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A 16SrII-D Phytoplasma strain associated with Tomato Witches'-Broom in Bushehr province, Iran

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Abstract: In 2010- 2012 surveys, witches'- boom disease of tomato was observed in Borazjan area (Bushehr province, Iran). Agent of the disease was transmitted from tomato to tomato and eggplant by grafting and to Madagascar periwinkle via dodder inoculation, inducing phytoplasma-type symptoms in inoculated plants. Presence of phytoplasma in naturally affected tomatoes and all symptomatic graft and dodder inoculated plants was confirmed by direct and nested polymerase chain reactions (PCR) using primer pairs P_1/P_7 and $R_{16}F_{2n}/R_{16}R_2$. BLAST search and phylogenetic analysis of 16SrDNA showed that detected phytoplasma belonged to peanut witches'- broom (16SrII) group. Phylogenetic analysis, percent homology and virtual RFLP indicated that, as a member of 16SrII group, Borazjan tomato witches'- broom (BTWB) phytoplasma together with Bushehr eggplant and alfalfa witches'- broom (BEWB and BAWB, respectively) phytoplasmas were classified with *Candidatus* Phytoplasma australasia, a phytoplasma related to 16SrII-D subgroup. Based on the same analysis, BTWB, BEWB and BAWB phytoplasmas were differentiable from three other Iranian 16SrII related phytoplasmas associated with alfalfa witches'- broom diseases in Yazd and Fars provinces and lime witches'- broom disease in southern Iran.This is the first report of tomato witches'-broom disease and characterization of its associated phytoplasma in Iran.

Keywords: *tomato diseases, phytoplasmas, witches'- broom, graft and dodder transmission, 16SrII-D subgroup*

Introduction

Phytoplasma diseases of tomato (PDT) have been reported in several countries around the world (EPPO/CABI 1996) under different names including big bud (Shaw *et al.,* 1993; Granett and Provvidenti, 1974; Dale and Smith, 1975; Del Serrone *et al.,* 2001; Varma 1979; Zimmermam-Gries and Klein, 1978; Anfoka *et al.,* 2003; Xu *et al.,* 2013), stolbur (Ploaie, 1981; BrcaK, 1979; Valenta *et al.*, 1961), mal azul (EPPO/CABI, 1996), tomato yellows (Holguín-Peña and Vázquez-Juárez, 2007; Tapia-Tussell *et al.,* 2o12), and hoja de perejil'(Arocha *et al.,* 2007). PDT have been ascribed to at least six distinct phytoplasma groups worldwide. These include aster yellows (16SrI) subgroups A (Lee *et al.,* 1998) and B (Okuda *et al.,* 1997; Marcone *et al.,* 1997; Archoa *et al.,* 2007), peanut witches'- broom (16srII) subgroups A (Xu *et al.,* 2013) and D (Omar and Foissac, 2012; Singh *et al.,* 2012), Western-X (16SrIII) (Del Serrone *et al.,* 2001; Tapia-Tussell *et al.,* 2012; Amaral mello *et al.,* 2006), elm yellows (16SrV) (Del Serrone *et al.,* 2001), clover proliferation (16SrVI) (Anfoka *et al.,* 2003, Lee *et al.,* 1998, Du *et al.,* 2013) and stolbur (16SrXII) subgroup A (Sertkaya *et al.,* 2007: Vellios and Lioliopoulou, 2007).

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Iran has a total annual production of 4,826,396 tons of tomato and ranks seventh in the world for tomato production (Anonymus, 2013). Symptoms of tomato big bud (TBB) have been previously reported from Iranian provinces of Fars (Salehi and Izadpanah, 1992), Isfahan, Ardabil, West Azarbaijan (Rashidi *et al.,* 2006), Khorasan (Jamshidi *et al.,* 2010) and Lorestan (Dehghani and Salehi, 2011). The Main symptoms of TBB in these provinces are abnormal flowers with enlarged and united calyx segments (big bud), and virescent petals. In 2010–2012 surveys severe witches'- broom disease of tomato was observed in Borazjan (Bushehr province). Absence of big bud symptoms, suggested that Borazjan tomato witches'- broom (BTWB) is a new phytoplasma disease in Iran. The aim of this study was to characterize this phytoplasma molecularly and biologically.

Materials and Methods

Source of disease and plant material

A tomato (*Solanum lycopersicum* L.) plant with typical symptoms of witches'- broom disease was selected in a Borazjan tomato field and used as the source of the disease agent for biological and molecular studies. BTWB was propagated and maintained in a red line of Madagascar periwinkle (*Catharanthus roseus*). Nine -week-old seed grown periwinkle, eggplant (*Solanum melongena*) and tomato plants, maintained in an insect-free greenhouse at 30 °C during the day and 20 ˚C at night, were used for dodder and graft inoculation.

Dodder and graft inoculation

For dodder transmission, seeds of dodder (*Cuscuta campestris* Yunk.) were germinated on moist paper and transferred to witches'- broom source plant. After three weeks, dodder strands were used to connect each source tomato plant to five healthy, seed grown periwinkle plants. Connections were maintained for 4 weeks, after which the test plants were freed of dodder strands and kept in an insect-free greenhouse. Controls were exposed to dodder grown on healthy sugar beet. Infection of dodder-inoculated plants was verified by nested PCR.

Graft inoculation was used to transmit tomato witches'- broom agent from naturally infected tomato plants to tomato and eggplant (five per species) and from a symptomatic dodder-inoculated periwinkle plant to five healthy 10-week-old periwinkle plants. Following graft inoculations, inoculated plants were placed in an insect-free greenhouse.

DNA extraction and PCR amplification

Total DNA was extracted from 0.25 gr of fresh midrib tissue of naturally witches'-broom affected tomato plants or graft and dodder inoculated plants, using the method described by Zhang *et al.,* (1998). Total DNA extracted from healthy seed grown tomato, eggplant and periwinkle plants were used as negative controls and that from a periwinkle plant infected with lime witches'- broom phytoplasma as positive control.

The universal phytoplasma primer pair P_1/P_7 (Schneider *et al.,* 1995) was used in PCR for amplifying a 1.8 kbp fragment of ribosomal operon consisting of the 16SrRNA gene, the 16S-23S spacer region and a portion of the 5' region of 23SrRNA gene. A 1:30 dilution of the direct PCR product amplified by the P1/P7 primer pair was used as template for nested PCR, utilizing the primer pair $R_{16}F_{2n}/R_2$ which amplifies an internal DNA fragment of 1250 bp from the 16SrRNA gene (Gunderson and Lee, 1996). With primer pair P_1/P_7, each 50 µl PCR reaction mixture contained 100 ng of extracted DNA from diseased or healthy plants, 0.4 µM of each primer, 0.2 mM of each dNTP, 1.25 U of Taq DNA polymerase (CinnaGen, Iran) and 1 X PCR buffer. PCR was performed for 35 cycles in a thermal cycler (Bio-Rad, USA) using 1 min (2 min for the first cycle) denaturation step at 94 °C, 2 min for annealing at 50 °C and 3 min (10 min for the last cycle) for primer extension at 72 °C. Conditions for nested PCR were the same, except for the annealing temperature that was 58 °C. PCR products were analyzed by electrophoresis in a 1% agarose gel in 1 X TBE buffer (67 mM Tris-HCl, 22 mM boric acid, 10 mM EDTA, pH 0.8) together with 100 bp DNA marker

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(Fermentas, Lithuania). DNA bands were stained with ethidiuim bromide and visualized with a UV transilluminator.

Restriction fragment length polymorphism (RFLP)

Eight microlitres of nested-PCR products (1.2 kbp from 16S rDNA) of three isolates from different tomato field in Bushehr province were separately digested with the restriction enzymes *Alu*I, *Hpa*II, *Hinf*I *and Rsa*I according to the manufacturer's instructions (Fermentas, Lithuania) at 37 °C overnight. Digestion products were electrophoresed through 2.5% agarose gels and visualized after staining with ethidium bromide by UV transillumination. The resulting RFLP patterns were compared with those previously published for 16S rDNA from other phytoplasmas (Lee *et al.,* 1998; Marcone *et al.,* 2000).

Cloning, sequencing and phylogenetic analyses

A DNA fragment amplified with P_1/P_7 primer pair from a BTWB associated phytoplasma isolate was ligated to pTZ57R/T vector and cloned in *Escherichia coli* strain DH5α cells using InsT/A cloneTM PCR Product Cloning Kit (Fermentas, Lithuania) according to the manufacturer's instructions. Recombinants were screened using the blue and white screening method (Sambrook *et al.,* 1989). Sequencing was performed by Macrogen (South Korea) on both strands. The resulting sequence was deposited in the GenBank database and the whole length of 16S rDNA was used for BLAST search (http://blast.ncbi.nlm. nih.gov/Blast.cgi) and phylogenetic analysis. A phylogenetic tree was constructed using the neighbor-joining (NJ) method, with MEGA5 software, (Tamura *et al.,* 2011) comparing 33 phytoplasmas including BTWB isolate. The reliability of the tree was assessed by bootstrap analysis with 100 replications (Efron, 1982). The sequence homology between strains was evaluated after alignments were generated by using homology matrix distance option of DNAMAN program version 4.02 (Lynon Corporation, Canada).

Virtual RFLP analysis

Virtual RFLP analysis using iPhyclassifier (Zhao *et al.,* 2009) was used to determine subgroup affiliation of BTWB and selected phytoplasmas. RFLP profile of 1.25 kb fragment (F2n/R2 region of 16S rRNA gene) of BTWB phytoplasma was compared to those of 16SrII-subgroups A to E and five Iranian 16SrII related phytoplasmas associated with alfalfa witches'- broom disease in Fars, Yazd and Bushehr (FAWB, YAWB, BAWB, respectively) and Bushehr eggplant witches'-broom (BEWB) using *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I enzymes. Agarose gel (3%) electrophoresis image was plotted and the virtual RFLP patterns were compared.

Results

Symptomatology and Transmission

Characteristic symptoms of witches'- broom disease of tomato in Borazjan fields (Busheher province) were excessive development of long, curved spindly shoots from axillary buds along the stem, small deformed leaves at the tips of shoots and witches'- broom (Fig. 1A). Such plants did not produce flowers.

Agent of BTWB was transmitted from naturally diseased tomato to all graft inoculated eggplant and tomato plants. The main symptoms in graft inoculated tomato plants were exhibition of intense shoot proliferation, severe reduction of leaf size, shortened internodes, yellowing, witches'-broom and stunting (Fig. 1B). Symptoms in eggplants were yellowing, internode shortening, flower virescence, phyllody and proliferation (Fig. 1C), small leaves and witches'- broom. Three of five periwinkle plants inoculated via dodder developed flower virescence, phyllody (Fig. 1D), yellowing and internode shortening.

Phytoplasma infection of symptomatic periwinkle and graft inoculated plants was confirmed by PCR assay.

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PCR amplification

With universal primer pairs, P_1/P_7 and $R_{16}F_{2n}/R_2$, target DNA fragments of approximately 1.8 and 1.250 kbp were amplified by direct and nested PCR, respectively, from total nucleic acid samples extracted from all naturally affected tomato plants, experimentally inoculated plants and positive control (Fig. 2).

No DNA bands were observed from similarly processed samples of healthy plants.

RFLP analyses

Digestion of nested PCR products (1.2 kbp) of three witches'-broom affected tomato plants from Borazjan with *Alu*1, *Hin*f1, *Hpa*I and *Rsa*I enzymes yielded identical RFLP patterns (Fig. 3) corresponding to the profile of the peanut-witches'-broom group phytoplasmas (Lee *et al.*, 1998).

Computer-simulated restriction analyses were carried out on R16F2n/R16R2 sequences from BTWB phytoplasma together with four Iranian 16SrII related phytoplasmas associated with BEWB, BAWB, FAWB, and YAWB diseases and six representative strains in 16SrII subgroups (A, B, C, D, E and F). Visualization and comparison of virtual gel plotted images (Fig. 4) revealed that RFLP patterns of BTWB, BEWB and BAWB phytoplasmas were identical to those of *Candidatus* Phytoplasma australasia, representative of 16SrXII-D subgroup. The same analysis showed that FAWB and YAWB phytoplasmas belonged to 16SrII-C subgroup.

Figure 1 (A) excessive development of long, curved spindly shoots with small deformed leaves from axillary buds along the stem in a witches'- broom affected tomato plant in Borazjan; (B, C and D) disease symptoms in plants graft or dodder inoculated with agent of Borazjan tomato witches'- broom: (B) little leaf, internode shortening, shoot proliferation, witches'- broom, yellowing and stunting in tomato. (C and D) virescence and phyllody in eggplant and periwinkle, respectively.

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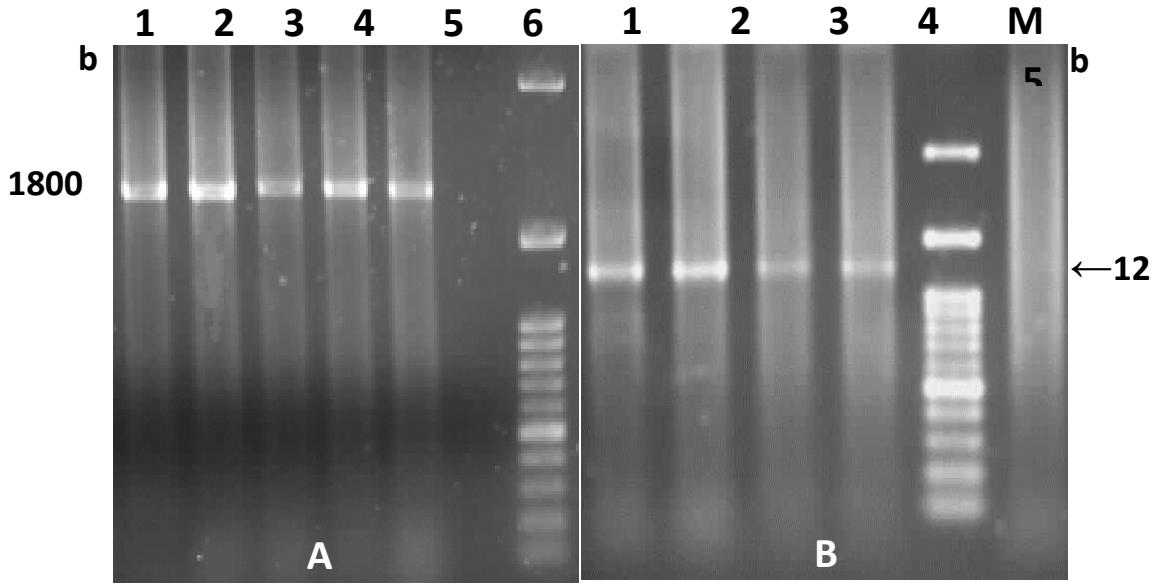

Figure 2 Electrophoresis pattern of PCR products: (A) Direct PCR using P_1/P_7 primer pair. Lanes 1-4, four witches'- broom affected tomato plants from field; lane 5, dodder inoculated periwinkle plant; (B) nested PCR using P_1/P_7 followed by $R_{16}F_{2n}/R_{16}R_2$ primer pairs. Lane 1, dodder inoculated periwinkle plant; Lanes 2 and 3, graft inoculated tomato and eggplant plants; Lane 4, naturally witches'- broom affected tomato plant. Lane 6 in A and Lane 5 in B, healthy tomato plant. (IT) Lanes M, 100 bp DNA marker.

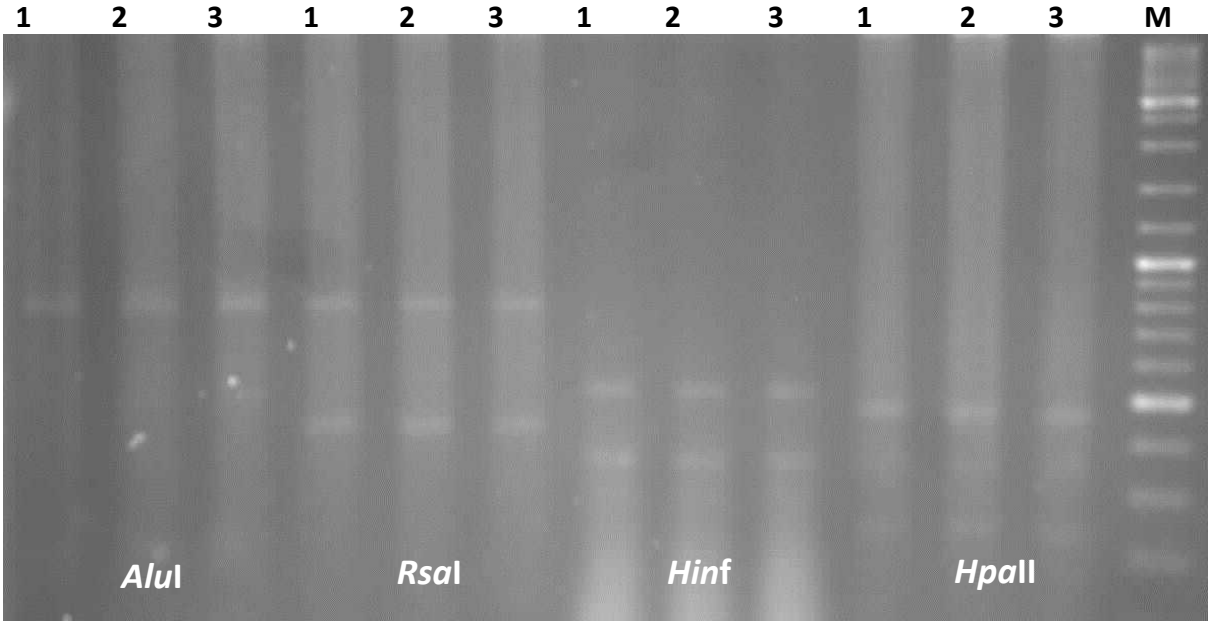

Figure 3 Restriction fragment length polymorphism (RFLP) analyses of 16SrDNA amplified by nested PCR from three different witches'- broom affected tomato samples using *Alu*I, *Hin*fI, *Hpa*II and *Rsa*I restriction enzymes. M: 100 bp DNA marker.

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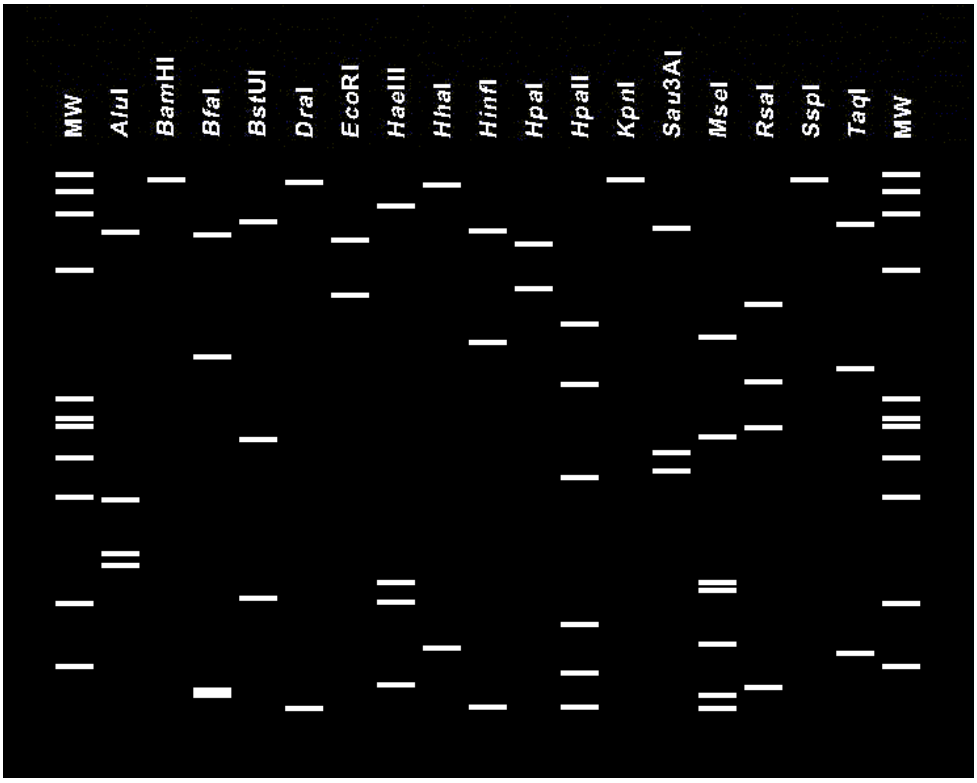

Figure 4 Virtual restriction fragment length polymorphism (RFLP) pattern of $R_{16}F_{2n}/R_2$ PCR product sequence from Borazjan (Iran) tomato witches'- broom phytoplasma. Recognition sites for the following 17 restriction enzymes were used in the simulated digestions: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I,*Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I.

Sequence analyses

A P_1/P_7 PCR amplicon of BTWB isolate was sequenced and submitted to the GenBank data base under the accession number KC182528. BLAST search showed that the 16SrDNA sequence of BTWB phytoplasma shared the highest homology with phytoplasma sequences belonging to members of the 16SrII group (*Candidatus* Phytoplasma aurantifolia)**.** Phylogenetic analysis of 16SrDNA sequences of 28 phytoplasmas including BTWB isolate and *Acholeplasma laidlawii* clustered BTWB, BAWB and BEWB isolates with *Ca*. P. australasia (Fig. 5), the reference of subgroup 16SrII-D. FAWB and YAWB strains were closer to Faba bean phyllody phytoplasma, representative of 16SrII-C subgroup.

The percentage homology between 16SrDNA sequences was determined. Among members of six 16SrII subgroups (A-F), BTWB had maximum homology of 100% with *Ca*. P. australasia (16SrII-D subgroup) and minimum homology of 98.5% with Faba bean phyllody strain (acc. no X83432) belonging to 16SrII-C subgroup (Table 1). Percentage homology of BTWB phytoplasma with other selected Iranian 16SrII related phytoplasmas including BEWB, BAWB, YAWB and FAWB phytoplasmas (acc. nos. JX483698, JN860711, DQ233656 and DQ233655, respectively) were 99.9, 99.7, 98.6 and 98.5%, respectively. Homology of BTWB phytoplasma with Saudi Arabian tomato witches'-broom phytoplasma (acc. No. HM584815) (Alhudaib and Razq 2011) was 99.6%.

[DOR: 20.1001.1.22519041.2014.3.3.2.1]

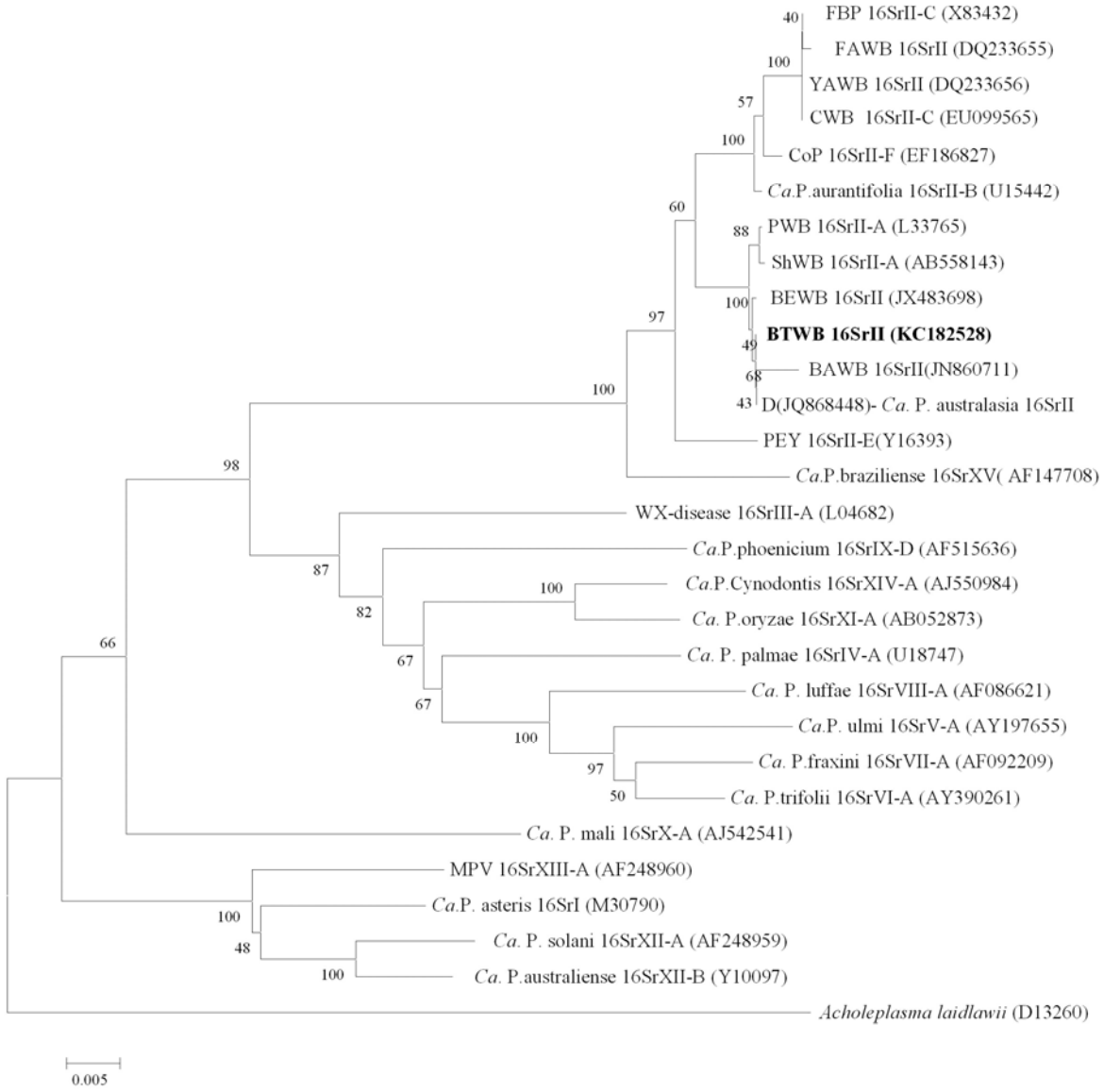

Figure 5 Phylogenetic tree constructed by the neighbor-joining method of 16S rRNA gene sequences from 22 phytoplasmas and *Acholeplasma laidlawii* as outgroup. The position of Borazjan tomato witches'- broom phytoplasmas is bolded. Numbers at the nodes are bootstrap values based on 100 repetitions. Abbreviations: BAWB, Borazjan alfalfa witches'- broom; BEWB, Borazjan eggplant witches'- broom; BTWB, Borazjan tomato witches'- broom; CWB,Cactus witches'- broom; Ca.P., *Candidatus* Phytoplasma; CoT, Cotton phyllody; FAWB, Fars alfalfa witches'- broom; FBP, faba bean phyllody; MPV, Mexican periwinkle virescence; PEY, *Picris echoides* phyllody; PWB, Peanut witches'- broom; ShWB, Sun hemp witches'- broom; WX, Western X; YAWB, Yazd alfalfa witches'- broom. GenBank accession numbers for sequences are given in parentheses.

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Table 1 Pairwise homology (%) between Borazjan tomato witches'- broom and other 16SrII related phytoplasmas as determined by analysis of 16SrDNA sequences.

	BTWB	***Ca*. P. aurantifolia**	***Ca*. P. Australasia**	**CoP**	**FBP**	**PEY**	**ShWB**
BTWB		98.9	100.0	98.7	98.6	98.5	99.8
***Ca*. P. aurantifolia**			98.9	99.7	99.5	98.3	98.7
***Ca*. P. australasia**				98.7	98.6	98.5	99.8
CoP					99.5	98.2	98.6
FBP						98.0	98.4
PEY							98.3
ShWB							

WB, witches'-broom, BTWB, Borazjan tomato WB; *Ca*. P. aurantifolia,*Candidatus* Phytoplasma aurantifolia (U15442); *Ca*.P. australasia, *Candidatus* Phytoplasma australasia (JQ868448); COP, cotton phyllody (EF186827); FBP, faba bean phyllody (X83432); PEY, *Picris echoides* phyllody (Y16393); ShWB, sun hemp WB (AB558143).

Discussion

The type of symptoms observed in Borazjan tomato fields was suggestive of phytoplasmal infection. Transmission of the disease agent by dodder and graft and positive reaction in PCR using phytoplasma universal primers confirmed that BTWB disease has phytoplasmal etiology. Symptoms of BTWB differed from those of tomato big bud reported from other regions of Iran. This is the first report of witches'- broom disease of tomato in Iran. The identity of the associated phytoplasma as a member of the 16SrII group, subgroup D in BTWB affected tomato plants was established through Blast, RFLP, phylogentic analysis and percent homology. Phytoplasmas belonging to eight rRNA groups (I, II, VI, IX, X, XI, XII, XIV) have been identified in different plants in Iran. Phytoplasmas of groups 16SrII are most prevalent and of great economical importance in this country (Salehi *et al.,* 2008). In the present study in addition to BTWB, subgroup affiliation of BEWB, BAWB, FAWB and YAWB phytoplasmas were also determined. BEWB and BAWB phytoplasmas were classified in 16SrII-D subgroup and AWB and YAWB phytoplasmas in 16SrII-C subgroup. BTWB, BEWB and BAWB are from the same geographical region (Bushehr province) and possibly caused by the same phytoplasma. In the present study transmission trial showed that BTWB phytoplasma is transmissible to eggplant. To our knowledge, this is the first report of a 16SrII-D phytoplasma strain infecting tomato, eggplant and alfalfa in Iran. Results of this study showed genetic diversity of phytoplasma agent of alfalfa witches'-broom disease in Iran, as BAWB belongs to 16SrII-D subgroup while FAWB and YAWB belong to 16SrII-C subgroup. Omani alfalfa witches'- broom phytoplasma also belongs to 16SrII-D subgroup (Khan *et al.,* 2002). Association of 16SrII-D related phytoplasmas with tomato phytoplasmal disease have also been reported from Australia (Pearce and Scott, 2011), Egypt (Omar and Foissac, 2012) and India (Singh *et al.,* 2012). Chickpea (*Cicer arietinum*) (Akhtar *et al*., 2009; Alfaro-Fernandez *et al.,* 2012), faba bean (*Vicia* faba) (Alfaro-Fernandez *et al.,* 2012), Sesame (Akhtar *et al.,* 2009), papaya (*Carica papaya*)

[DOR: 20.1001.1.22519041.2014.3.3.2.1]

(White *et al.,* 1998), sweet potato (*Ipomoea batatas*) and pale purple coneflower, (Pearce and Scott, 2011) are also reported as hosts of 16SrII-D subgroup phytoplasmas. Faba bean and sesame phyllody as two important phytoplasma diseases were previously reported from Iran (Salehi and Izadpanah, 1998; Salehi *et al.,* 2008). Further investigations are required to determine whether 16SrII-D phytoplasma strains are associated with faba bean and sesame phyllody diseases. The means of natural spread of BTWB phytoplasma remains to be determined.

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همراهی یک فیتوپلاسما از زیرگروه 16SrII-D با بیماری جاروک گوجه‌فرنگی در استان بوشهر

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چکیده: در بازدیدهای سال‌های۱۳۹۰ تا ۱۳۹۲ از مزارع گوجه‌فرنگی برازجان (استان بوشهر) بیماری جاروک مشاهده گردید. عامل جاروک گوجه‌فرنگی با استفاده از پیوند به گوجه و بادنجان و به‌وسیله سس (*Cuscuta campestris* Yank.) به پروانش انتقال داده شد و در گیاهان مایه‌زنی شده علائم بارز بیماری‌های فیتوپلاسمایی شامل ریزبرگی، کاهش فاصله میانگره‌ها، فیلودی، گلسبزی و جاروک ظاهر شد. واکنش دی‌ان‌ای کل استخراج شده از گوجه‌فرنگی دارای علائم جاروک از مزرعه و گیاهان مایه‌زنی شده با پیوند و سس، در آزمون پی‌سی‌آر مستقیم با استفاده از جفت آغازگر P1/P7 و پی‌سی‌آر دو مرحله‌ای با استفاده از جفت آغازگرهایP1/P7 (دور اول) و R16F2n/R16R2 (دور دوم) مثبت بود و با آنها باند مورد انتظار به‌ترتیب ۱۸۰۰ و ۱۲۰۰ جفت باز ار اپرون ارانای ریبوزومی تکثیر شد. محصول پی‌سی‌آر مستقیم جدایه برازجان همسانه‌سازی و تعیین ترادف شد و تحت رس شمار KC182528 در بانک جهانی ترادف‌ها ثبت گردید. جستجو با برنامه بلاست و آنالیز فیلوژنتیکی با استفاده از ترادف کامل ژن ارانای ریبوزومی ۱۶S نشان داد که فیتوپلاسمای جاروک گوجه‌فرنگی برازجان متعلق به گروه جاروک بادام زمینی (۱۶SrII) می‌باشد. مقایسه چند شکلی طولی قطعات برشی (RFLP) واقعی با استفاده از محصول پی‌سی‌آر دو مرحله‌ای وRFLP مجازی با استفاده از ترادف ناحیه تکثیری جفت آغازگر R16F2n/R16R، آنالیز فیلوزنتیکی و میزان تشابه نوکلئوتیدی نشان داد که فیتوپلاسمای جاروک گوجه‌فرنگی برازجان به‌همراه فیتوپلاسماهای جاروک بادنجان و یونجه در استان بوشهر با *Candidatus* Phytoplasma australasia که فیتوپلاسمایی متعلق به زیرگروه D از گروه 16SrII است طبقه‌بندی می‌شود. همین آنالیزها نشان دادند که این فیتوپلاسماها با سه فیتوپلاسمای مهم و اقتصادی دیگر از گروه جاروک بادام‌زمینی در ایران شامل فیتوپلاسماهای جاروک یونجه در استان‌های فارس و یزد و جاروک لیموترش در مناطق جنوبی متفاوتند. این اولین گزارش از بیماری جاروک گوجه‌فرنگی در ایران و تعیین ویژگی‌های بیولوژیکی و مولکولی فیتوپلاسمای همراه با آن می‌باشد.

واژگان کلیدی: بیماری‌های گوجه‌فرنگی، فیتوپلاسما، انتقال با پیوند، سس، زیرگروه 16SrII-D

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