahan, South Khorasan, Khuzestan, Hamedan and Bushehr provinces were members of -D and -C subgroups. These phytoplasma subgroups were widespread in Iran, and different plant species like sesame, pot marigold, garden beet and sunflower were reported as their hosts (Esmailzadeh Hosseini *et al.*, 2007; 2011a; 2011b; 2015a; 2015c; Mirzaie *et al.*, 2007; Salehi *et al.*, 2015). Alfalfa is a perennial plant that may play the role of alternative host of 16SrII–C and –D phytoplasma subgroups. With increasing in harvesting time disease severity and rate of mortality of infected plant increased. Despite the increasing of incidence and severity in infected fields, no correlation was observed between them; moreover, in several fields, a low infection percentage was observed indicating the complexity of AWB epidemiology. Considering the reported occurrence of 16SrII-D AWB strains in Sistan-Baluchestan province (South East of Iran) (Esmailzadeh Hosseini *et al.*, 2015b) and the results of these studies the predominant 16SrII phytoplasmas associated with AWB disease in Iran appears to belong to the 16SrII-D subgroup.

Due to the distribution of 16SrII AWB phytoplasma disease all over the country and especially in central, southern and east parts of Iran, and its natural transmission by *Orosius albicinctus* and *Neoaliturus haematoceps* (Salehi *et al.*, 1993a; 1995; Esmailzadeh Hosseini *et al.,* 2015a; 2015b) it seems that AWB strains play important roles also in the epidemiology of other phytoplasma diseases.

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بررسی خصوصیات فیتوپلاسماهای آران‌ای ریبوزومی گروه ۲ همراه با بیماری جاروک یونجه در مناطق مختلف ایران

سیدعلیرضا اسمعیل‌زاده حسینی[۱،۲]، غلام خداکرمیان[۱*]، محمد صالحی[۳] و آسوآنتا برتاچینی[۴]

۱- گروه گیاه‌پزشکی، دانشگاه بوعلی سینا همدان، همدان، ایران.
۲- بخش تحقیقات گیاه‌پزشکی، مرکز تحقیقات و آموزش کشاورزی و منابع طبیعی استان یزد، سازمان تحقیقات، آموزش و ترویج کشاورزی، یزد، ایران.
۳- بخش تحقیقات گیاه‌پزشکی، مرکز تحقیقات و آموزش کشاورزی و منابع طبیعی استان فارس، سازمان تحقیقات، آموزش و ترویج کشاورزی، زرقان، ایران.
۴- آزمایشگاه بیماری‌شناسی گیاهی، بخش علوم کشاورزی دانشگاه بولونیا ایتالیا.
* پست الکترونیکی نویسنده مسئول مکاتبه: khodakaramian@yahoo.com
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چکیده: جاروک یکی از مهم‌ترین بیماری‌های یونجه در ایران است. به‌منظور بررسی ویژگی‌های فیتوپلاسماهای گروه آران‌ای ریبوزومی جاروک بادام زمینی (16SrII) همراه با این بیماری، طی سال‌های ۱۳۹۲ تا ۹۴ گیاهان یونجه دارای علایم بیماری و بدون آن جمع‌آوری و در آزمون‌های پی‌سی‌آر مستقیم و دو مرحله‌ای با استفاده از جفت آغازگرهای P1/P7، R16mF2/R16mR2 و R16F2n/R16R2 مورد بررسی قرار گرفتند. محصول پی‌سی‌آر ۱/۸، ۱/۴ و ۱/۲۵ کیلو جفت باز حاصل از این آغازگرها فقط در نمونه‌های گیاهان دارای علایم تکثیر شد. آزمون چندشکلی طولی قطعات برشی محصول پی‌سی‌آر با جفت آغازگر R16F2n/R16R2 نشان داد که فیتوپلاسماهای همراه با بیماری جاروک یونجه اعضای زیر گروه‌های D و C گروه 16SrII می‌باشند. آنالیز بلاست با استفاده از توالی‌های به‌دست آمده و میزان تشابه نوکلئوتیدی استرین‌های در دست مطالعه و استرین‌های گرفته شده از بانک جهانی ترادف‌ها نشان داد که فیتوپلاسماهای جاروک یونجه جمع‌آوری شده از بافق، اردکان، بهاباد و هرات در استان یزد، نیک‌شهر در سیستان و بلوچستان، بم، زرند و جیرفت در استان کرمان، بوشهر در استان بوشهر، طبس در استان خراسان جنوبی، جوکار در استان همدان و زردنجان در استان اصفهان در زیر گروه D و استرین‌های چاهگیر در استان یزد و جویم در استان فارس در زیر گروه C قرار می‌گیرند. براساس این نتایج استرین‌های متعلق به زیرگروه **D** غالب می‌باشند. در اشکذر و ابرکوه در استان یزد تمامی مزارع یونجه به بیماری جاروک آلوده بودند. در اشکذر مزارع یونجه ۳ ساله به‌دلیل میزان آلودگی بالا به بیماری جاروک شخم زده شدند.

واژگان کلیدی: فیتوپلاسما، زیر گروه آران‌ای ریبوزومی D و C، PCR، RFLP

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