



Association of 16SrII-C subgroup phytoplasma related strains with witches'-broom symptoms in Sweet orange and Orlando tangelo in Iran

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Abstract

Sweet orange (*Citrus sinensis*) and Orlando tangelo (*C. paradisi* × *C. reticulata*) trees showing witches'-broom symptoms were observed during 2018 in southern Kerman province, Iran. Detection of phytoplasmas in the symptomatic trees was confirmed by nested PCR assays using the phytoplasma specific primers. Sequence analysis indicated that the phytoplasmas associated with the witches'-broom affected trees belong to the 16SrII-C subgroup phytoplasma. This is the first report of a 16SrII-C phytoplasma associated with witches'-broom symptoms of Sweet orange and Orlando tangelo trees in the world.

Keywords Orlando tangelo · Phytoplasma · Sweet orange · Witches'-broom

Citrus production is an important agricultural industry with a great economic importance in Iran. The witches'-broom disease of lime (WBDL) is one of the most destructive citrus diseases in Iran and the Arabian Peninsula. WBDL, caused by 'Candidatus Phytoplasma aurantifolia' (16SrII-B), was first observed in Oman and then within two decades reported from the United Arab Emirates (Garnier et al. 1991), Iran (Bové et al. 2000) and India (Ghosh et al. 1999, 2017, 2019). It is destroying the acid lime (*Citrus aurantifolia*) trees in the two main lime-producing provinces of Iran, viz., Kerman and Hormozgan and thus heavily impacting lime production in the country (Azadvar 2016). Furthermore, 'Ca. Phytoplasma aurantifolia' has previously been reported associated with acid lime, bakraee (*Citrus* sp.), grapefruit (*C. paradisi*), citron (*C. medica*), sweet lime (*C. limettioides*) and limequat (*C. aurantifolia* × *Fortunella margarita*) trees showing witches'-broom symptoms in Iran (Djavaheri and Rahimian 2004; Bagheri

et al. 2010; Azadvar et al. 2015; Azadvar 2016; Faghihi et al. 2017).

During a survey in summer 2018, four Sweet orange (*C. sinensis* var. Olinda Valencia) and five Orlando tangelo (*C. paradisi* × *C. reticulata*) trees with typical witches'-broom symptoms (Fig. 1) were observed in Southern Kerman (Anbarabad and Manoojan regions), Iran. Leaf samples from the symptomatic as well as asymptomatic trees were collected and the total genomic DNA was extracted from leaf midribs using a CTAB method (Zhang et al. 1998). The extracted DNA was used for phytoplasma detection with specific primer pairs P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) in direct PCR and R16F2n/ R16R2 (Gundersen and Lee 1996) in nested PCR to amplify a fragment of 16S rDNA as previously described by Azadvar et al. (2011). The amplified PCR products (~ 1.25 kb) were directly sequenced (Macrogen, South Korea) on both forward and reverse directions and consensus sequences were assembled with the BioEdit v 7.1.9 program (Hall 1999) and deposited in GenBank. The partial 16S rDNA sequences were analyzed by BLAST (<http://www.ncbi.nlm.nih.gov/blast>) and iPhyClassifier tool (<https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>) (Zhao et al. 2013) and virtual gel was generated by pDRAW32 software (AcaClone software, <http://www.acaclone.com>) for identification of phytoplasma group and subgroup. The phylogenetic tree was constructed using MEGA X software (Kumar et al. 2018).

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Fig. 1 Witches'-broom symptoms on Sweet orange **a** and Orlando tangelo **b** trees in Southern Kerman province, Iran

Expected 1.8 kb and ~ 1.25 kb amplicons corresponding to sequences of the phytoplasma 16S rRNA gene were amplified from all the symptomatic trees in direct and nested PCR, but not from the asymptomatic ones (Fig. 2).

BLAST analyses of the sequences indicated that the phytoplasmas associated with Sweet orange (*C. sinensis* var. Olinda Valencia) witches'-broom, 'SOWB' (MK973070 and MW000920) and Orlando tangelo (*C. paradisi* × *C. reticulata*) witches'-broom, 'OTWB' (MK990222 and MW000919) diseases in Iran showed the highest similarity with the 16S rDNA sequence of 16SrII group phytoplasma members. Virtual restriction fragment length polymorphism analyses of the 16S rDNA sequences using the *iPhyClassifier* tool showed that the phytoplasmas associated with SOWB and OTWB in Iran were closely related to the reference pattern of 16Sr group II, subgroup C (Cactus witches'-broom phytoplasma, AJ293216) with a similarity coefficient of 0.99. Moreover, *in silico* RFLP analysis of the sequences obtained in this work and with pDRAW32 program using *AluI*, *BamHI*, *BfaI*, *BstUI*, *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *MseI*, *RsaI*, *Sau3AI*, *SspI* and *TaqI* endonucleases further confirmed that the SOWB and OTWB phytoplasmas are the strains related to subgroup 16SrII-C (Fig. 3). The phylogenetic tree constructed based on the partial 16S rDNA sequences (Fig. 4) confirmed that the SOWB and OTWB phytoplasmas are clustered with Cactus witches'-broom phytoplasma (AJ293216) a member of 16SrII subgroup C.

To the best our knowledge, this is the first report of the association of a 16SrII-C phytoplasma with witches'-broom symptoms of Sweet orange and Orlando tangelo trees in the world. WBDL was limited to acid lime and some of the less economically important citrus varieties for last decades. The spread of 16SrII-C phytoplasma to wide distribute and more economically important citrus cultivars, such as Sweet orange and Orlando tangelo, is an emerging great threat to the citrus industry and underscores the need to revise the current management strategies for the control of this phytoplasma in Iran.

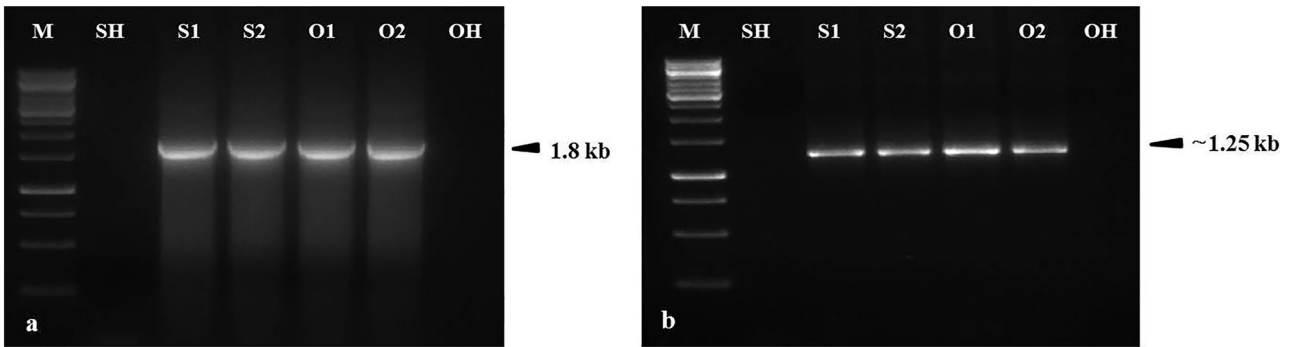


Fig. 2 Agarose gel photograph of PCR products from the 16S rRNA gene using P1/P7 **a** and R16F2n/R16R2 **b** primer pairs. S1 and S2: Sweet orange witches'-broom symptomatic plants, O1 and O2:

Orlando tangelo witches'-broom symptomatic plants, SH: healthy Sweet orange, OH: healthy Orlando tangelo, M: 1 kb DNA Ladder

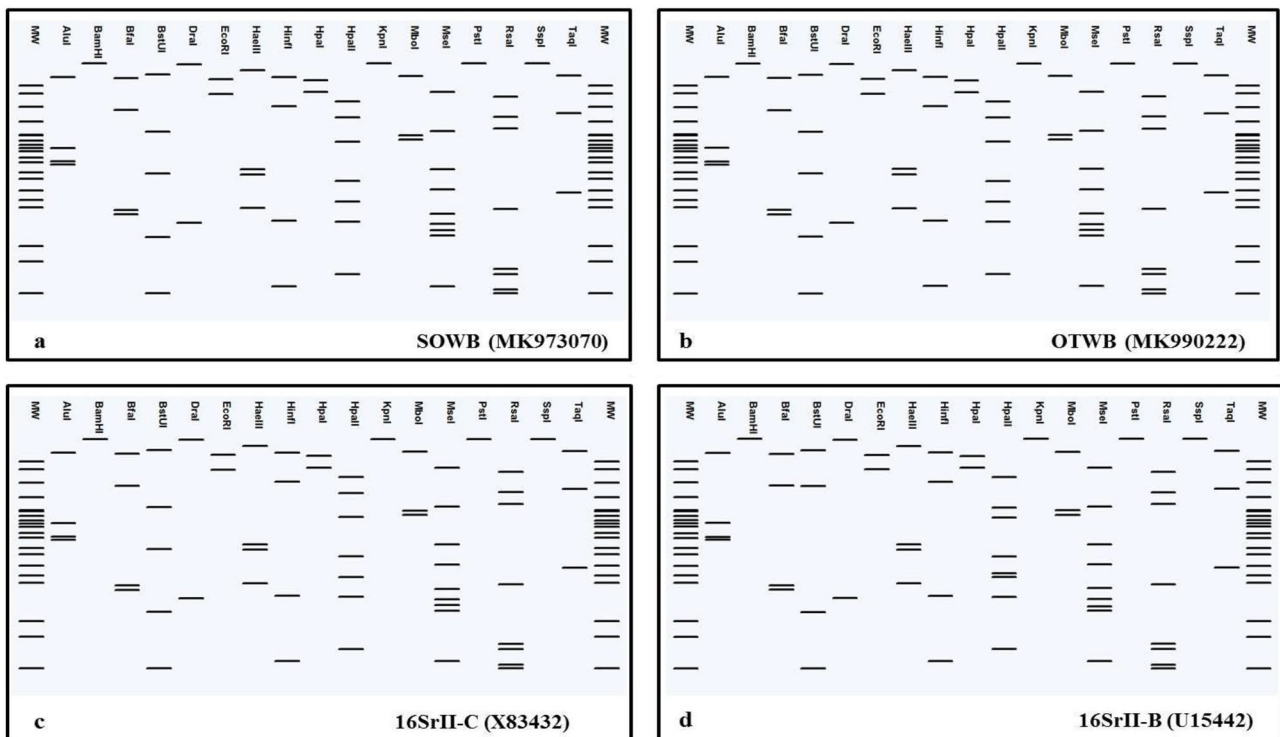


Fig. 3 Virtual RFLP generated with program pDRAW32 from in silico digestion of the 16S rDNA R16F2n/R16R2 fragments of the phytoplasmas associated with Sweet orange witches'-broom (SOWB) and Orlando tangelo witches'-broom (OTWB) and reference strain phytoplasma subgroups (16SrII-B and 16SrII-C) using 17 restriction enzymes. **a** Sweet orange witches'-broom (SOWB) phytoplasma

(MK973070), **b** Orlando tangelo witches'-broom (OTWB) phytoplasma (MK990222), **c** Faba bean phyllody phytoplasma (X83432), 16SrII-C (reference strain) **d** Lime witches'-broom phytoplasma (U15442), 16SrII-B (reference strain). MW: pBR322 DNA-*MspI* digest ladder (New England Biolabs)

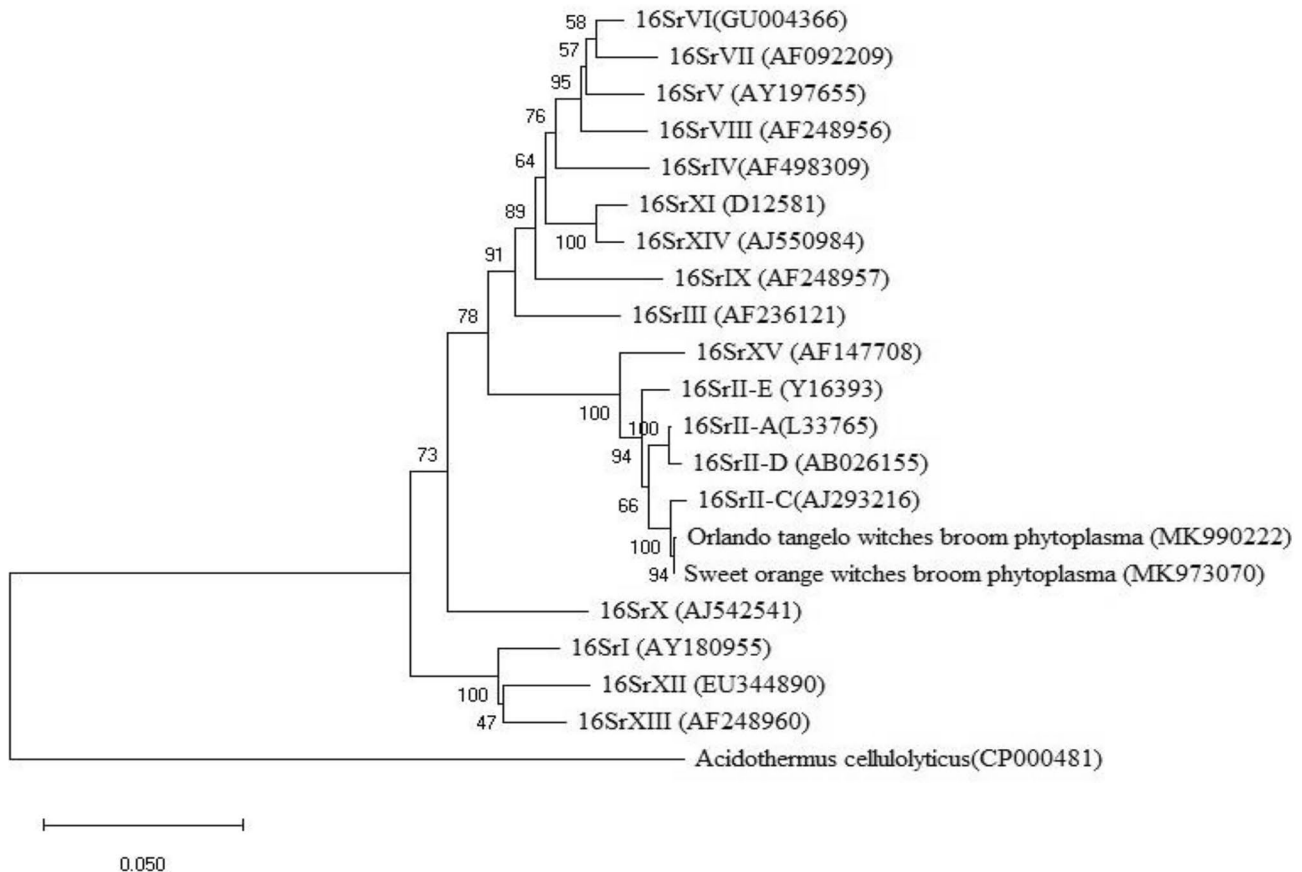


Fig. 4 Phylogenetic tree constructed by neighbor-joining analysis of partial 16S rDNA sequences of the phytoplasma strains associated with witches'-broom affected Sweet orange and Orlando tangelo trees

and some of the group representative phytoplasmas from GenBank. *Acidothermus cellulolyticus* was used as outgroup

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