



First report of '*Candidatus Phytoplasma trifolii*' (16SrVI group) in Nagpur mandarin (*Citrus reticulata*) showing huanglongbing symptoms in central India

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Huanglongbing (HLB) or citrus greening disease causes decline and/or death of millions of citrus trees in production areas worldwide, including India. Although the disease is primarily attributed to '*Candidatus Liberibacter asiaticus*', an unculturable α -proteobacterium, phytoplasmas belonging to the groups 16SrIX in Brazil (Teixeira *et al.*, 2008), 16SrII in China (Lou *et al.*, 2014), and 16SrI in China (Chen *et al.*, 2009) and Mexico (Arratia-Castro *et al.*, 2014) have been associated with HLB. The present study examined whether in addition to '*Ca. L. asiaticus*', phytoplasmas were also associated with HLB-like symptoms in Indian citrus groves.

Nagpur mandarin (*Citrus reticulata*), a commercially important citrus cultivar, is grown on around 200,000 hectares in central India. Leaf samples of Nagpur mandarin trees showing HLB-like yellow shoot (Fig. 1) and blotchy mottle symptoms (Fig. 2) were collected from Nagpur district (Kalmeshwar, Katol and Saoner localities) of Maharashtra state, central India between October 2015 and March 2016. Total genomic DNA was isolated from 150 mg of 15 symptomatic and five asymptomatic samples (leaf midribs) using a Qiagen DNeasy Plant Mini Kit. The presence of '*Ca. Liberibacter asiaticus*' was tested by real-time PCR (ABI 7300, Applied Biosystems, USA) using specific 16S rDNA-based TaqMan primer-probe sets (HLBasr/HLBp) (Li *et al.*, 2006). To ascertain the association of phytoplasma, PCR was performed using P1/P7 universal primers specific to the 16S rRNA gene of phytoplasmas, followed by a nested PCR from a 1:30 dilution of the P1/P7 PCR products using primer sets R16F2n/R16R2 (Gundersen & Lee, 1996) and Fu5/Ru3 (Lou *et al.*, 2014).

Out of the 15 HLB-symptomatic mandarin plants, all were positive for '*Ca. L. asiaticus*' (real time PCR Ct values ranged from 19.2 to 26.7) and three were positive for phytoplasma (nested PCR amplicons of the expected sizes, ~1.25 Kb for R16F2n/R16R2 and ~0.88 Kb for Fu5/Ru3 primers were obtained). The five symptomless samples were negative both for '*Ca. L. asiaticus*' and phytoplasma. The three R16F2n/R16R2 amplicons were purified and directly sequenced (Eurofins, Bangalore, India) and showed 100% sequence identity to each other. The consensus sequence of Nagpur mandarin (NM)-HLB phytoplasma was deposited in GenBank (Accession No. KX588712). A BLAST search showed that the NM-HLB phytoplasma had 99% sequence identity with those of members of the 16SrVI ('*Ca. Phytoplasma trifolii*') group. Phylogenetic analysis (MEGA software version 6.0, using the neighbour joining method with 1000 times bootstrapping) of 16S rRNA sequences confirmed the NM-HLB phytoplasma as a '*Ca. Phytoplasma trifolii*'-related strain (Fig. 3). Computer-simulated restriction analysis of R16F2n/R16R2 sequence with 17 restriction endonucleases (*AluI*, *BamHI*, *BfaI*, *BstUI*, *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *Sau3AI*, *MseI*, *RsaI*, *SspI* and *TaqI*)

using the iPhyClassifier online tool (<http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>) revealed that the virtual RFLP pattern of the NMHLB phytoplasma was identical (similarity coefficient 1.00) to the reference pattern of 16Sr group VI, subgroup D (X83431).

To our knowledge, this is the first report of the association of a '*Ca. Phytoplasma trifolii*' (16SrVI)- related strain with HLB in India. This is also the first time this phytoplasma has been detected in citrus which underlines the need to expand the current HLB management strategy.

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Figure 1



Figure 2

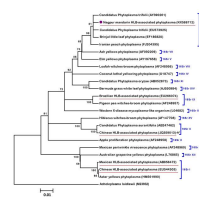


Figure 3

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