

Research Article

Molecular characterization of a 16SrIX phytoplasma associated with *Convolvulus glomeratus* witches' broom and with an insect vector in Iran

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Abstract: During a survey in 2016, *Convolvulus glomeratus* showing symptoms resembling those caused by phytoplasmas were observed in Bandar Abbas, Hormozgan province, Iran. These plants were examined for phytoplasma presence by nested-PCR assays using universal primer pair P1/P7 followed by R16F2n/R16R2. All the infected plants indicated positive results using universal primer pairs of P1/P7 followed by R16F2n/R16R2. Results of phylogenetic and virtual RFLP analyses of the *16S rRNA* gene sequences revealed that the phytoplasma associated with *Convolvulus glomeratus* witches' broom (CgWB) was a strain of '*Candidatus* Phytoplasma phoenicium'. The phytoplasma was successfully transmitted to healthy plants by leafhopper, *Orosius albicinctus* (Distant) which account as a vector of this phytoplasma. This is the first report on the presence of '*Candidatus* Phytoplasma phoenicium' (phytoplasma group 16SrIX-J) in *C. glomeratus* and its insect vector in Iran.

Keywords: 16SrIX, *Convolvulus glomeratus*, *Orosius albicinctus*, Bandar Abbas, witches' broom

Introduction

Phytoplasmas are pleomorphic, cell wall-less phytopathogenic bacteria, falling under the class Mollicutes (Firrao *et al.*, 2005) that can cause significant yield loss in diverse, high value crops worldwide including food, vegetable, and fruit crops; ornamental plants, trees and medicinal and aromatic plants (Du Toit, 2014). Phytoplasmas can also infect many wild plant species (Mori *et al.*, 2015). Phytoplasmas associated with these diseases, can be transmitted via dodder and grafting in laboratory conditions and by phloem-feeding insects like

leafhoppers and planthoppers (Hemiptera) in nature (Weintraub and Beanland, 2006), especially those classified in Opsiini tribe. In addition, the phytoplasmas have a persistent and propagative relationship with insects, similar to other symbiotic prokaryotes found in Hemiptera (Weintraub, 2008).

Convolvulus is a genus of about 200 to 250 species of flowering plants in the bindweed family Convolvulaceae, with a cosmopolitan distribution. They are annual or perennial climbing or creeping herbaceous plants and a few species of woody shrubs. Many of the species are problematic weeds, which can smother other more valuable plants by climbing over them, but some are also cultivated for their attractive flowers (Carin and Robba, 2010).

In July 2016, typical symptoms of phytoplasma diseases, including witches' broom and little leaf

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were observed in *Convolvulus glomeratus* in Bandar Abbas, Hormozgan province, Iran (Fig. 1A). Accordingly, they were suspected of

phytoplasma infection. So, this study was carried out to identify the phytoplasma associated with *C. glomeratus* and the insect vector in Iran.



Figure 1 (A) Symptoms of witches' broom, little leaf and yellowing (B) in comparison with healthy *Convolvulus glomeratus*.

Materials and Methods

Samples of both symptomatic and asymptomatic *Convolvulus glomeratus* were collected from Bandar Abbas growing as weeds in municipal lands and parks in Bandar Abbas (N27°11'40"; E56°19'58"), Hormozgan province, Iran. Total DNA was extracted from leaves of the three symptomless and five symptomatic samples using cetyltrimethylammonium bromide (CTAB) extraction procedure described by Doyle and Doyle (1990). A nested polymerase chain reaction (PCR) was employed for the detection of phytoplasmas using the universal primers P1/P7 (Deng and Hiruki 1991) followed by

R16F2n/R16R2 (Gundersen and Lee, 1996) which amplify a phytoplasma *16S rRNA* fragment (approximately 1.25kbp). The PCR was performed in 20 μ l of reaction mixture containing 10 μ l PCR Master Mix (Amplicon), 1 μ l of each primer (10 μ M), 2 μ l of template DNA and 6 μ l sterile distilled water. The thermocycling program was done as described by Hemmati and Nikooei (2017). The P1/P7 primed PCR product was diluted at 1: 10 ratio in sterile water and 2 μ l was used in nested-PCR as a template. The nested-PCR cycles were the same as for the first round PCR. '*Candidatus Phytoplasma aurantifolia*' and DNA template free were used as positive and negative controls, respectively.

Insects feeding on the symptomatic *C. glomeratus* were collected using a D-Vac aspirator (Echo-ES210; Japan). Insects were separated and specimens preserved in acetone (Fukatsu, 1999) and stored at -20 °C. The nucleic acids from individual leafhoppers were extracted using a cetyltrimethyl-ammonium-bromide (CTAB) method in accordance with the protocol of Reineke *et al.* (1998) with some modifications. The primers and PCR conditions used for phytoplasma detection in insects were the same as above.

To confirm the presence of phytoplasma and for phylogenetic analysis, two fragments (one plant, one insect) were randomly selected from the nested PCR round and sequenced bidirectional using primer P1/P7 and R16F2n/R16R2 by Macrogen Sequencing Service (Republic of Korea). Sequences received in this study were assembled and aligned using software: DNASTar and ClustalX. Phylogenetic analyses were conducted by neighbor joining (NJ) method using MEGA 6.0 software (Tamura *et al.*, 2013) with 10,000 replicates for bootstrap analysis. *Acholeplasma laidlawii* was used as out-group to root the tree. The sequences of 16S rRNA of different phytoplasma groups used in comprehensive phylogenetic analyses were downloaded from GenBank database.

Catharanthus roseus was used for transmission trials. Before transmission trials, all plants were tested for phytoplasma presence by Nested-PCR using the same primer pairs. For transmission trial, insects were collected from infected plants using a D-Vac aspirator and *Orosius albicinctus* (Distant, 1908) were separated. The leafhoppers (5-7 individuals per plants) were released under the insect-proof net where five healthy *C. roseus* (5-6 week old) were maintained. The plants with leafhoppers were maintained in the growth chamber (25 °C, 16: 8 L: D and 70 ± 5 %RH) for monitoring disease symptoms development and sampling for PCR assays. After 8-10 weeks after inoculation, plants were molecularly tested for phytoplasma presence. Symptoms of disease development also were observed weekly. Five

individual plants, that had no contact with insects, kept in the same conditions, of the growth chamber, were used as control.

Results

A correct size PCR product was detected in all five symptomatic plants tested by nested PCR assays. Two of the obtained PCR products was directly sequenced by P1/P7 and R16F2n/R16R2 primers. Similarly, correct amplicons were obtained from positive control samples but not from non-symptomatic plant samples and negative control. BLAST analysis revealed that the phytoplasma associated with *Convolvulus glomeratus* witches' broom (CgWB) was a phytoplasma belonging to group 16SrIX, 'Candidatus Phytoplasma phoenicium' with the highest identity (99%) to the Eggplant big bud phytoplasma (accession no. JX483702), *Lactuca serricola* phytoplasma (DQ889749) and 'Candidatus Phytoplasma phoenicium' (JX857827). The representative nucleotide sequence of the detected phytoplasma was deposited in the GenBank database (accession no. MG569789). A phylogenetic tree constructed was in accordance with the outcome of BLAST analysis and the sequence from present study was clustered in group 16SrIX (Fig. 2). This result was further confirmed by the analysis using the iPhyClassifier online tool (<http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>) (Zhao *et al.*, 2009) where it was determined that the *C. glomeratus* phytoplasma was related to 16SrIX group, subgroup J.

Insects collected on the symptomatic plants were morphologically identified (according to the key of Pakarpour Rayeni and Seraj (2016)). To confirm species identification, specimens were sent out to Dr. Pakarpour Rayeni and final confirmation was done. PCR assay was conducted to test if phytoplasma was present in the body. *Orosius albicinctus* was the only leafhopper species which was collected from the infected plants in the field. Four out of ten individuals (40%) tested positive in both, direct and nested PCR tests. The sequence

obtained from insect was aligned in Clustal omega with that obtained from symptomatic plants and showed 100% similarity. The

sequence obtained from *O. albicinctus* was also deposited in GenBank database (Acc. No. MG570468).

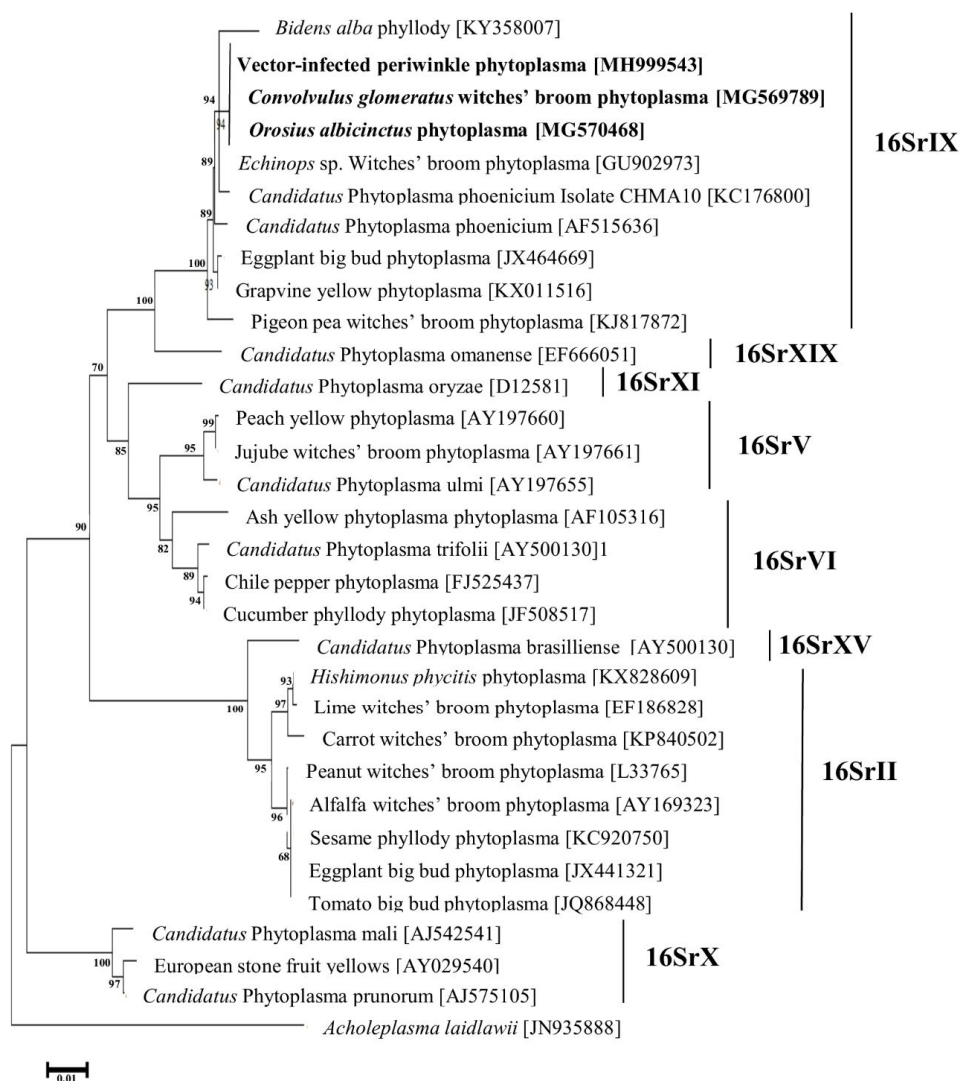


Figure 2 Phylogenetic tree of partial *16S rRNA* gene sequences from *Convolvulus glomeratus* witches' broom phytoplasma isolates (marked by boldface type) and phytoplasma reference sequences belonging to different *16S rRNA* groups. GenBank accession numbers shown in brackets and 16Sr groups are annotated to the right. *Acholeplasma laidlawii* was used as out-group to root the tree. The tree was constructed by the neighbour-joining method using MEGA 6 software. The bar indicates the number of nucleotides substitution per site. Bootstrab values are shown at nodes with 50% support.

In transmission trial, after 2-4 months of inoculation, four out of five *C. roseus* plants fed on by the *O. albicinctus* showed phytoplasma disease symptoms such as yellowing, little leaf, stunting, phyllody and witches' broom (data not shown). Infection of symptomatic insect-inoculated plants were verified by Nested-PCR and sequences were deposited in GneBank under acc. No. MH999453. The plants that were not exposed to *O. albicinctus* were grown normally. Based on these results, *O. albicinctus* is a vector of the phytoplasma 16SrIX-J subgroup, causing disease of *Convolvulus glomeratus* in Bandar Abbas. As reported by other researchers (Esmailzadeh-Hosseini *et al.*, 2007; Omidi *et al.*, 2010; Ikten *et al.*, 2014; Salehi *et al.*, 2015), *O. albicinctus* can vector many diverse phytoplasmas to diverse plants.

Discussion

In Iran, group 16SrIX phytoplasmas have often been identified in some hosts including, grapevine (Salehi *et al.* 2016), eggplant (Tohidi *et al.*, 2015), *Chrysanthemum* (Bayat *et al.*, 2013), sainfoin (Esmailzadeh-Hosseini *et al.*, 2007). *C. glomeratus* is a widespread weed of crop fields in Iran, occurring in association with economically important species, including some of those listed previously, as well as citrus which are widely planted in southern parts of Iran. In nature, in the absence of preferential host(s), leafhoppers may feed on alternative host and transmit pathogens to new plants expanding the host range of a phytoplasma to cultivated crops. This phenomenon can be a potential threat to agriculture. For instance, *O. albicinctus* has been reported as the experimental vector of cucumber phyllody (Azadvar *et al.*, 2005), a disease which is suspected to have originated from sesame. It is believed that during the autumn, when sesame, the preferential host of the insect, has already been harvested, *O. albicinctus* may have been forced to feed on alternative plants, such as

greenhouse cucumbers and has transmitted the disease.

Our findings implicate that *C. glomeratus* can be a reservoir for a relevant group of phytoplasmas that occur in diverse Iranian crops. In addition, we could indicate that *O. albicinctus* is a insect vector of the 16SrIX-J phytoplasma. In summary, the availability of the diverse phytoplasma 16SrIX host plants in Hormozgan province and wide distribution of *O. albicinctus* in this region, together with warm and humid climate, suitable for the insect to be active in all seasons, this pathogen can be a new threat to all plants planted in the region. In addition, to the best of our knowledge, our results reveal for the first time that *C. glomeratus* is a host of phytoplasmas in Iran or worldwide.

Statement of conflicting interest

The authors state that there is no conflict of interest.

Author contribution

All authors contribute equally in this research.

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خصوصیات مولکولی فیتوپلاسمای 16SrIX مرتبط با بیماری جاروک پیچک *Convolvulus glomeratus* و ناقل آن در ایران

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چکیده: طی بازدیدهای به عمل آمده در سال ۱۳۹۵ از فضای سبز شهری بندرعباس، علایمی نظیر بیماری‌های فیتوپلاسمایی شامل جارویی شدن، توقف رشد و کوچک شدن برگ‌ها در برخی از گیاهان پیچک *Convolvulus glomeratus* مشاهده شد. به همین منظور، پژوهش حاضر جهت مشخص شدن فیتوپلاسمای همراه بیماری و شناسایی ناقل آن انجام شد. از نمونه‌ها DNA کل استخراج گردید و جهت بررسی آلودگی فیتوپلاسمایی آن‌ها، از روش PCR و جفت آغازگرهای عمومی P1/P7 (دور اول) و آغازگرهای آشیانه‌ای R16F2n/R16R2 (دور دوم) استفاده شد. نتایج تجزیه و تحلیل فیلوژنتیک و RFLP مجازی از توالی ژن 16S rRNA نشان داد که فیتوپلاسمای مرتبط با بیماری جاروک *Convolvulus glomeratus* (CgWB) متعلق به یک گونه از 'Candidatus Phytoplasma phoenicium' می‌باشد. همچنین نتایج نشان داد که زنجریک *Orosius albicinctus* (Distant) می‌تواند با موفقیت فیتوپلاسمای را به گیاهان سالم منتقل نماید. در نتیجه این زنجریک به عنوان ناقل بیماری شناخته شد. براساس اطلاعات حاضر، این اولین گزارش از وجود استرینی از فیتوپلاسمای 'Candidatus *Phytoplasma phoenicium*' از زیرگروه 16SrIX-J روی پیچک و ناقل آن از ایران می‌باشد.

واژگان کلیدی: فیتوپلاسمای گروه 16SrIX، پیچک *Convolvulus glomeratus*، زنجریک *Orosius albicinctus*، بندرعباس، بیماری جاروک