Identification of a group 16SrIX ‘Candidatus Phytoplasma phoenicium’ phytoplasma associated with sweet orange exhibiting decline symptoms in Iran

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Abstract

During a survey in an orange (*Citrus sinensis*) garden in the south of the Kerman province of Iran, some trees were observed showing symptoms of yellowing and decline. Nested PCR analysis using phytoplasma universal primers identified symptomatic samples as phytoplasma-positive, whereas the symptomless samples gave no PCR products. Sequence similarity and phylogenetic studies using 16S rRNA gene sequences demonstrated that the sweet orange decline associated phytoplasma belongs to the 16SrIX ‘Candidatus Phytoplasma phoenicium’ group. To the best of the authors’ knowledge this is the first report of association of a phytoplasma belonging to the ‘Candidatus Phytoplasma phoenicium’ group with decline symptoms in sweet orange in Iran.

Keywords Iran · Phytoplasma · Yellows · Decline · Sweet orange

Field surveys in recent years have revealed the presence of a significant number of citrus trees showing disease symptoms including leaf yellows, little leaf, leaf defoliation and sudden decline in summer. In China, phytoplasmas related to ‘Candidatus Phytoplasma asteris’ have been identified in sweet orange, pomelo and mandarin with greening (Huanglongbing (HLB))-like symptoms (Chen et al. 2009) and related to the peanut witches’ broom (16SrII) phytoplasma group in grapefruit with similar symptoms (Lou et al. 2013). A phytoplasma from the peanut witches’ broom (16SrII) group has also been found associated with HLB symptoms in sweet orange and lime in Iran (Sabeti et al. 2017) and in 2019, it was reported that ‘Candidatus Liberibacter asiaticus’ and ‘Candidatus Phytoplasma aurantifolia’ (16SrII) related strains are associated with a new citrus decline disease (CDD) in Iran (Passera et al. 2018). However, the symptoms observed in this present study

differ from those reported previously for HLB. Here, the fruits of symptomatic trees did have not HLB fruit symptoms, and molecular analysis did not indicate infection of these samples with ‘Candidatus Liberibacter asiaticus’, *Spiroplasma citri* or citrus tristeza virus and the leaves of infected trees showed severe yellows. In this article, the phytoplasma associated with these yellowing and decline symptom in sweet orange in Iran has been identified.

A total of 84 leaf samples were collected in 2016 and 2017 from 74 symptomatic and 10 symptomless sweet orange plants (cultivars Valencia, and navel) in orchards and domestic gardens located in the south of Kerman province, Iran. Symptomless leaves from the same sweet orange cultivars grown in a greenhouse were used as the healthy control samples.

The specimens from sweet orange plants showing decline symptoms in the garden were grafted onto healthy trees that have been tested for major sieve tube pathogens, and the specimens from healthy sweet orange trees were grafted to other healthy trees as a control in greenhouse conditions. The grafted plants were tested for phytoplasma infections as described below, 6 months after grafting.

Total DNA was extracted from 0.1 g of leaf samples according to previously described protocols (Doyle and Doyle 1987). Two universal phytoplasma nested primer sets P1/Tint (Smart et al. 1996; Deng and Hiruki 1991) and R16F2n/R16R2 (Gundersen and Lee 1996) were used for direct and nested PCR. Almond witches’ broom phytoplasma (16SrIX-

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Fig. 1 Sweet orange decline symptoms on (a) a tree in the garden; (b) HLB symptoms on infected fruit; (c) sweet orange decline phytoplasma on infected fruits

B) was used as a standard positive controls in molecular analyses in this study. The PCR products were analyzed in 1.2% agarose gels in TAE buffer.

Positive PCR products that were amplified from 5 sweet orange trees from different orchards, using R16F2n/R2 primers in nested PCR were sequenced directly. The sequences obtained were assembled, aligned and compared with nucleotide sequences in the GenBank database, using BLAST (NCBI, Bethesda, MD, USA). Phylogenetic analyses were performed with maximum parsimony (MP) analysis by using

16S rDNA sequences from sweet orange samples SO84, SO47, SO18, SO24 and SO65 and from 56 ‘*Candidatus* phytoplasma’ reference strains (Wei et al. 2007), using *Bacillus subtilis* as the outgroup. The analysis was replicated 1000 times (Tamura et al. 2004).

All samples showing decline symptoms (Fig. 1) resulted in positive nested PCR amplification with the R16F2n/R2 primers. No amplification was obtained from the negative controls without DNA template and from the symptomless sweet orange samples. Results of grafting in two sweet orange

Fig. 2 Grafted sweet orange (1: native type and 2: Valencia cultivar) showing disease symptoms 8 month after grafting and healthy Valencia (right)

Fig. 3 Phylogenetic tree using the Maximum parsimony method on 16S rRNA sequences of representative ‘*Candidatus Phytoplasma*’ species with the sweet orange decline phytoplasma sequences (SO84, SO47, SO18, SO24 and SO65), indicating their presence in the ‘*Ca.*

Phytoplasma phoenicium’ 16SrIX-B/16SrIX-D grouping. Accession numbers are shown in brackets and *Bacillus subtilis* was used as the outgroup

cultivars (1: native type and 2: Valencia) with 5 replicates showed that disease symptoms appear in graft-inoculated

plants 8 month after grafting (Fig. 2) and phytoplasma infections were confirmed using nested PCR analysis.

The sequences of the selected strains SO84, SO47, SO18, SO24 and SO65 have been deposited in GenBank (NCBI) with accession numbers SAMN10409248, SAMN10409249, SAMN10409250, SAMN10409251 and SAMN10409252 respectively. The BLAST analysis showed all the 5 phytoplasma 16S rDNA sequences from the present study had 99% homology with '*Prunus scoparia*' witches'-broom phytoplasma isolate Seerjan (accession number KM235729) (Salehi et al. 2015). The obtained aligned sequences of the sweet orange decline phytoplasma strains were classified into the 16SrIX-B/16SrIX-D group, also referred to as '*Candidatus Phytoplasma phoenicium*' (Fig. 3).

It has been reported before that there is genetic diversity among '*Ca. Phytoplasma phoenicium*' strains (Barbosa et al. 2012; Lova et al. 2011), which could be related to different ecological or climatic niches of the phytoplasma populations. The sweet orange decline phytoplasma could be transmitted to other citrus species from external sources of inocula, which could include crop plants, ornamental plants or weeds, but correlation studies of sweet orange decline phytoplasma strains with symptom specificity, efficiency, severity and the vector transmission of strains should be performed in future studies in Iran.

Compliance with ethical standards

Funding This study has not received any funding.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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