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Manuscript Draft

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Title: Peach witches'-broom, an emerging disease associated with 'Candidatus Phytoplasma phoenicium' and 'Candidatus Phytoplasma aurantifolia' in Iran

Article Type: Full Length Article

Keywords: 16SrIX; 16SrII; almond witches'-broom; SNP genetic lineages

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Abstract: During field surveys carried out from 2013 to 2017 in the eight main peach producing provinces of Iran, symptoms of a phytoplasma-like peach witches'-broom disease (PWIB), inducing severe yellowing, little leaf, internode shortening, crown and stem witches'-broom, decline, and death, were observed. The aim of this work was to identify and characterize the agent(s) associated with PWIB by biological assays and molecular analyses. PWIB agents were successfully transmitted under controlled conditions from scions of in field-affected peach trees, exhibiting severe or mild symptoms, to peach and bitter almond seedlings inducing phytoplasma-like symptoms. A 16S rDNA fragment of 1,250 bp was amplified by nested-PCR from all PWIB-affected trees and grafted seedlings. Nucleotide sequence identity, presence of species-specific signature sequences, in silico RFLP, single nucleotide polymorphisms, and phylogenetic analyses of 16S rDNA allowed the assignation of the phytoplasma strains identified in seven Iranian provinces in peach trees with severe PWIB symptoms to four SNP genetic lineages of 'Ca. P. phoenicium' (subgroup 16SrIX-B and its variant). PWIB phytoplasma strains identified in Abarkooh (Yazd province) in peach trees with mild symptoms were attributed to the species 'Ca. P. aurantifolia' (subgroup 16SrII-C). This report of a wide spread of 'Ca. P. phoenicium' in association with PWIB in Iran supported its capability of adaptation to a broad range of fruit tree species, such as peach, nectarine, and apricot. As 'Ca. P. phoenicium' and 'Ca. P. aurantifolia' are the etiological agents of other important plant diseases in Iran and neighbouring countries, further investigations are needed to determine the role played by peach in their epidemiological pathways.

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DiSAA

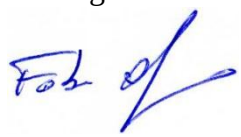
Milan, June 18, 2019
Object: Revised manuscript submission

Dear Editor,

we are re-submitting the manuscript no. CROPRO-D-19-00105 by Salehi and colleagues, revised in accordance with the useful comments and suggestions by the referees. In the attached file "Response to reviewers", you can find our point-by-point reply to the comments made by the referees.

We hope that the new revised version of our manuscript could be considered for publication in Crop Protection.

Thank you for your kind consideration of our work
Best regards



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Manuscript no. CROPRO-D-19-00105
Response to reviewers

Reviewer #1

Comment. Line 103: I think Fig 1 should be moved to the line 102.
Answer. We modified the text as suggested.

Comment. Line 103, I guess Fig 1 should be replaced by Fig 2.
Answer. We modified the text as suggested.

Comment. Lines 103-105: you collected 8 samples from 4 orchards for each province (8 provinces in total). You should have 256 samples per year, right? please specify.
Answer. For clarity, we rephrased the sentence as follows: "In each of the eight provinces, four affected peach orchards were surveyed. In each orchard, a small symptomatic branch with at least ten leaves was collected from two affected trees randomly selected (eight trees per province), for a total of 64 peach trees". (lines 105-108)

Comment. Line 178: Fig 2 or Fig 3? please check.
Answer. We checked and confirmed that Fig.2 is right.

Comment. Line 211: I suggest to remove "highly".
Answer. We modified the text as suggested.

Comment. In the Material and Methods are not specified the procedure you adopted for the DNA extraction, please specify.
Answer. As indicated in the text, the procedure used for total nucleic acids extraction was as published by Zhang and colleagues in 1998 without modifications. Generally, if a protocol previously published is applied without modifications, it is suggested to cite the reference. Thus, we prefer to maintain the sentence in its original version.

Comment. Fig. 6 seems not to be reported in the text, please check.
Answer. We checked and Fig. 6 is reported as (Figs. 5 and 6). Anyway, in agreement with the suggestions by reviewer #3 we decided to eliminate the Fig. 5 which duplicates the data already presented in Table 1. Thus, the original Fig. 6 is now renamed as Fig. 5.

Comment. In Results you specified the 16S rDNA nucleotide sequences you obtained for each province and Tables 1 and 2 clearly indicate genetic differences in phytoplasma strains you identified. The same

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clarity was not adopted for describe possible differences you found over the years (2013-2017). If is it possible, please specify.
Answer. In each province, we collected samples from eight trees throughout the years 2013-17. Phytoplasma strains, detected within each province, share identical 16S rDNA nucleotide sequences. Thus, genetic variability of phytoplasma populations during the examined years did not change, within each province and among provinces.

Comment. In the text you talk about mild and severe symptoms. How did you evaluate symptoms in fields and greenhouse.
Answer. The symptom evaluation was conducted by visual observation both in the field (naturally infected peach trees) and in the greenhouse (grafted plants). Severe symptoms include evident leaf yellowing, little leaf, internode shortening and stem witches'-broom, observed on the majority of the canopy, along with crown witches'-broom, decline and death. Mild symptoms include partial leaf yellowing, little leaf and internode shortening, observed in well delimited parts of the canopy, along with limited stunting. Accurate details concerning symptom description were added throughout the text (lines 179-185).

Reviewer #2

Comment. A better description of the survey would have been helpful for reader to have an indication of the extent of this disease issue. Eg. 32 orchard were surveys, what is the damage from this disease that is observed - range of incidence and severity. Are these orchards similar in size (area and # of plants) and each practice very similar cultural and management practices?
Answer. As suggested, we added details about the surveys (orchard size, number of trees, management) in the section Materials and Methods [The sentence reads: "Each orchard was nearly one hectare in size, presented a similar number of peach trees (almost 600), and was managed by conventional cultural practices" (lines 103-105)]. Moreover, the average disease incidence per each area was inserted in the section Results [The sentence reads: "In 2017, the average PWIB incidence (average % of symptomatic peach trees in four orchards per area) in Abarkooh, Bardseer, Borugerd, Chenaran, Naeen, Sanandaj, Urmia, and Zarghan areas was 14.5, 6.5, 30.7, 8.5, 11.2, 24.4, 11.3, and 45.4%, respectively" (lines 186-189)].

Comment. Symptoms of this disease was noted and then described as MILD and SEVERE. Although images were provided, but it would be helpful for authors to indicate their explicit definition of MILD and SEVERE.
Answer. As reported in the reply to reviewer #1: Severe symptoms include evident yellowing, little leaf, internode shortening and stem witches'-broom, observed on the majority of the canopy, along with crown witches'-broom, decline and death. Mild symptoms include partial yellowing, little leaf and internode shortening, observed in well delimited parts of the canopy, along with limited stunting. For clarity, we reported this description in the section Results (lines 179-185).
.
Comment. Grafting transmission (biological assay) was only performed using symptomatic peach shoots from 2 areas: Zarghan and Abarkooh. What about the specimens from the other regions? There was also no indication of replication in the experimental design. Details in the execution of the experiment would be most helpful. Eg. How were the 8 seedlings of bitter almond and the 8 seedlings of peach used in this experiment? Also, description of the length of time for this grafting study would have been helpful to indicate the observation period OR should one assume that study was terminated when symptoms were

observed (8 and 11 months for Zarghan and Abarkooh symptomatic scions respectively). Did these plants appear to be similar to the healthy control until symptom development? This was not clearly observable or evident in the photos (fig. 3).
Answer. As suggested, we modified the text by adding more details (replication, length of the observation after grafting, etc…) on grafting-mediated transmission trials in the section Materials and Methods (lines 112-121). We would like to clarify that, in such trials, we utilized peach shoots (scions) from Zarghan as representative of trees exhibiting severe symptoms, and peach shoots from Abarkooh as representative of trees showing mild symptoms.

Comment. There was mention of the use of periwinkle leaves infected with WDBL strain as positive control in molecular analysis. Abarkooh disease agent was identified as a WDBL strain. It would be helpful for authors to show that the Abarkooh detection was unique and not due to potential cross contamination.
Answer. The phytoplasma strain associated with WBDL (positive control) belongs to taxonomic subgroup 16SrII-B. In Abarkooh, diseased peach trees were found infected by a phytoplasma strain belonging to subgroup 16SrII-C (a strain quite common in Abarkooh). Moreover, grafting-mediated transmission trials using symptomatic peach scions from Abarkooh demonstrated that a 16SrII-C phytoplasma strain was transmitted to peach and almond seedlings. Based on such evidences, cross contamination should be excluded.

Comment. Lines 201-214: Items described in this paragraph needs to be clarified. First sentence indicates that the 16S sequence was identical from all 8 province but later distinct strains were described. This is confusing and need to be made clear to avoid perception of contradicting statements.
Answer. The meaning of the original sentence would be that our results showed that 16S rDNA nucleotide sequences of phytoplasma strains identified within the same province are identical (not that sequences of strains detected in all the province are identical). We re-phrased the sentence for clarity (line 213).

Reviewer #3

Comment. The title is adequate but could be more informative (e.g. naming the species of phytoplasma detected in peach in Iran)
Answer. We modified the title as suggested. The new title reads as follows: "Peach witches'-broom, an emerging disease associated with '*Candidatus* Phytoplasma phoenicium' and '*Candidatus* Phytoplasma aurantifolia' in Iran".

Comment. The highlight no. 1 (line 51) is unnecessary, because it just repeats the title. In this study, 2 new distinct SNP genetic lineages (a1 and f4) of phytoplasma Ca. P. phoenicium were discovered by SNP analysis of 16S rDNA. This could be accented among the highlights.
Answer. We modified the highlights as suggested (lines 51-53).

Comment. Line 105-106 (sentence: The collected symptomatic shoots were utilized for disease transmission trials by grafting and molecular analysis) leaves unspecified principles applied for sample selection (from how many plants per province and with what exactly symptoms).
Answer. As indicated in our previous answer to Reviewer #2, we modified the text by adding more details (replication, length of the observation after grafting, etc…) on grafting-mediated transmission trials in the section Materials and Methods (lines 112-121). We would like to clarify that, in such trials,

we utilized peach shoots (scions) from Zarghan as representative of trees exhibiting severe symptoms, and peach shoots from Abarkooh as representative of trees showing mild symptoms.

Comment. Line 121, 142: too many "and" makes a sentence not clear enough.
Answer. We modified the text as suggested.

Comment. The word "further" in line 127 is not necessary before "negative control".
Answer. We modified the text as suggested.

Comment. Several analyzes were dealing with sequences, and it remains unclear whether all sequences are of the same length and reliability. Thus, it is worth to mention the length of a reliable sequence obtained from this study and from NCBI for a comparable analysis.
Answer. We inserted the size of sequences obtained in the present study (1250 nt) (line 212) and other details in Materials and Methods (lines 155-156, line 163).

Comment. Fig. 5 duplicates the information provided in table 1 in some aspects so could be omitted.
Answer. We agree with this point; we can eliminate Fig. 5.

Comment. Line 178: It is not clear what the authors mean by "in certain areas, such as Zarghan", and why only in some areas (e.g. Zarghan) PWIB disease destroyed orchards, in spite that all 'Ca. P. phoenicium' infected gardens exhibited severe symptoms (lines 174-175).
Answer. In all the seven areas where PWIB was associated with infection by '*Ca*. P. phoenicium', diseased trees exhibited severe symptoms, but the disease incidence (% of symptomatic trees) was different in such areas. In details, in Borujerd, Sanandaj, and Zarghan areas, the disease incidence was higher. Variations in disease incidence can be related to disease progression steps, weather conditions, vector population, sources of disease agents other than peach and import of asymptomatic infected peach seedlings. We added details in the sections Results (lines 189-190) and Discussion (lines 283-287).

Comment. Line 181-192 "Disease transmission by grafting": no information is provided on the sequences obtained from grafting trials which were cloned (according to lines 142 to 145), so what was the goal of cloning remains unclear.
Answer. We inserted the results of 16S rDNA nucleotide sequences amplified from grafted peach and almond seedlings (lines 226-229).

Comment. The triangles in Figure 1 do not provide information on the peculiarities of sampling. Since only one phytoplasma sequence was detected within each province, maybe it could be more informative to mark by dots the places of orchards in order to make more clear the distribution of sampled places in the province.
Answer. In each province only one location with four orchards was chose for sampling. Thus, surveyed orchards were not so scattered to separate them by dots.

Comment. Since two highlights and the specificity of the journal deals with the focusing on epidemiology, the more in-depth discussion could be provided by focusing on the peculiarities of the sites where genetically distinct phytoplasmas were detected e.g.:

Was Abarkooh area the only place in Yazd province with PWIB infected peach orchards or just one place used as a representative of the province?

Answer. Abarkooh area is the only one in Yazd province with peach orchards infected by '*Ca*. P. aurantifolia' (16SrII-C); moreover, apricot chlorotic leaf roll disease, associated with the same phytoplasma, has bean previously reported in Abarkooh (Rasoulpour et al., 2019) (lines 279-280; 420-422).

Could the very different phytoplasma (by symptoms expression and genome) detected in Abarkooh area be associated with any other differences (such as peach age, variety, origin or environmental conditions, alfalfa, carrots or cucurbits more widely cultivated in this province) or not?
Answer. In Abarkooh area 16SrIX group related phytoplasma has not been reported yet. Unlike other areas, in Abarkooh wild almond (*Prunus scoparia*), the natural source of 16SrIX-B strains, is not found. On the other hand, in Abarkooh, alfalfa and apricot, sources of 16SrII-C related strains, are wildly cultivated.

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Highlights

- '*Ca.* Phytoplasma phoenicium' and '*Ca*. P. aurantifolia' are associated respectively with severe and mild symptoms of peach witches'-broom disease (PWIB)
- New distinct SNP genetic lineages (a1 and f4) of '*Ca*. P. phoenicium' were discovered
- PWIB epidemiology can be extremely complex
- PWIB etiological agents can be related to other important plant diseases

Peach witches'-broom, an emerging disease associated with '*Candidatus* Phytoplasma phoenicium' and '*Candidatus* Phytoplasma aurantifolia' in Iran

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Abstract

During field surveys carried out from 2013 to 2017 in the eight main peach producing provinces of Iran, symptoms of a phytoplasma-like peach witches'-broom disease (PWIB), inducing severe yellowing, little leaf, internode shortening, crown and stem witches'-broom, decline, and death, were observed. The aim of this work was to identify and characterize the agent(s) associated with PWIB by biological assays and molecular analyses. PWIB agents were successfully transmitted under controlled conditions from scions of in field-affected peach trees, exhibiting severe or mild symptoms, to peach and bitter almond seedlings inducing phytoplasma-like symptoms. A 16S rDNA fragment of 1,250 bp was amplified by nested-PCR from all PWIB-affected trees and grafted seedlings. Nucleotide sequence identity, presence of species-specific signature sequences, *in silico* RFLP, single nucleotide polymorphisms, and phylogenetic analyses of 16S rDNA allowed the assignation of the phytoplasma strains identified in seven Iranian provinces in peach trees with severe PWIB symptoms to four SNP genetic lineages of '*Ca*. P. phoenicium' (subgroup 16SrIX-B and its variant). PWIB phytoplasma strains identified in Abarkooh (Yazd province) in peach trees with mild symptoms were attributed to the species '*Ca*. P. aurantifolia' (subgroup 16SrII-C). This report of a wide spread of '*Ca*. P. phoenicium' in association with PWIB in Iran supported its capability of adaptation to a broad range of fruit tree species, such as peach, nectarine, and apricot. As '*Ca*. P. phoenicium' and '*Ca*. P. aurantifolia' are the etiological agents of other important plant diseases in Iran and neighbouring countries, further investigations are needed to determine the role played by peach in their epidemiological pathways.

Key words: 16SrIX; 16SrII; almond witches'-broom; SNP genetic lineages

Highlights

- '*Ca.* Phytoplasma phoenicium' and '*Ca.* P. aurantifolia' are associated respectively with severe and mild symptoms of peach witches'-broom disease (PWIB)
- New distinct SNP genetic lineages (a1 and f4) of '*Ca.* P. phoenicium' were discovered
- PWIB epidemiology can be extremely complex
- PWIB etiological agents can be related to other important plant diseases

1. Introduction

Phytoplasmas are cell wall-less obligate plant pathogenic bacteria of the class *Mollicutes* with a small genome size (Marcone, 2014). In diseased plants, they are restricted to the phloem sieve tubes and are transmitted between plants by phloem-sap-feeding leafhoppers, planthoppers or psyllids in a persistent manner (Weintraub and Beanland, 2006). Based on unique molecular and biological features phytoplasmas have been classified into about 40 species within the provisional genus '*Candidatus* Phytoplasma' (IRPCM, 2004); moreover, taxonomic groupings have been delimited according to the similarity coefficients derived from the comparison of collective restriction profiles of 16S rRNA gene sequence digested with a selected pool of endonucleases (Lee et al., 1998; Wei et al., 2007). Phytoplasmas have been associated with several hundred diseases affecting economically important crops, such as ornamentals, vegetables, fruit trees and grapevine (Bertaccini et al., 2014).

Peach (*Prunus persica*) is highly susceptible to phytoplasma infection. In fact, several peach diseases have been reported worldwide in association with genetically and biologically distinct phytoplasmas belonging to at least eleven 16Sr taxonomic groups (16SrI, II,

III, V, VI, VII, IX, X, XII, XV, and XVII) (Marcone et al., 2014). In North America, western X-disease, peach rosette, and peach yellows, the most important phytoplasma diseases with destructive effects on the peach production, are associated with '*Candidatus* Phytoplasma pruni' (mainly subgroup 16SrIII-B) (Scott and Zimmerman, 2001; Seemüller and Schneider, 2004; Ragozzino, 2011; Marcone et al. 2014); moreover, peach yellow leaf roll (PYLR) disease is associated with a '*Ca*. Phytoplasma pyri' subtype (subgroup 16SrX-C) (Marcone et al., 2014). In Europe, European stone fruit yellows (ESFY) is the main phytoplasma disease of *Prunus* species, including peach, and is associated with '*Ca*. P. prunorum' (subgroup 16SrX-B) (Jarausch et al., 2001; Seemüller and Schneider, 2004). In Lebanon, a destructive disease of peach is caused by '*Ca*. P. phoenicium' (subgroup 16SrX-B), the etiological agent of almond witches'-broom disease (Abou-Jawdah et al., 2009). Grafting-mediated transmission trials, carried out in Iran under controlled conditions, confirmed that '*Ca*. P. phoenicium' from almond-infected scions can be transmitted and induce symptoms in peach trees (Salehi et al., 2006).

Peach is one of the most important fruit crops in Iran, with 500,000 tons produced annually. Previous studies reported the presence of peach diseases inducing leaf rolling, little leaf, rosetting, yellowing, and decline in association with infection by '*Ca*. P. aurantifolia', '*Ca*. P. trifolii', '*Ca*. P. solani', and '*Ca*. P. omanense' in Iran (Zirak et al., 2010; Esmailzadeh Hosseini et al., 2017). Moreover, in the Iranian provinces of Kurdistan, North Khorosan and Razavi Khorosan stunting and leaf yellowing have been observed on peach trees infected by phytoplasmas provisionally identified as belonging to16SrIX group (Ghayeb Zamharir, 2014; Tarighi et al., 2018). During field surveys carried out from 2013 to 2017 in the eight main peach producing provinces of Iran, a phytoplasma-like peach witches'-broom disease (PWIB) was

observed. The aim of this work was to identify and characterize the agent(s) associated with this disease by both biological assays and molecular analyses.

2. Materials and Methods

2.1 Field surveys and plant sampling

During field surveys carried out from 2013 to 2017 in 32 orchards localized in Abarkooh, Bardseer, Borugerd, Chenaran, Naeen, Sanandaj, Urmia, and Zarghan areas, respectively in Yazd, Kerman, Lorestan, Razavi Khorasan, Isfahan, Kurdistan, West Azerbaijan, and Fars provinces of Iran (Fig. 1), peach (*Prunus persica*, Zafarani variety) trees exhibiting phytoplasma-like witches'-broom symptoms were observed (Fig. 2). Each orchard was nearly one hectare in size, presented a similar number of peach trees (almost 600), and was managed by conventional cultural practices. In each of the eight provinces, four affected peach orchards were surveyed. In each orchard, a small symptomatic branch with at least ten leaves was collected from two affected trees randomly selected (eight trees per province), for a total of 64 peach trees. The collected symptomatic shoots were utilized for disease transmission trials by grafting and molecular analyses. Symptomless peach trees were collected as controls.

2.2 Grafting-mediated transmission trials

Two-year old seedlings of bitter almond (*Prunus amygdalus*) and peach (*P. persica*, variety Zafarani) both on GF-677 rootstock were purchased from a local nursery in Eghleed, a stone fruit phytoplasma disease free area in Fars province. All seedlings were verified as phytoplasma-free by nested PCR reactions using the protocol described below. Four seedlings of each species were side grafted (two scions per seedling) with peach symptomatic shoots collected in Zarghan

(representative of PWIB-affected trees showing severe symptoms) and four seedlings with peach symptomatic shoots collected in Abarkooh (representative of PWIB-affected trees showing mild symptoms). Four seedlings grafted with scions prepared from symptomless peach trees and four ungrafted seedlings were used as controls. Grafted and ungrafted seedlings were maintained in an insect-proof greenhouse for 24 months for symptoms observation and molecular analyses.

2.3 Phytoplasma detection

Total nucleic acids were extracted from 0.3 g of fresh midrib tissues of field-collected symptomatic and symptomless peach trees, and bitter almond and peach seedlings used in disease transmission trials using the procedure of Zhang et al. (1998). Total nucleic acids extracted from leaf midribs of eight peach trees grown from seeds and maintained in the greenhouse (healthy trees) and from leaves of a periwinkle plant (*Catharanthus roseus*) infected with the phytoplasma strain causing the witches'-broom disease of lime (WBDL, taxonomic subgroup 16SrII-B) were used as negative and positive controls, respectively. PCR mixture devoid of DNA was also employed as a negative control. Extracted total nucleic acids were used as templates in nested PCR reactions for the amplification of a 1.2 kbp-long 16S rDNA fragment, using the universal phytoplasma primer pairs P1/P7 **(**Deng and Hiruki, 1991; Schneider et al., 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996) in, respectively, direct and nested PCR assays. PCRs were performed in 25 µl reaction volume containing 50 ng of template, 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 in the buffer supplied by the manufacturer (CinnGen, Tehran, Iran). PCRs were conducted in a thermal cycler (Bio-Rad, USA**)** for 35 cycles as follows: 45 sec denaturation at 94°C (3 min for

the first cycle), 45 sec annealing at 55°C (58°C in nested PCR), and 2 min of extension at 72°C. In the final cycle the extension step was extended to 10 min. PCR products were separated in 1% agarose gel in 1 x TBE buffer, stained with Gel Red and visualized by a UV transilluminator. The PCR products size was estimated by comparison with 100 bp DNA ladder (Fermentas, Vilnius, Lithuania).

2.4 Phytoplasma molecular characterization

R16F2n/R16R2 primed PCR products, amplified from witches'-broom affected peach trees, and from bitter almond and peach seedlings grafted with witches'-broom affected peach scions, were ligated into pTZ57R/T vector and cloned into *Escherichia coli* DH5α cells using InsT/A clone™ PCR Product Cloning Kit (Fermentas) according to the manufacturer's instructions. The recombinant plasmids were confirmed by colony PCR. For each PCR product, plasmid DNA from three recombinant colonies was purified using GF-1 PCR Clean-Up Kit (Vivantis, Malaysia), according to the manufacturer's instructions, and the cloned F2n/R2 fragment was sequenced by a commercial DNA sequencing service (BioNeer, South Korea). The 16S rDNA nucleotide sequences were assembled using the "Contig Assembling" application, trimmed to the annealing sites of the primers F2n and R2, aligned using the "ClustalW Multiple Alignment" application, and analyzed for sequence similarity determination using the "Sequence Identity Matrix" application of the software BioEdit version 7.0.5 (Hall, 1999).

For ribosomal group/subgroup attribution, a virtual RFLP analysis of 16S rDNA nucleotide sequences of representative PWIB phytoplasma strains was carried out using the *i*PhyClassifier tool (Wei et al., 2007; Zhao et al., 2009).

For species attribution, 16S rDNA nucleotide sequences of representative PWIB

phytoplasma strains were compared with F2n/R2 trimmed nucleotide sequences (1250 nt) of reference strains of '*Ca*. Phytoplasma' species, retrieved from NCBI GenBank, as described above. The alignment of 16S rDNA nucleotide sequences from this and previous studies (Salehi et al., 2018) was utilized, firstly, to confirm the '*Ca*. Phytoplasma' species attribution by the presence of species-specific unique signature sequences and determining the sequence similarity values, and secondly, to evaluate the diversity among peach witches'-broom phytoplasma strains and closely related strains belonging to distinct single nucleotide polymorphism (SNP) genetic lineages recently published (Salehi et al., 2018). Nucleotide sequences of 16S rRNA gene of peach witches'-broom phytoplasma strains and reference strains of '*Ca*. Phytoplasma' species were used for phylogenetic analyses. The Minimum-Evolution method was employed using the Jukes-Cantor model and bootstrap replicated 1,000 times with the software MEGA7 to obtain a phylogenetic tree (Kumar et al., 2016).

3. Results

3.1 Peach witches'-broom symptoms

In all the Iranian areas surveyed from 2013 to 2017, witches'-broom disease of peach (PWIB) was observed. In Bardseer, Borugerd, Chenaran, Naeen, Sanandaj, Urmia, and Zarghan areas, the characteristic PWIB symptoms were severe and included evident leaf yellowing, little leaf, internode shortening and stem witches'-broom (observed on the majority of the canopy) along with crown witches'-broom, decline and death (Fig. 2). In Abarkooh area (Yazd province), diseased peach trees showed mild symptoms including partial leaf yellowing, little leaf and internode shortening (observed in well delimited parts of the canopy), along with limited stunting; clear witches'-broom was not observed (not shown). In all areas affected parts of

185 diseased trees do not set fruits. In 2017, the average PWIB incidence (average % of symptomatic
186 peach trees in four orchards per area) in Abarkooh, Bardseer, Borugerd, Chenaran, Naeen,
187 Sanandaj, Urmia, and Zarghan areas was 14.5, 6.5, 30.7, 8.5, 11.2, 24.4, 11.3, and 45.4%,
188 respectively. In certain areas, like Borugerd, Sanandaj and Zarghan, the high disease incidence
189 combined with the symptom severity completely destroyed the peach orchards (Fig. 2).
190
191 *3.2 Disease transmission by grafting*
192 PWIB-affected peach scions from Zarghan (severe symptoms observed in field) and Abarkooh
193 (mild symptoms observed in field) remained alive after grafting and produced phytoplasma-like
194 symptoms in all the peach and bitter almond seedlings. The agent of PWIB from Zarghan caused
195 severe symptoms of yellowing, little leaf, internode shortening and witches'-broom in both
196 peach and bitter almond (Fig. 3). The agent of PWIB from Abarkooh caused mild symptoms of
197 yellowing, reddening (only in bitter almond), little leaf and internode shortening (Fig. 3). The
198 time elapsed between the seedling inoculation to the first appearance of disease symptoms was
199 eight months for Zarghan PWIB agent and 11 months for Abarkooh PWIB agent. All graft
200 inoculated plants showed positive results in nested PCR assays. Control plants (not grafted with
201 PWIB-affected scions) were negative for phytoplasma-like symptoms, and analyses by nested
202 PCR gave negative results.
203
204 *3.2 Phytoplasma identification and characterization*
205 A fragment of approximately 1.25 kbp (F2n/R2 fragment) was amplified in nested PCR from all
206 field-collected symptomatic peach trees, all symptomatic graft inoculated peach and bitter
207 almond seedlings, and periwinkle plant infected with WBDL phytoplasma (positive control). No

PCR products were obtained from symptomless peach trees, control (not grafted or grafted with symptomless peach scions) peach and bitter almond seedlings, and PCR mixture devoid of DNA (negative control).

The obtained 16S rDNA nucleotide sequences (R16F2n/R2 fragment, 1250 nt), amplified from field PWIB-affected peach trees within the same province, were identical to each other (sequence identity 100%). One sequence per province was therefore submitted to NCBI GenBank database, under the accession numbers MH363612 (Abarkooh, Yazd province), MH363613 (Bardseer, Kerman province), MH363614 (Urmia, West Azerbaijan province), MH363615 (Borujerd, Lorestan province), MH363616 (Chenaran, Razavi Khorasan province), MH363617 (Naeen, Isfahan province), MH363618 (Sanandaj, Kurdistan province), and MH363619 (Zarghan, Fars province). Moreover, PWIB phytoplasma strains from Abarkooh are clearly distinct (average 16S rDNA sequence identity 89.99%) from strains identified in the other seven surveyed areas. On the other hand, such PWIB phytoplasma strains, identified in Bardseer, Urmia, Borujerd, Chenaran, Naeen, Sanandaj, and Zarghan orchards, were closely related (average 16S rDNA sequence identity 99,65%). In detail, strains from Borujerd and Zarghan, and strains from Chenaran, Naeen and Sanandaj, were identical based on 16S rDNA sequence identity (Table 1).

Phytoplasma strains detected in peach and bitter almond seedlings, graft inoculated by PWIB-affected peach scions from Zarghan (severe symptoms) and Abarkooh (mild symptoms), shared identical 16S rDNA nucleotide sequences with strains identified in field PWIB-affected peach trees in Zarghan (Acc. No. MH363619) and Abarkooh (Acc. No. MH363612).

The *i*PhyClassifier analyses (Fig. 4) revealed that: (i) the virtual RFLP patterns derived from the PWIB phytoplasma strains, identified in Borujerd, Chenaran, Naeen, Sanandaj, and

Zarghan, were undistinguishable and identical (similarity coefficient 1.00) to the reference pattern of 16Sr group IX, subgroup B; (ii) the virtual RFLP patterns derived from the PWIB phytoplasma strains, identified in Bardseer and Urmia, were undistinguishable and identical to the pattern of a variant of subgroup 16SrIX-B (previously classified as 16SrIX-D); (iii) the virtual RFLP pattern derived from the PWIB phytoplasma strains identified in Abarkooh was a variant (similarity coefficient of 0.99) of the reference pattern of the 16Sr group II, subgroup C. Bardseer and Urmia PWIB strains shared a similarity coefficient of 0.97 with the reference pattern of subgroup 16SrIX-B, and this difference was due to the restriction pattern produced using the enzyme *Taq*I.

BlastN search showed that 16S rDNA nucleotide sequences of PWIB phytoplasma strains of subgroup 16SrIX-B (Borujerd, Chenaran, Naeen, Sanandaj, and Zarghan) and its variant (Bardseer and Urmia) shared closest homology (> 99.6%) with the '*Ca*. Phytoplasma phoenicium' reference strain A4 (AF515636) (Verdin *et al.*, 2003) (Table 1). Such strains also harboured the species-specific signature sequences (5'-CCTTTTTCGGAAGGTATG-3', nt 58…75; 5'-TTGATAAGTCTATAGTTTAAT-3', nt 441…461; 5'-TACCGCTATAGAAACT-3', nt 479…494, from the annealing site of the primer F2n) of '*Ca*. P. phoenicium' (Verdin et al., 2003; Salehi et al., 2018) (data not shown). Abarkooh PWIB phytoplasma strains, belonging to subgroup 16SrII-C, shared closest homology (99%) with the '*Ca*. Phytoplasma aurantifolia' reference strain WBDL (U15442), and harbored its species-specific signature sequence (5'-GCAAGTGGTGAACCATTTGTT-3', nt 309…329, from the annealing site of the primer F2n) (Zreik et al., 1995) (data not shown).

Phylogenetic analyses positioned with high confidence values the PWIB phytoplasma strains of the subgroup 16SrIX-B and its variant in a cluster including 16SrIX phytoplasmas in

which the PWIB phytoplasma strains belong to a distinct subcluster including the '*Ca*. P. phoenicium' strain A4 (data not shown). Based on all these evidences, the PWIB phytoplasmas, identified in this study, belong to '*Ca*. P. phoenicium', for which strain members (subgroup 16SrIX-B) are clearly distinct from phytoplasma strains of other 16SrIX subgroups. Moreover, phylogenetic clustering with high confidence value reinforced the affiliation of Abrakooh PWIB phytoplasma strains (subgroup 16SrII-C) to the species '*Ca*. P. aurantifolia' (Fig. 5).

Alignment of 16S rDNA nucleotide sequences of '*Ca*. P. phoenicium' (16SrIX-B) strains associated with PWIB with representative strains of SNP genetic lineages recently reported within subgroup 16SrIX-B (Salehi et al., 2018), evidenced that PWIB phytoplasma strains carried distinguishing SNPs found exclusively within Iranian '*Ca*. P. phoenicium' strains, and belong to four SNP genetic lineages. PWIB phytoplasma strains from Urmia, Borujerd and Zarghan belong to previously described lineages f1 and g; on the other hand, strains from Bardseer, Chenaran, Naeen, and Sanandaj belong to SNP lineages a1 and f4, reported for the first time in the present study (Table 2).

4. Discussion

Overall results of field surveys, transmission trials and molecular analyses proved that severe symptoms observed in peach trees cultivated in orchards localized in seven Iranian provinces are associated with '*Ca*. P. phoenicium' (subgroup 16SrIX-B and its variant), the etiological agent of almond witches'-broom (on almond, peach, and nectarine) and apricot yellows diseases in Lebanon and Iran (Abou-Jawdah et al., 2002, 2009; Salehi et al., 2018). On the other hand, mild symptoms observed in peach trees cultivated in orchards localized in the Yazd province (Abarkooh area) are associated with '*Ca*. P. aurantifolia' (subgroup 16SrII-C), the etiological

agent of alfalfa and carrot witches'-broom (Esmailzadeh Hosseini et al., 2015; Salehi et al., 2016a), cucurbits phyllody (Salehi et al., 2015b) and apricot chlorotic leaf roll (Rasoulpour et al., 2019) in Abarkooh area, and several other plant diseases in other parts of Iran (Salehi et al., 2016b). This evidence reinforces the recent reports of the association of severe and mild peach witches'-broom (PWIB)-like diseases respectively with phytoplasmas of taxonomic groups 16SrIX (Ghayeb Zamharir, 2014) and II (Zirak et al., 2010) in Iran. In Borugerd, Sanandaj and Zarghan areas, the disease incidence was higher than in the other surveyed areas where PWIB was associated with '*Ca*. P. phoenicium'. Variation in disease incidence can be related to weather conditions, vector population, sources of etiological agent(s) other than peach and import of asymptomatic infected peach seedlings.

Concerning '*Ca*. P. phoenicium', its previous association with PWIB-like disease was only provisional, due to the short size of the 16S rDNA nucleotide sequence analyzed, and limited to the Iranian provinces of Kurdistan, North Khorasan and Razavi Khorasan (Ghayeb Zamharir, 2014; Tarighi et al., 2018). Here, nucleotide sequence identity, presence of species-specific signature sequences, and phylogenetic analysis of 16S rDNA allowed the certain assignation of PWIB phytoplasma strains, identified in affected peach trees from seven Iranian provinces, to the species '*Ca*. P. phoenicium'. Moreover, analysis of single nucleotide polymorphisms (SNPs) within 16S rDNA sequences highlighted that PWIB phytoplasma strains are distinguished by SNPs found exclusively within Iranian '*Ca*. P. phoenicium' strains and belong to four distinct SNP genetic lineages, two (f1 and g) previously described (Salehi et al., 2018) and two (a1 and f4) firstly reported in this study. Interestingly, this result confirms the usefulness of molecular markers within the conserved 16S rDNA to resolve the genetic complexity within phytoplasma populations (Cheng et al., 2015; Quaglino et al., 2017), and

evidences that climatic and geographic features in the ecosystems may be significant, directly or indirectly, in determining the strain composition of phytoplasma populations in different regions (Cai et al., 2008; Salehi et al., 2018). The report of specific '*Ca*. P. phoenicium' genetic lineages associated with PWIB disease in Iran opens a new intriguing scenario on the epidemiology of AlmWB phytoplasma, suggesting its possible adaptation to other fruit trees species, as previously reported for peach and nectarine in Lebanon, and for apricot in Iran (Abou-Jawdah et al., 2009; Salehi et al., 2018). It is reasonable to hypothesize that *Prunus scoparia*, a wild almond species harboring '*Ca*. P. phoenicium' and scattered in Iranian provinces examined in the present study, could play a role in the phytoplasma transmission pathways to fruit trees, including peach. In fact, previous study demonstrated that '*Ca*. P. phoenicium' naturally infecting wild almond can be transmitted to peach through grafting-mediated transmission trials (Salehi et al. 2015a). Furthermore, based on detection of '*Ca*. P. phoenicium' in insect body and saliva and the presence of consistent populations, the leafhopper (Cicadellidae, Typhlocybinae) *Frutioidea bisignata* can be considered as potential vector of this phytoplasma in Iran (Taghizadeh and Salehi, 2002; Siampour et al., 2004). On the other hand, in spite of the presence of high population, the leafhopper *Asymmetrasca decedens* Paoli, vector of '*Ca*. P. phoenicium' in Lebanon (Abou-Jawdah et al., 2014), was not able to transmit '*Ca*. P. phoenicium' in Iran (Taghizadeh and Salehi, 2002).

Concerning '*Ca*. P. aurantifolia', its previous association in Iran with peach diseases inducing mild symptoms, such as leaf rolling, little leaf, rosetting, and yellowing, is in agreement with the results of the present study, expect for the presence of witches'-broom in infected peach trees (Zirak et al., 2010). In the Yazd province, where PWIB was associated with '*Ca*. P. aurantifolia', alfalfa witches'-broom associated with the same phytoplasma is a

widespread and economically important disease (Esmailzadeh Hosseini et al., 2016), transmitted by the leafhopper *Orosious albicinctus* (Salehi et al., 1995). Thus, alfalfa and *O. albicinctus* could be involved in the transmission of '*Ca*. P. aurantifolia' to peach.

Interestingly, the different PWIB symptom severity observed in both field peach trees and grafted peach and bitter almond seedlings infected by '*Ca*. P. phoenicium' and '*Ca*. P. aurantifolia' can be related to differences in the virulence of the pathogens and/or in the susceptibility of infected peach varieties, as previously proposed for other phytoplasma-associated plant diseases (Quaglino et al., 2016; Pierro et al., 2018).

Further investigations are needed to (i) determine the epidemiological pathways of '*Ca*. P. phoenicium' and '*Ca*. P. aurantifolia' associated with PWIB in Iran, (ii) survey PWIB in other Iranian provinces, as the disease seems to be present but symptomatic plants were found not infected by phytoplasmas, (iii) investigate accurately susceptibility of peach varieties against PWIB associated phytoplasmas.

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Conflict of interest

346 No conflict exists: the authors declare that they have no conflict of interest.

347

348 **References**

349 Abou-Jawdah, Y., Abdel Sater, A., Jawhari, M., Sobh, H., Abdul-Nour, H., Bianco, P.A.,
350 Molino Lova, M., Alma, A., 2014. *Asymmetrasca decedens* (Cicadellidae,
351 Typhlocybinae), a natural vector of '*Candidatus* Phytoplasma phoenicium'. Ann. Appl.
352 Biol. 165, 395–403.

353 Abou-Jawdah, Y., Karakashian, A., Sobh, H., Martini, M., Lee, I.-M., 2002. An epidemic of
354 almond witches'-broom in Lebanon: Classification and phylogenetic relationships of
355 the associated phytoplasma. Plant Dis. 86, 477−484.

356 Abou-Jawdah, Y., Sobh, H., Akkary, M., 2009. First report of almond witches' broom
357 phytoplasma ('*Candidatus* Phytoplasma phoenicium') causing a severe disease on
358 nectarine and peach in Lebanon. EPPO Bull. 39, 94−98.

359 Bertaccini, A., Duduk, B., Paltrinieri, A., Contaldo, N., 2014. Phytoplasmas and
360 phytoplasma diseases: A severe threat to agriculture. Am. J. Plant Sci. 5, 1763−1788.

361 Cai, H., Wei, W., Davis, R.E., Chen, H., Zhao, Y., 2008. Genetic diversity among phytoplasmas
362 infecting *Opuntia*: virtual RFLP analysis identifies new subgroups in the peanut witches'-
363 broom phytoplasma group. Int. J. Syst. Evol. Microbiol. 58, 1448–1457.

364 Cheng, M., Dong, J., Lee, I.-M., Bottner-Parker, K.D., Zhao, Y., Davis, R.E., Laski, P.J., Zhang,
365 Z., McBeath, J.B., 2015. Group 16SrXII phytoplasma strains, including subgroup
366 16SrXII-E ('*Candidatus* Phytoplasma fragariae') and a new subgroup, 16SrXII-I, are
367 associated with diseased potatoes (*Solanum tuberosum*) in the Yunnan and Inner Mongolia
368 regions of China. Eur. J. Plant Pathol. 142, 305–318.

Deng, S., Hiruki, C., 1991. Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. J. Microbiol. Meth. 14, 53−61.

Esmailzadeh Hosseini, S.A., Khodakaramian, G., Salehi, M., Bertaccini, A., 2016. Characterization of 16SrII group phytoplasmas associated with alfalfa (*Medicago sativa*) witches' broom disease in diverse areas of Iran. J. Crop. Prot. 5, 581−590.

Esmailzadeh Hosseini, S.A., Salehi, M., Babaie, G., Mohammadi, S., Purmohamadi, S., Bertaccini, A., 2017. Occurrence and molecular characterization of a '*Candidatus* Phytoplasma omanense'-related strain associated with *Prunus persica* yellowing and decline in Iran. In Proceedings, 6th Asian Conference on Plant Pathology, 2017, Jeju, South Korea, p. 233.

Esmailzadeh Hosseini, S.A., Salehi, M., Khodakaramian, G., Mirchenari, S.M., Bertaccini, A., 2015. An up to date status of alfalfa witches' broom disease in Iran. Phytopath. Moll. 5, 9–18.

Ghayeb Zamharir, M., 2014. Molecular study of a new disease of peach in Iran associated with a phytoplasma. Adv. Microbiol. 4, 20–24.

Gundersen, D.E., Lee, I.-M., 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer sets. Phytopathol. Mediterr. 35, 144–151.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acid. S. 41, 95–98.

IRPCM, 2004. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. Int. J. Syst. Evol. Microbiol. 54, 1243–1255.

Jarausch, W., Jarausch-Wertheim, B., Danet, J.L., Broquaire, J.M., Dosba, F., Saillard, C., Garnier, M., 2001. Detection and identification of European stone fruit yellows and other

392 phytoplasmas in wild plants in the surroundings of apricot chlorotic leaf roll-affected
393 orchards in southern France. Eur. J. Plant Pathol. 107, 209–217.
394 Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis
395 version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
396 Lee, I-M., Gundersen-Rindal, D.E., Davis, R.E., Bartozyk, I.M., 1998. Revised classification
397 scheme of phytoplasmas based on RFLP analysis of 16S rRNA and ribosomal protein
398 gene sequences. Int. J. Syst. Bacteriol. 48, 1153–1169.
399 Marcone, C., 2014. Molecular biology and pathogenicity of phytoplasmas. Ann. Appl. Biol., 165,
400 199–221.
401 Marcone, C., Guerra, L.J., Uyemoto, J.K., 2014. Phytoplasma diseases of peach and
402 associated phytoplasma taxa. J. Plant Pathol. 96, 15–28.
403 Pierro, R., Passera, A., Panattoni, A., Casati, P., Luvisi, A., Rizzo, D., Bianco, P.A., Quaglino, F.,
404 Materazzi, A., 2017. Molecular typing of 'bois noir' phytoplasma strains in the Chianti Classico
405 area (Tuscany, central Italy) and their association with symptom severity in *Vitis vinifera* L. cv.
406 Sangiovese. Phytopathology 108, 362−373.
407 Quaglino, F., Maghradze, D., Casati, P., Chkhaidze, N., Lobjanidze, M., Ravasio, A., Passera, A.,
408 Venturini, G., Failla, O., Bianco, P.A. (2016). Identification and characterization of new
409 '*Candidatus* Phytoplasma solani' strains associated with bois noir disease in *Vitis vinifera* L.
410 cultivars showing a range of symptoms severity in Georgia, the Caucasus region. Plant Dis.
411 100, 904−915.
412 Quaglino, F., Murolo, S., Zhao, Y., Casati, P., Durante, G., Wei, W., Bianco, P.A., Romanazzi,
413 G., Davis, R.E., 2017. Identification of new -J and -K 16SrXII subgroups and distinct
414 single nucleotide polymorphism genetic lineages among '*Candidatus* Phytoplasma solani'

415 strains associated with “bois noir” in central Italy. Austral. Plant Pathol. 46, 31−34.
416 Ragozzino, A., 2011. Peach rosette phytoplasma, in: Hadidi, A., Barba, M., Candresse, T.,
417 Jelkmann, W. (Eds.), Virus and Virus-Like Diseases of Pome and Stone Fruits, APS Press,
418 St. Paul, MN, USA, pp. 251−253.
419 Rasoulpour, R., Salehi, M., Bertaccini, A., 2019. Association of a ‘*Candidatus* Phytoplasma
420 aurantifolia’-related strain with apricot showing European stone fruit yellows symptoms in
421 Iran. 3 Biotech. 9, 65.
422 Salehi, M., Esmailzadeh Hosseini, S.A., Salehi, E., Bertaccini, A., 2016a. Molecular and
423 biological characterization of a 16SrII phytoplasma associated with carrot witches’ broom
424 in Iran. J. Plant Pathol. 98, 83−90.
425 Salehi, M., Izadpanah, K., Ebrahim–Nesbat, F., 1995. Etiology, transmission and host range of
426 alfalfa witches’ broom in southern Iran. Iran. J. Plant Pathol. 31, 1−4.
427 Salehi, M., Izadpanah, K., Heydarnejad, J., 2006. Characterization of a New Almond Witches’
428 Broom Phytoplasma in Iran. J. Phytopathol. 154, 386−391.
429 Salehi, M., Rasoulpour, R., Izadpanah, K., 2016b. Molecular characterization, vector
430 identification and partial host range determination of phytoplasmas associated with faba
431 bean phyllody in Iran. Crop Prot. 98, 12−20.
432 Salehi, M., Salehi, E., Abbasian, M., Izadpanah, K., 2015a. Wild almond (*Prunus scoparia*), a
433 potential source of almond witches’ broom phytoplasma in Iran. J. Plant Pathol. 97,
434 377−381.
435 Salehi, M., Salehi, E., Siampour, M., Quaglino, F. Bianco, P.A., 2018. Apricot yellows associated
436 with ‘*Candidatus* Phytoplasma phoenicium’ in Iran. Phytopathol. Mediterr. 57, 269−283.

Salehi, M., Siampour, M., Esmailzadeh Hosseini, S.A., Bertaccini, A., 2015b. Characterization and vector identification of phytoplasmas associated with cucumber and squash phyllody in Iran. Bull. Insectol. 68, 311−319.

Schneider, B., Seemüller, E., Smart, C.D., Kirkpatrick, B.C., 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas, in: Razin, S., Tully, J.G. (Eds.), Molecular and Diagnostic Procedures in Mycoplasmology, Academic Press, San Diego, CA, USA, pp. 369−380.

Scott, S.W., Zimmerman, M.T., 2001. Peach rosette, little peach, and red suture are diseases induced by a phytoplasma closely related to western X-disease. Acta Hortic. 550, 351−354.

Seemüller, E., Schneider, B., 2004. '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. Int. J. Syst. Evol. Microbiol. 54, 1217−1226.

Siampour, M., Izadpanah, K., Afsharifar, A., Salehi, M., Taghizadeh, M., 2004. Study of piercing sucking insects from almond grooves in relation to the transmission of almond brooming phytoplasma. In Proceedings, 16th Iranian Plant Protection Congress, 2004, Tabriz, Iran, p. 390.

Taghizadeh, M., Salehi, M., 2002. Leafhoppers of subfamily Typhlocybinae found on almond (*Prunus amygdalus*) in Fars Province. In Proceedings, 15th Iranian Plant Protection Congress, 2002, Kermanshah, Iran, pp. 92–93.

Tarighi, S., Dehghan, M., Ebadi, E., 2018. Identification and molecular characterization of the phytoplasma associated with leaf stunting and yellowing disease in Razavi and North

460 Khorasan provinces. J. Agric. Biotech. 10, 124−131.
461 Verdin, E., Salar, P., Danet, J.L., Choueiri, E., Jreijiri, F.E., Zammar, S., Gelie, B., Bove, J.,
462 Garnier, M., 2003. '*Candidatus* Phytoplasma phoenicium' sp. nov, a novel phytoplasma
463 associated with an emerging lethal disease of almond trees in Lebanon and Iran. Int. J.
464 Syst. Evol. Microbiol. 53, 833–838.
465 Wei, W., Davis, R.E., Lee, I.-M., Zhao, Y., 2007. Computer-simulated RFLP analysis of 16S
466 rRNA genes: identification of ten new phytoplasma groups. Int. J. Syst. Evol. Microbiol.
467 57, 1855−1867.
468 Weintraub, P., Beanland, L., 2006. Insect vectors of phytoplasmas. Annu. Rev. Entomol. 51,
469 91−111.
470 Zhang, Y.P., Uyemoto, J.K., Kirkpatrick, B.C., 1998. A small-scale procedure for extracting
471 nucleic acids from woody plants infected with various phytoplasmas for PCR assay. J.
472 Virol. Meth. 71, 45−50.
473 Zhao, Y., Wei, W., Lee, I.-M., Shao, J., Suo, X., Davis, R.E., 2009. Construction of an
474 interactive online phytoplasma classification tool, *i*PhyClassifier, and its application in
475 analysis of the peach X-disease phytoplasma group (16SrIII). Int. J. Syst. Evol. Microbiol.
476 59, 2582–2593.
477 Zirak, L., Bahar, M., Ahoonmanesh, A., 2010. Molecular characterization of phytoplasmas
478 associated with peach diseases in Iran. J. Phytopathol. 158, 105–110.
479 Zreik, L., Carle, P., Bové, J.M., Garnier, M., 1995. Characterization of the mycoplasmalike
480 organism associated with witches'-broom disease of lime and proposition of a *Candidatus*
481 taxon for the organism, "*Candidatus* phytoplasma aurantifolia". Int. J. Syst. Bacteriol. 45,
482 449−453.

Table 1. Sequence identity of PWIB phytoplasma strains versus '*Ca*. Phytoplasma' reference strains

	Strain	1	2	3	4	5	6	7	8
1	Abarkooh (MH363612)	ID	0,898	0,899	0,901	0,9	0,9	0,9	0,901
2	Bardseer (MH363613)	0,898	ID	0,996	0,995	0,995	0,995	0,995	0,995
3	Urmia (MH363614)	0,899	0,996	ID	0,996	0,996	0,996	0,996	0,996
4	Borujerd (MH363615)	0,901	0,995	0,996	ID	0,996	0,996	0,996	1
5	Chenaran (MH363616)	0,9	0,995	0,996	0,996	ID	1	1	0,996
6	Naeen (MH363617)	0,9	0,995	0,996	0,996	1	ID	1	0,996
7	Sanandaj (MH363618)	0,9	0,995	0,996	0,996	1	1	ID	0,996
8	Zarghan (MH363619)	0,901	0,995	0,996	1	0,996	0,996	0,996	ID
9	'*Ca*. Phytoplasma solani' (AF248959)	0,892	0,885	0,886	0,885	0,885	0,885	0,885	0,885
10	'*Ca*. Phytoplasma aurantifolia' (U15442)	0,994	0,898	0,898	0,9	0,899	0,899	0,899	0,9
11	'*Ca*. Phytoplasma asteris' (AY265210)	0,896	0,883	0,884	0,884	0,884	0,884	0,884	0,884
12	'*Ca*. Phytoplasma australasiae' (Y10097)	0,983	0,903	0,904	0,905	0,905	0,905	0,905	0,905
13	'*Ca*. Phytoplasma ulmi' (AY197655)	0,889	0,936	0,937	0,937	0,938	0,938	0,938	0,937
14	'*Ca*. Phytoplasma trifolii' (AY390261)	0,895	0,937	0,938	0,939	0,939	0,939	0,939	0,939
15	'*Ca*. Phytoplasma costaricanum' (HQ225630)	0,899	0,892	0,893	0,894	0,894	0,894	0,894	0,894
16	'*Ca*. Phytoplasma phoenicium' (AF515636)	0,901	0,996	0,996	0,997	0,999	0,999	0,999	0,997
17	'*Ca*. Phytoplasma mali' (AJ542541)	0,886	0,901	0,904	0,903	0,903	0,903	0,903	0,903

Table 2. Single nucleotide polymorphism genetic lineages of PWIB and 16SrIX-B phytoplasma strains

Strain	Origin	Lineage	SNPs in 16S rDNA[a] (position from the annealing site of the primer R16F2n)																				
			159	**287**	**460**	**473**	572	639	**646**	**690**	**691**	713	**759**	781	806	**863**	**901**	**940**	983	**1089**	**1107**	1152	1171
Bardseer (MH363613)	Iran	f4	C	A	T	G	G	G	-	T	G	A	A	A	C	-	-	C	G	G	G	T	T
Urmia (MH363614)	Iran	f1	C	A	T	G	G	G	C	T	G	A	A	A	C	A	-	C	G	G	A	T	T
Borujerd (MH363615)	Iran	g	T	A	C	G	G	G	-	T	G	A	G	A	C	-	-	C	G	G	A	T	T
Chenaran (MH363616)	Iran	a1	T	G	T	G	G	G	-	T	G	A	A	A	C	-	-	C	G	T	A	T	T
Naeen (MH363617)	Iran	a1	T	G	T	G	G	G	-	T	G	A	A	A	C	-	-	C	G	T	A	T	T
Sanandaj (MH363618)	Iran	a1	T	G	T	G	G	G	-	T	G	A	A	A	C	-	-	C	G	T	A	T	T
Zarghan (MH363619)	Iran	g	T	A	C	G	G	G	-	T	G	A	G	A	C	-	-	C	G	G	A	T	T
A4 (AF515636)	Lebanon, Iran	a	T	A	T	G	G	G	-	T	G	A	A	A	C	-	-	C	G	T	A	T	T
N27-2 (HQ407532)	Lebanon	b1	T	A	T	G	C	G	-	T	G	A	A	A	C	-	-	C	G	T	A	T	T
N5 (HQ407522)	Lebanon	b2	T	A	T	G	C	G	-	T	G	A	A	A	C	-	-	C	G	T	A	G	T
N28-1 (HQ407526)	Lebanon	b3	T	A	T	G	G	G	-	T	G	A	A	A	C	-	-	C	G	T	A	G	T
N29-1 (HQ407529)	Lebanon	c1	T	A	T	G	G	T	-	T	G	A	A	A	C	-	-	C	G	T	A	T	T
Al4 (HQ407521)	Lebanon	c2	T	A	T	G	G	G	-	T	G	A	A	A	T	-	-	C	G	T	A	T	T
Smasp (KP851773)	Lebanon	c3	T	A	T	G	G	G	-	T	G	A	A	C	T	-	-	C	G	T	A	T	T
P3-1 (HQ407518)	Lebanon	c4	T	A	T	G	G	T	-	T	G	A	A	C	T	-	-	C	G	T	A	T	T
Smilax12 (KF583756)	Lebanon	d	T	A	T	G	G	G	-	T	G	A	A	A	C	-	-	C	A	T	A	T	T
Smilax13 (KF583757)	Lebanon	e	T	A	T	G	G	G	-	T	G	G	A	A	C	-	-	C	G	T	A	T	C
A21 (AF515637)	Iran	f1	C	A	T	G	G	G	C	T	G	A	A	A	C	A	-	C	G	G	A	T	T
Breijan (KY014991)	Iran	f2	C	A	T	A	G	G	-	C	T	A	A	A	C	-	-	C	G	G	A	T	T
Kavar (KM235725)	Iran	f3	C	A	T	G	G	G	-	T	G	A	A	A	C	-	G	C	G	G	G	T	T
Meymand (KM235727)	Iran	g	T	A	C	G	G	G	-	T	G	A	G	A	C	-	-	C	G	G	A	T	T
Moshkan (JN565017)	Iran	h1	T	A	T	G	G	G	-	T	G	A	A	A	C	-	-	A	G	G	A	T	T
Kerman II (JN565013)	Iran	h2	T	A	T	G	G	G	-	T	G	A	A	A	C	-	-	A	G	T	A	T	T

[a] nucleotide positions n normal and bold characters are present exclusively in 16SrIX-B phytoplasma strains from Lebanon and Iran, respectively

Fig. 1. Maps of the Iranian provinces in which field surveys and plant sampling were conducted.

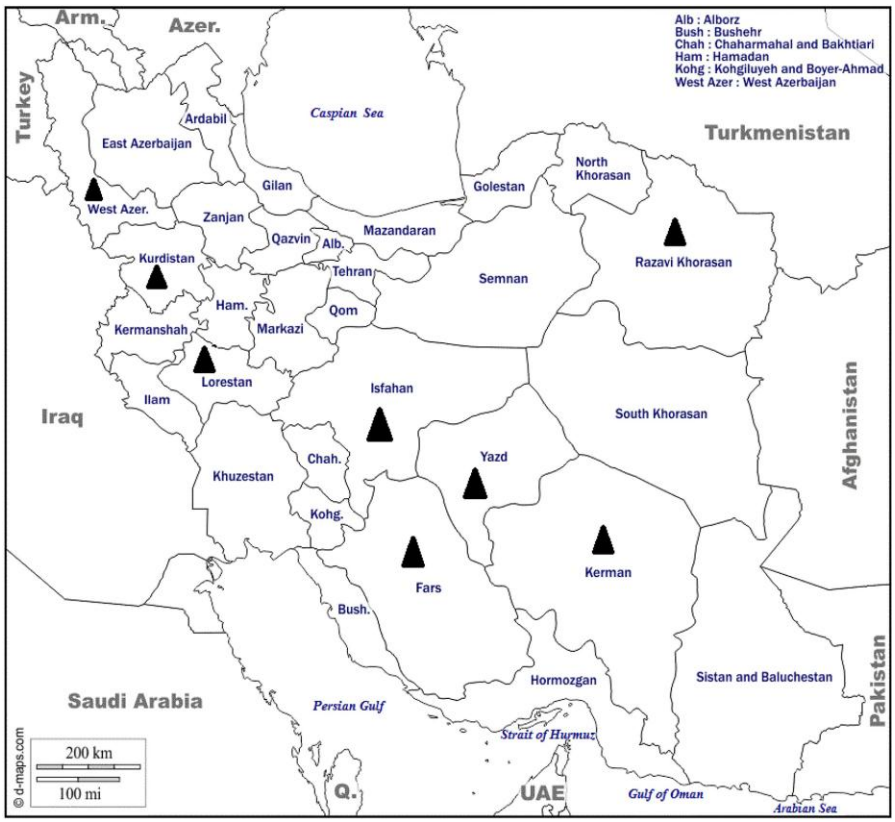


Fig. 2. Symptoms of peach witches'-broom disease in Zarghan. Witches'-broom affected trees in a peach orchard (A); healthy (right) and witches'-broom affected (left) peach branch (B); witches'-broom on main stem and crown region (C); peach tree showing severe witches'-broom and decline (D).

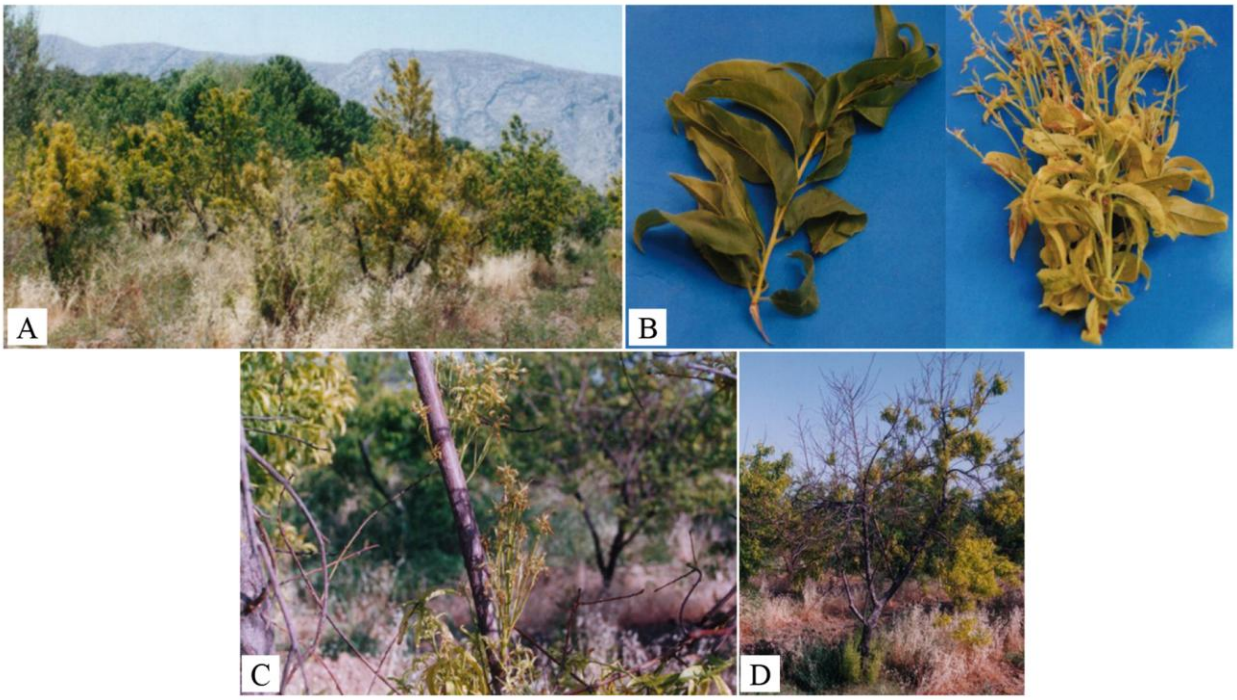
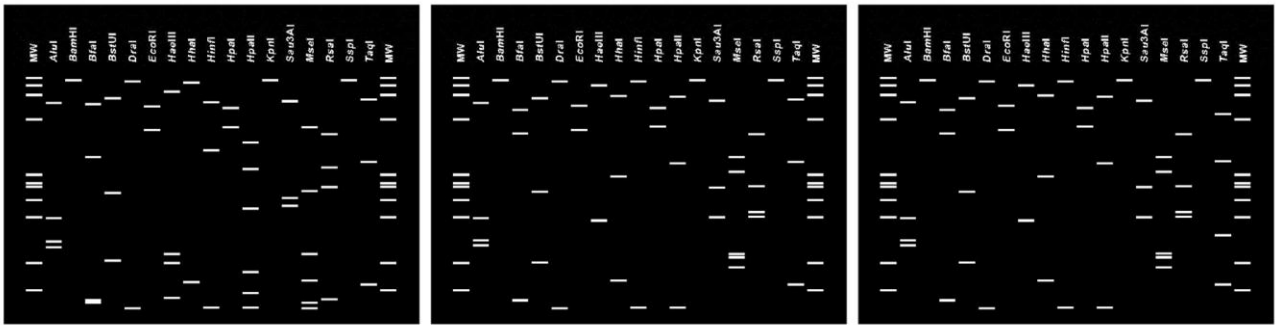


Fig. 3. Symptoms on peach and bitter almond seedlings grafted with witches'-broom affected peach scions. Mild and severe symptoms observed on peach seedlings grafted respectively with PWIB-affected peach scion from Abarkooh (left) and Zarghan (right); healthy peach seedling (centre) (A); mild and severe symptoms observed on bitter almond seedlings grafted respectively with PWIB-affected peach scion from Abarkooh (right) and Zarghan (centre); healthy bitter almond seedling (left) (B).

Fig. 4. *In silico* RFLP profiles of PWIB phytoplasma strains



Abarkooh WBDP strain
16SrII-C

Borujerd WBDP strain
16SrIX-B

Bardseer WBDP strain
16SrIX-B variant

Fig. 5. Phylogenetic tree inferred from 16S rDNA nucleotide sequences of phytoplasma strains of group 16SrIX [including PWIB phytoplasma (PWIBp) strains, identified in this study]. Minimum-Evolution analyses were carried out using the Jukes-Cantor model and bootstrap replicated 1,000 times. GenBank accession number of each sequence is given in parentheses, and gene sequences obtained in the present study are indicated in bold font.