خصوصیات مولکولی، سرولوژیکی و بیولوژیکی دو فیتوپلاسمای متعلق به زیر گروه C درگروه آران ای ریبوزومی 16SrII همراه با بیماری جاروک یونجه در استان های یزد و فارس

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چکیده

جاروک یونجه یک فاکتور محدود کننده ی رشد و تولید یونجه در ایران به ویژه در مناطق گرمسیری می باشد. استرین های فیتوپلاسمای همراه با بیماری جاروک یونجه در دو منطقه شدیدا" آلوده ی چاهگیر (استان یزد) و جویم (استان فارس) از نظر خصوصیات بیولوژیکی، سرولوژیکی و مولکولی مقایسه شدند. بر اساس علایم بیماری در مزارع یونجه جاروک یونجه چاهگیر و جویم قابل تفکیک نبودند. در پروانش و گوجه مایه زنی شده، فیتوپلاسمای جاروک یونجه جویم ریزبرگی بیشتری در مقایسه با فیتوپلاسمای جاروک یونجه چاهگیر ایجاد کرد. از بین زنجرک هایی که قبلا به عنوان ناقل بیماری جاروک یونجه گزارش شده بودند، فیتوپلاسمای جاروک یونجه جویم فقط با گونه *Orosius albicinctus* و فیتوپلاسمای جاروک یونجه چاهگیر فقط با گونه *Circulifer haematoceps* انتقال داده شد. بر اساس توالی ژن آر ان ای ریبوزومی 16S و ناحیه ی بین ژنی 16S-23S، جاروک یونجه جویم و چاهگیر قابل تفکیک نبودند، اگرچه ارتباط سرولوژیکی بین دو فیتوپلاسما در آزمون های الایزا و دیبا با استفاده از آنتی بادی های چند همسانه ای تولید شده بر علیه دو فیتوپلاسما مشاهده نشد. به دلیل تفاوت در ناقل، فقدان ارتباط سرولوژیکی و ایجاد علایم متفاوت در پروانش و گوجه فرنگی، فیتوپلاسما های جاروک یونجه چاهگیر و جویم دو استرین متفاوت از زیر گروه C در گروه آران ای ریبوزومی16SrII می باشند.

کلیدواژه: آر اف ال پی، الایزا، پی سی آر، دیبا، سرولوژی

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Biological, serological and molecular characteristics of two 16SrII-C-related phytoplasma strains associated with alfalfa witches' broom disease in Yazd and Fars provinces, Iran

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Abstract

Alfalfa witches' broom (AWB) is a limiting factor for alfalfa growth and production in Iran, especially in the tropical regions of the country. AWB phytoplasma strains from two severely affected areas, Chahgeer (Abarkooh, Yazd province) and Juyom (Larestan, Fars province), were compared for main biologic, serologic and molecular characteristics. Based on disease symptoms in alfalfa farms, Chahgeer and Juyom AWB (CAWB and JAWB, respectively) strains were not differentiable. In dodder inoculated periwinkle and tomato plants JAWB phytoplasma induced stronger little leaf compared to the one induced by CAWB phytoplasma. In these experiments the two JAWB and CAWB phytoplasma strains are confirmed as vectored by different leafhopper species, *Orosius albicinctus* and *Circulifer haematoceps* respectively. Based on 16S rRNA gene and 16S-23S intergenic spacer region sequences, CAWB and JAWB were not differentiable, however no serologic relationship was observed between the two phytoplasmas in ELISA and DIBA tests using polyclonal antibodies prepared against each of them. Due to the lack of serological relationship, different insect vectors and induction of different symptoms in common host plants, CAWB and JAWB phytoplasmas should be considered as two different AWB strains both belonging to 16SrII-C subgroup.

Keywords: DIBA, ELISA, PCR, RFLP, serology

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Introduction

Phytoplasmas, members of class *Mollicutes*, are cell wall-less prokaryotes inhabiting the phloem of a wide range of plant species and are associated with different destructive plant diseases worldwide (Bertaccini *et al.,* 2014). They are transmitted mainly by leafhoppers and psyllids; typical symptoms include yellowing, discoloration, witches' broom, dwarfing, virescence and phyllody. Alfalfa has been reported as host of different phytoplasmas worldwide. At least eight distinct phytoplasmas have been identified associated with alfalfa including '*Candidatus* Phytoplasma asteris' (Peters *et al.,* 1999; Jomantiene *et al.,* 2000; Jones *et al.* 2005), '*Ca.* P. trifolii' and '*Ca.* P. fraxini' (Conci *et al.* 2005), '*Ca*. P. solani' (Marzachì *et al.,* 2000; Starović *et al.,* 2012), '*Ca*. P. ulmi' (Li *et al.,* 2012), '*Ca.* P. australiense' (Getachew *et al.,* 2007), '*Ca*. P. pruni' (Starović *et al.* 2012), and '*Ca*. P. aurantifolia' (Esmailzadeh Hosseini *et al.,* 2015c; Khan *et al.,* 2001; 2002; Al-Saleh *et al.,* 2014). Alfalfa witches' broom (AWB) disease is widespread all over Iran (Esmailzadeh Hosseini *et al.,* 2015a; 2015b; 2015c; 2016a; 2016b; 2016c), and its major disease symptoms include excessive branching, little leaf, internode shortening, flower malformation, dwarfing, yellowing and eventually death of the infected plant (Esmailzadeh Hosseini *et al.,* 2015a; 2015c). Whole alfalfa field's infection was observed in 2 and 3 years stand alfalfa in different parts of Iran (Esmailzadeh Hosseini *et al.,* 2015a; 2016a). In the early 1990s, occurrence of AWB phytoplasma disease was observed in Sistan-Baluchestan, Fars and Kerman provinces (Salehi and Izadpanah 1993), and subsequently in all alfalfa growing areas on different cultivars of *Medicago sativa* L., especially in central and southern provinces of Iran (Salehi *et al.,* 1995; Esmailzadeh Hosseini *et al.,* 2015a; 2015b; 2015c). Among several leafhopper species collected in alfalfa fields in Yazd and Fars provinces, *Orosius albicinctus* Distant and *Circulifer haematoceps* (Mulsant & Rey) were identified as insect vectors (Salehi *et al*. 1995; Esmailzadeh Hosseini *et al.,* 2015a; 2015b). The aim of the present work was to study biological, serological and molecular properties of two 16SrII-C AWB phytoplasmas from two severely AWB affected areas in Iran, Chahgeer (Abarkooh, Yazd province) and Juyom (Larestan, Fars province).

Material and Methods

Phytoplasmas used in this study

Juyom (Fars province) and Chahgeer (Yazd province), alfalfa-witches' broom phytoplasmas (JAWBP and CAWBP, respectively) maintained in periwinkle [*Catharanthus roseus* (L.) G. Don] by dodder inoculation (Salehi *et al.,* 1995) were used for biological and molecular studies. P1/P7 primed PCR products (Deng and Hiruki, 1991; Schneider *et al.,* 1995) of JAWB and CAWB strains were cloned, sequenced, and 1,803 and 1,806 bp sequences were deposited in GenBank under the accession numbers DQ233656 and DQ233655, respectively.

Dodder transmission

Dodder transmission was employed for propagation and maintenance of the two strains and also for the assessment of symptom expression in periwinkle and tomato (*Lycopersicum esculentum* Mill.) (Salehi *et al.,* 1995). Seeds of dodder (*Cuscuta campestris* Yunk.) were germinated on moist filter paper, and seedlings were transferred to healthy seed grown eggplants under insect-proof conditions. After 3-4 weeks connections were established between dodder strands from healthy eggplants and infected periwinkle plants previously dodder inoculated with JAWB or CAWB phytoplasmas. One month later, newly developed strands of dodder from infected periwinkle plants were connected to seed grown 15 healthy periwinkle and 10 healthy tomato plants. The connection was maintained for 30 days, after which the plants were freed from dodder strands and kept in an insect-proof greenhouse for symptom development. Negative controls were represented by 10 healthy periwinkle plants and 10 healthy tomato plants inoculated with dodder grown on healthy eggplants.

Graft transmission

For propagation and long-term maintenance of disease sources, ten-week old seed grown periwinkle plants for each phytoplasma strain were side grafted with small axillary shoots from newly infected periwinkle plants (Salehi *et al.,* 1995). The graft area was wrapped with parafilm and each grafted plant was separately covered with a plastic bag for a week to maintain humidity.

Vector transmission

C. haematoceps and *O. albicinctus*, which previously reported as AWB phytoplasma vectors in Iran (Salehi *et al.* 1995; Esmailzadeh Hosseini *et al.,* 2015a; 2015b) were tested to verify their ability to transmit CAWB and JAWB phytoplasmas considering their close molecular relationships. From each alfalfa field in Chahgeer and Juyom, insects were collected by D-vac aspirator; *C. haematoceps* and *O. albicinctus* leafhopper species were separated and selected for transmission trials. The two leafhopper species were identified by comparison to the voucher specimens identified previously by the British Museum (UK). Non-inoculative colony of each species was developed by caging single fertilized females on healthy seed grown sugar beet (*Beta vulgaris* subsp. *vulgaris*) plants, a suitable host plant for rearing, egg deposition, and subsequent hatching of both insect species. Samples of leafhoppers (five samples per each colony) and sugar beet plants were checked for phytoplasma absence by nested PCR assays (Zhang *et al.,* 1988). Inoculative colonies of each species were developed by transferring adult leafhoppers (five per each colony) separately on three CAWBP or JAWBP infected periwinkle plants. Total nucleic acids were extracted from five leafhopper samples per each colony by the Doyle and Doyle (1990) method and phytoplasma presence in inoculative colonies was tested by nested PCR before inoculation trials. To assess *C. haematoceps* and *O. albicinctus* ability to transmit CAWB and JAWB strains, from each inoculative colony 80 leafhoppers were caged on 20 periwinkle plants (4 insects per plant and 4 plants in each cage) for four weeks inoculation access period (Salehi *et al.,* 1995). The plants were then sprayed with *metasystux-R* insecticide and maintained in insect-proof greenhouse for symptom expression.

For all transmission tests acquisition access period (AAP) and inoculation access period (IAP) were 3 and 4 weeks, respectively. All the transmission tests were carried out at the same time on 8 week-old periwinkles.

Enzyme linked immunosorbent assay (ELISA) and dot immunobinding assay (DIBA) tests

Serologic comparison between CAWB and JAWB phytoplasmas was carried out in ELISA and DIBA tests using polyclonal antisera raised against JAWB (Salehi *et al.,* 2011) and CAWB (Esmailzadeh Hosseini *et al.,* 2015c) considering the very close relationships of the two phytoplasmas at the molecular level and considering that they are sharing the same host plant. Healthy extract was produced from seed grown periwinkle in insect proof greenhouse. ELISA tests were carried out by using the Clark and Adams (1977) method using samples from 8 infected periwinkle plants for each phytoplasma strain and 5 replications. The DIBA test was carried out by the method described by Hibi and Saito (1985) using 8 samples of each plant extract represented by Borazjan faba bean phyllody (positive control), CAWB, JAWB, and healthy faba bean and periwinkle (negative controls) with 6 replications. To carry out DIBA test, 0.1 g of midrib tissue was homogenate with a pestle and mortar in 1 ml of TBS-T [.0.05% Tween 20 in 20 mM-Tris HC1 containing 0.5 M-NaC1, pH 7.5 (TBS)]. After centrifugation of the homogenized at 7,000 rpm for 10 min (VISION centrifuge, South Korea), the supernatant was used for dot blot test at room temperature. Aliquots of 5-7 µl of antigen were blotted on a nitrocellulose sheet and after blocking with blocking buffer (TBS-T containing 2% PVP and 3% skimmed milk), 1.5 µl/ml of polyclonal antiserum of each of the two strains were again applied following described procedures (Hibi and Saito 1985). After addition of 5-7 µl of blue tetrazolium (NBT, Sigma-Aldrich Co.) to each spot, the nitrocellulose was maintained for 10 minutes in a dark room and appearance of well-defined purple spots was regarded as positive reaction.

DNA extraction and polymerase chain reaction (PCR)

Total DNA was extracted from 0.2 g midrib tissues of infected symptomatic periwinkle and tomato plants grafted and dodder inoculated with JAWBP and CAWBP using the method described by Zhang *et al.* (1988). Total nucleic acids were extracted from leafhoppers species using Doyle and Doyle procedure (1990). Total DNA extracted from healthy periwinkles and a symptomatic periwinkle plant infected with lime witches' broom phytoplasma (Salehi *et al.,* 2005) were used as negative and positive controls, respectively. DNA samples were tested for phytoplasma presence by direct PCR using P1/P7 (Deng and Hiruki, 1991; Schneider *et al.,* 1995) and nested PCR using

R16F2n/R16R2 primer pair (Gundersen and Lee, 1996). PCR was performed in a 25 µl reaction volume containing 50 ng DNA, 0.4 µM 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. Amplification was carried out in a thermal cycler (Bio-Rad T100, USA) for 35 cycles as follows: 45 sec denaturation at 94°C (3 min for the first cycle), 45 sec annealing at 55°C and 2 min of extension at 72°C. In the final cycle the extension step was extended to 10 min. PCR conditions for the nested PCR were the same except that the annealing temperature was 58°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). DNA bands were stained with ethidium bromide and visualized with a UV transilluminator. The molecular weight of the PCR products was estimated by comparison with 100 bp DNA ladder (Fermentas, Vilnius, Lithuania).

Virtual RFLP analysis of sequences

Virtual RFLP analysis using *i*PhyClassifier (Zhao *et al.,* 2009) was used to determine subgroup affiliation of JAWB and CAWB strains. RFLP profile of 1,247 bp R16F2n/R16R2 primed sequences of JAWB phytoplasma (JAWBP, GenBank Accession No. DQ233655) and CAWB phytoplasma (CAWBP, GenBank Accession No. DQ233656) were compared to each other and to those of 16SrII-subgroup phytoplasmas. Each aligned DNA fragment was digested *in silico* with the 17 restriction enzymes available in the program and that have been used for the phytoplasma 16S rDNA RFLP analysis (Lee *et al.,* 1998).

Sequence homology and phylogenetic analysis

The 16S rDNA sequences of 19 phytoplasmas including JAWB and CAWB were aligned and phylogenetic trees and sequence homologies were generated using MEGA software version 6 (Tamura *et al.,* 2013). *Acholeplasma laidlawii* was used as an outgroup to root the tree. Bootstrapping was performed 1,000 times to estimate the stability and support for the branches.

Results

Symptomatology of sources of phytoplasmas employed in this study

In Juyom and Chahgeer areas the disease symptoms in alfalfa were not differentiable. Main disease symptoms were little leaf, internode shortening, virescence, phyllody, witches'-broom, leaf yellowing and curling, stunting, decline and death.

Graft and dodder transmission

In graft inoculation trials, all 10 periwinkle plants graft inoculated with CAWBP and JAWBP, developed disease symptoms in 4-6 weeks.

In dodder inoculation with JAWB and CAWB phytoplasmas, 11 out of 15 and 9 of 15 periwinkle plants, respectively showed phytoplasma symptoms and among 20 tomato plants (10 plants each JAWB and CAWB), 8 and 7 tomato plants respectively showed phytoplasma symptoms.

The main symptoms exhibited by experimentally infected periwinkle and tomato plants were little leaf, virescence, phyllody, yellowing and stunting. In periwinkle and tomato plants, little leaf, stunting and yellowing symptoms were stronger and more marked in JAWB strain compared to CAWB strain. All inoculated symptomatic plants reacted positively in direct and nested PCR assays. None of the control plants parasitized by dodder grown on healthy eggplants showed disease symptoms. Nested PCR assay also verified the phytoplasma absence in all control plants.

Leafhopper transmission

From 20 periwinkle plant inoculated by *O. albicinctus* reared on Juyom AWB strain and Chahgeer AWB strain separately, JAWB was transmitted in 14 plants that all showed phytoplasma symptoms, while CAWB-inoculated periwinkle remained symptomless for 6 months. In separate trials 11 out of 20 periwinkle plants exposed to *C. haematoceps* fed on CAWB infected periwinkles showed disease symptoms, but none of those reared on JAWB infected periwinkle could induce disease symptoms. In all cases, the non-symptomatic plants were phytoplasma negative when checked by nested PCR.

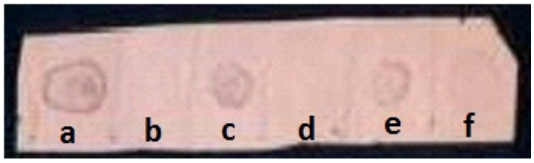

Fig. 1. Reaction of CAWB and JAWB in dot immunobinding assay test using polyclonal antiserum to JAWB phytoplasma. a: Borazjan faba bean phyllody (homologous reaction); b: CAWB; c: JAWB; d: negative control, healthy plant; e: JAWB; f: CAWB.

Phytoplasma detection by ELISA and DIBA

In ELISA tests, with CAWB antiserum the means of absorbance of 8 wells with CAWB and JAWB antigens was 0.251 and 0.084 respectively. Also mid average of absorbance of 8 wells using JAWB antiserum with JAWB and CAWB antigens was 0.197 and 0.058 respectively. Means + 3 sd (standard deviation) of OD of 4 healthy wells was 0.097. Each sample well with OD higher than 0.097 was assumed to be positive (Sutula *et al*., 1986). In DIBA test, using JAWB antiserum appearance of well-defined purple spots on nitrocellulose sheets were obvious in JAWB antigen and positive control (BFBP, Borazjan faba bean phyllody) (16SrII group) (Salehi *et al.,* 2005), but no reaction was observed in healthy plant and with CAWB antigen. These results confirmed the ELISA data (Fig. 1).

Polymerase chain reaction and RFLP analysis

DNA fragments of approximately 1800 and 1250 bp were amplified in direct and nested PCR respectively, from all graft and vector inoculated symptomatic plants and positive control. No amplification was observed in DNA samples from symptomless plants in direct and nested PCR assays. Restriction patterns of nested PCR amplicon from JAWB and CAWB were identical to that of peanut witches' broom phytoplasmas (16SrII group) (data not shown).

Nucleotide sequence analyses

In BLAST search using 1.8 kbp sequences the relatedness of CAWB and JAWB phytoplasmas to group 16SrII was confirmed. Phylogenetic analysis of 1,247 bp of CAWB and JAWB with other phytoplasma sequences yielded the phylogenetic tree shown in Fig. 2. These phytoplasmas grouped in the phylogenetic branch enclosing the phytoplasma strains classified in 16SrII group and were most closely related to the faba bean phyllody (X83432), cactus witches' broom phytoplasma strains YNO1 (AJ293216) both representatives of subgroup 16SrII-C. Pairwise homology (%) of 16S rRNA gene sequences and 16S-23S intergenic spacer region (SR) among CAWB and JAWB showed 99.9 and 100% homology respectively (Tables 1 and 2).

Virtual RFLP analysis

After virtual digestion of the 1,247 bp 16S rDNA, CAWB and JAWB exhibited RFLP profiles identical with each other and to that of cactus witches' broom phytoplasma strain YNO1

Table 1. Pairwise homology (%) among CAWB and JAWB and phytoplasmas in group 16SrII using 16S rRNA gene sequences

	CAWB*	JAWB**	CWBP YN20	CWBP YN01	FBP	'*Ca*. P. aurantifolia'	PnWB	'*Ca*. P. australasia'	'*Ca*. P. asteris'
CAWB *	100								
JAWB**	99.9	100							
CWB YN20	100	99.9	100						
CWBP YN01	100	99.9	100	100					
FBP	100	99.9	100	100	100				
'*Ca*. P. aurantifolia'	99.4	99.4	99.4	99.4	99.4	100			
PnWB	98.4	98.3	98.4	98.4	98.4	98.5	100		
'*Ca*. P. australasia'	98.5	98.4	98.5	98.5	98.5	98.7	99.8	100	
'*Ca*. P. asteris'	90.4	90.3	90.4	90.4	90.4	90.7	90.3	90.3	100

*CAWB, Chahgeer AWB (DQ233656); **JAWB, Juyom AWB (DQ233655); CWB YN20, cactus witches' broom phytoplasma strain YN20 (EU099565); CWBP YNO1, cactus witches' broom phytoplasma strain YNO1 (AJ293216); FBP, faba bean phyllody (X83432); *'Ca.* P. aurantifolia' (U15442); PnWB, peanut witches' broom (L33765); *'Ca.* P. australasia' (JQ868448); *'Ca.* P. asteris' (M30790).

Table 2. Pairwise homology (%) among 16S-23S intergenic spacer region of CAWB, JAWB and phytoplasmas in group 16SrII

	JAWB**	CAWB*	FBP	ShP	'Ca. P. australasia'	'Ca. P. aurantifolia'	'Ca. P. trifolii'
JAWB**	100						
CAWB *	100	100					
FBP	99.6	99.6	100				
ShP	98.8	98.8	98.4	100			
'*Ca*. P. australasia'	98.8	98.8	98.4	100	100		
'*Ca*. P. aurantifolia'	98.4	98.4	97.9	97.1	97.1	100	
'*Ca*. P. trifolii'	80.2	80.2	80.2	79.8	79.8	79.4	100

JAWB**, Juyom AWB (DQ233655); CAWB*, Chahgeer AWB (DQ233656); FBP, faba bean phyllody (X83432); ShP, sunhemp witches' broom (AB558143); *'Ca.* P. australasia' (JQ868448); *'Ca.* P. aurantifolia' (U15442); *'Ca.* P. trifolii' (AY390261).

(GenBank accession No. AJ293216) representative of 16SrII subgroup –C confirming that no differences at the RFLP level were present after evaluation with the *i*PhyClassifier tool that could be related to the reported serological differences.

Discussion

AWB phytoplasma disease distributed all over the country especially in central, southern and east parts of Iran, and its natural transmission by *O. albicinctus* and *C. haematoceps* was confirmed (Salehi *et al.*, 1993a; 1995; Esmailzadeh Hosseini *et al.,* 2015a; 2015b). In recent studies, 16SrI (aster yellows) group, 16SrII (peanut witches' broom) subgroup C and D, 16SrVI (clover proliferation) subgroup A and 16SrXII ("stolbur") subgroup A were identified in symptomatic alfalfa all over the country with different incidence and severity (Esmailzadeh Hosseini *et al.,* 2015a; 2015b; 2015c; 2016a; 2016b; 2016c). The majority of 16SrII-related AWB phytoplasmas detected were members of –D and -C subgroups (Esmailzadeh Hosseini *et al.,* 2016c). 16SrII subgroup C were predominant strains in destructive epidemics of AWB in Chahgeer (Abarkooh, Yazd province) where the AWB disease reduced alfalfa production to less than one-third, alfalfa plantations were destroyed completely, and farmers switched on to other crops (Esmailzadeh Hosseini *et al.,* 2015c).

Because alfalfa is a perennial plant it is an appropriate host for survival of phytoplasmas and play an important role in epidemiology of phytoplasma diseases.

According to the present and previous studies (Esmailzadeh Hosseini *et al.,* 2015a; 2015c; 2016a; 2016c) symptomatology in alfalfa could not be employed as a criterion for phytoplasma strain separation, also the pairwise homology of 16S rRNA gene sequences and 16S-23S intergenic spacer region (SR) among CAWB and JAWB showed that they were not differentiable; however the present study shows that some biological properties allow distinguishing the two phytoplasmas.

In previous studies (Salehi *et al*., 1995; Esmailzadeh Hosseini *et al*., 2015a; 2015b.) different leafhopper species including *C. haematoceps, O. albicinctus, Neoaliturus fenestratus, Psammotettix striatus, Neoaliturus guttulatus, Austroagallia sinuate, Anaceratagallia laevis* and *Empoasca descipiens* were identified in alfalfa farms in Chahgeer and Juyom. Among leafhoppers collected in these fields, *O. albicinctus* and *C. haematoceps* were identified as vectors of JAWB and CAWB phytoplasmas, respectively (Salehi *et al.*, 1995). All previous attempts for transmission of JAWB phytoplasma by *C. haematoceps* and CAWB phytoplasma by *O. albicinctus* were negative (Salehi *et al.*, 1995, Esmailzadeh Hosseini *et al.,* unpublished). This result is very interesting and deserve further specific investigation to verify the possible presence of phytoplasma infection in the two vector species with different strains having some kind of reciprocal inhibition for phytoplasma transmission in each single species.

Based on vector specificity, CAWB and JAWB phytoplasma strains were differentiable from each others having different insect vector in experimental transmission and also from '*Ca.* P. aurantifolia' that is transmitted by *Hishimonus phycitis* in a different plant host species (Salehi *et al*., 2007). Sequence-based related phytoplasmas could be differentiated using serology (IRPCM, 2004; Salehi *et al.*, 2011) and CAWB and JAWB strains were dis

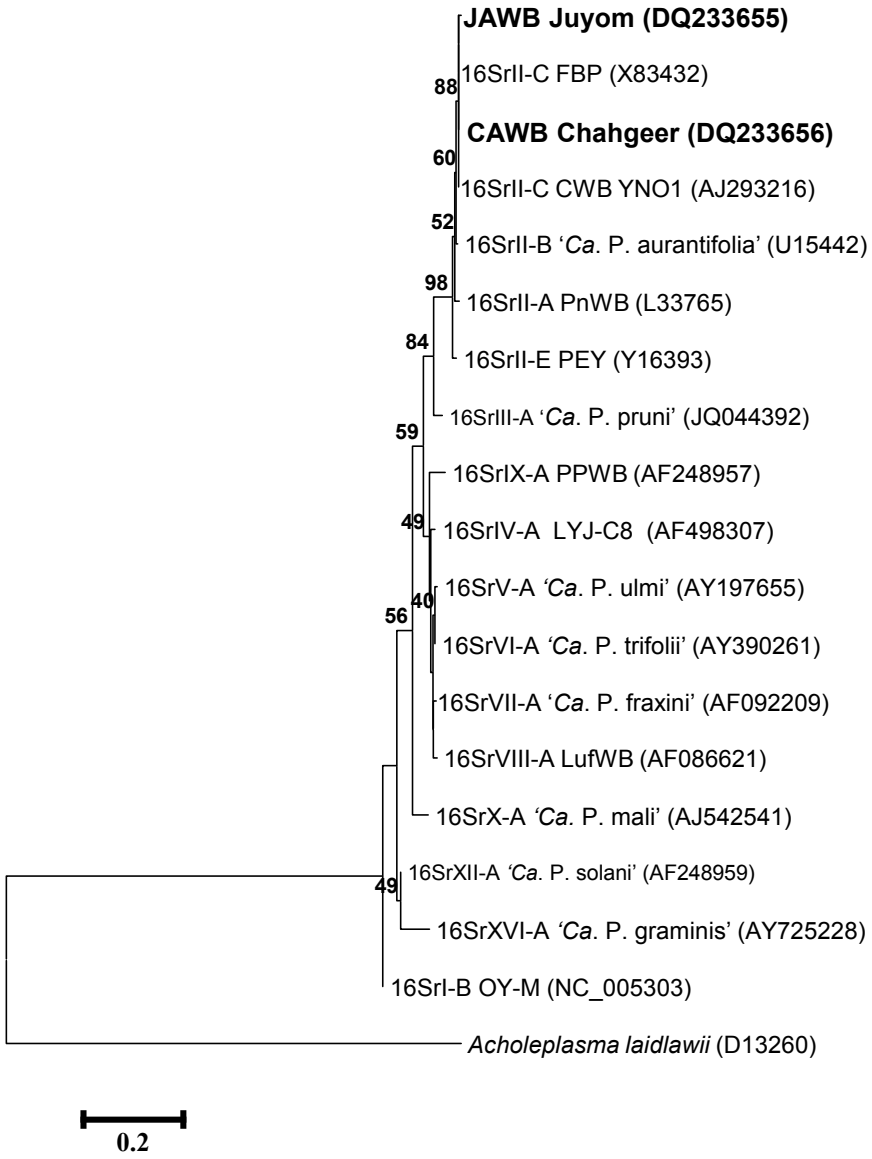

Fig. 2. Phylogenetic tree constructed by the Neighbor-Joining method of 1,247 bp of *16S rRNA* gene sequences from 18 phytoplasmas and *A. laidlawii*, as outgroup. Chahgeer and Juyom AWB phytoplasmas are bolded. '*Ca.* P.': '*Candidatus* Phytoplasma'. Ribosomal grouping is indicated (16Sr) before phytoplasma name or acronym. GenBank accession numbers for sequences are given in parentheses after the phytoplasma acronyms. Numbers at the nodes are bootstrap (confidence) values based on 1,000 repetitions (only values above 40 are shown). Abbreviations: JAWB: alfalfa witches' broom Juyom; FBP: faba bean phyllody; CAWB: alfalfa witches' broom Chahgeer; CWB YNO1: Cactus witches' broom; PnWB: peanut witches' broom; PEY: *Pichris echioides* phyllody; PPWB: pigeon pea witches' broom; LYJ-C8: Coconut lethal yellowing; LufWB: loofah witches' broom; OY-M: onion yellows mild strain.

tinguishable by ELISA and DIBA tests using specific polyclonal antisera. Collectively due to the lack of serologic relationship, difference in insect vector and induction of different symptoms in experimental common hosts, JAWB and CAWB should therefore to be regarded as two different phytoplasma strains associated with alfalfa witches' broom disease in Iran in two different locations.

This is the first experimental evidence of AWB phytoplasma strains molecularly indistinguishable in *16S rRNA* gene showing diverse biological and

serological properties; further studies should be carried out to allow their characterization as diverse biological variant of the same phytoplasma.

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