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First report of occurrence of *Candidatus* Phytoplasma trifolii-related strain causing witches' broom disease of chilli in India

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Abstract During February 2017, 20% of the chilli plants in fields at the Indian Agricultural Research Institute, New Delhi, India showed witches' broom symptoms of suspected phytoplasma etiology. Phytoplasma association was confirmed by utilizing a nested PCR assay with universal primer pairs P1/P7 and 3F/3R that target the phytoplasma *16S rRNA* gene. The chilli witches' broom phytoplasma (CWBP) sequence (Acc. No. KY612251) shared 100% nucleotide sequence identity with the 16S rDNA sequences of identified strains of '*Candidatus* Phytoplasma trifolii' subgroup D (16SrVI-D) phytoplasmas associated with *Lens culinaris'* witches'-broom (Acc. No. KY439869), brinjal little leaf (Acc. No. KX284698), *Brassica oleracea var. capitata* witches' broom (Acc. No. KX671553) and *Apium graveolens* white leaf (Acc. No. KX671551) in pair wise sequence comparisons. The CWBP strain also clustered with strains of '*Candidatus* Phytoplasma trifolii' subgroup D in phylogenetic comparison analysis. This is the first report of association of 'Ca. *P. trifolii*' subgroup D with witches' broom disease of chilli in India.

Keywords Phytoplasma · Identifiation · Chilli · India

Chilli (*Capsicum annum*, fam: Solanaceae) is one of the most valuable spice crops of India and is grown largely for its fruits all over India. It is also used for vegetables, spices, condiments, sauces and pickles. India is the largest producer, consumer and exporter of chilli. Chilli is grown in 0.17 million hectares with production of 1.98 million tonnes (Anonymous 2015). Major factors that limit chilli production besides its narrow genetic base are extreme susceptibility to biotic and abiotic stresses. Phytoplasmas are non-culturable prokaryotic microorganisms that cause several hundred diseases of various plants and are transmitted by phloem-feeding insects. Plants infected with phytoplasmas exhibit a variety of symptoms that suggest profound disturbances to the normal plant behaviour and physiology. Phytoplasma diseases are important worldwide, with a high economic impact on crop production and quality (Bertaccini et al. 2014). Chilli is affected by several diseases of which little leaf caused by phytoplasmas is one of the important factors causing considerable economic losses (Singh and Singh 2000). Limited information is available on phytoplasma infection of chilli crops in India. Hence, the present work included PCR screening for the presence of phytoplasmas, *16S rRNA* gene sequence comparison and phylogeny clustering based on the *16S rRNA* gene in samples collected from chilli and brinjal plants.

During a survey of experimental chilli fields at IARI, New Delhi in February 2017, an incidence of 20% of phytoplasma suspected symptoms of witches' broom and little leaf were observed in chilli plants (Fig. 1b). Besides chilli fields, little leaf symptoms were also observed in brinjal plants (Fig. 1d). Total genomic DNA from four symptomatic chilli plants (leaf veins and stem portions) and four symptomatic brinjal plants (leaf vein tissue) along with three symptomless samples of both chilli (Fig. 1a) and brinjal plants (Fig. 1c) were extracted following the procedure described by Ahrens and Seemuller (1992). The DNA concentration was determined spectrophotometrically and the quality evaluated by agarose gel electrophoresis. For the detection of phytoplasmas, nested PCR reactions were performed using primers P1/P7 in the first round (Deng and Hiruki 1991), followed by the 3F/3R primer pair (Manimekalai et al. 2010). PCR reactions were performed in a

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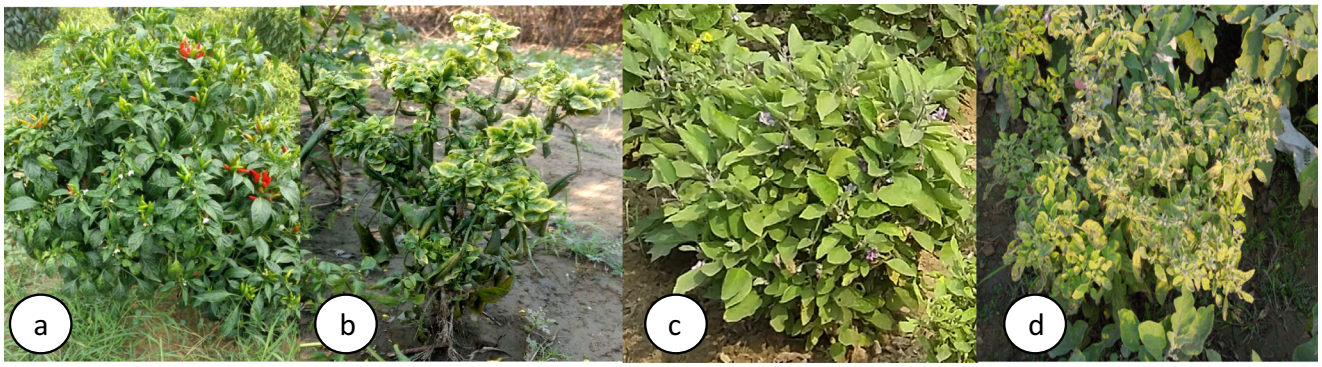

Fig. 1 **a**, Symptomless chilli plant **b**, Symptomatic chilli plant showing witches' broom and little leaf symptoms, **c**, Symptomless brinjal plant and d, Symptomatic brinjal plant showing little leaf symptoms

Mastercycler (Eppendorf, Germany) and the cycling protocol followed as described by Rao et al. (2014). The DNA isolated from *Datura stramonium* leaf tissue infected with witches' broom (16SrVI-D) was used as positive control. The PCR products (5 μl) were analyzed by electrophoresis in 1% agarose gel in 1 X TAE buffer, stained with good view dye, and visualized using a UV transilluminator. The amplified 16S rDNA phytoplasma fragments were purified using the Wizard[R] SV Gel and PCR Clean-up System (Promega, Madison, USA).

PCR amplification with primer pair P1/P7 did not yield the expected 1.8 kb product of the *16S rRNA* gene region from any of the symptomatic chilli and brinjal samples. However, 1.3 kb DNA products were amplified from symptomatic chilli and brinjal plants and the positive control of *D. stramonium* by using the 3F/3R primer pair, in nested PCR. No

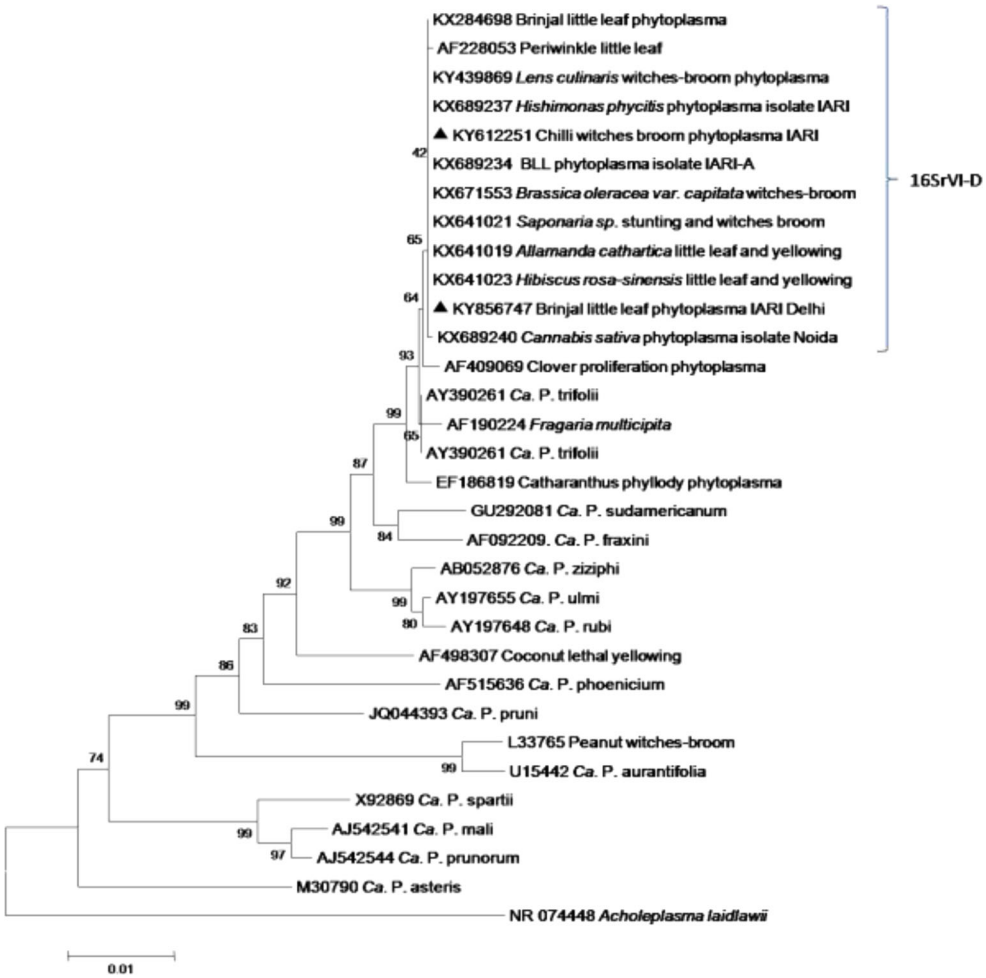

Fig. 2 Phylogenetic tree constructed by neighbor-joining method showing the relationships between CWBP, BLL phytoplasma and the reference phytoplasma strains. Accession numbers are specified in the tree. *Acholeplasma laidlawii* was used as the outgroup. Mega 6.0 software was used to construct the tree. Numbers on branches are bootstrap values obtained for 1000 replicates

amplifications were observed in any of the symptomless chilli and brinjal samples. All of the four amplicons obtained with the 3F/3R primers from symptomatic samples of chilli and brinjal were purified and sequenced bi-directionally. The 16S rDNA sequences were assembled and analysed using the BLAST and Bioedit programs (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The representative sequences of phytoplasmas from chilli and brinjal in the present study were submitted to GenBank with Acc. No. KY612251 (chilli) and KY856747 (brinjal). BLAST comparison revealed that the phytoplasma identified in chilli and brinjal exhibited over 99% sequence identity of its 16S rDNA sequence with those of '*Ca. P. trifolii*' and related strains and 100% sequence identity with *Lens culinaris* witches' broom (Acc. No. KY439869), brinjal little leaf (Acc. No. KX284698), *Brassica oleracea var. capitata* witches' broom (Acc. No. KX671553) and *Apium graveolens* white leaf (Acc. No. KX671551). The phylogenetic tree was constructed using 16S rDNA sequences belonging to phytoplasmas classified into distinct groups, the sequences of strains representative of the subgroups that constitute the 16SrVI group and the sequences of strains of chilli and brinjal under study, using MEGA6 software (Tamura et al. 2013) using the neighbour-joining method with default values and 1000 replicates for boostrap sanalysis. *Acholeplasma laidlawii* 16S rDNA sequence (Acc. No. NR074448) was used as the outgroup to root the phylogenetic tree. The phylogenetic tree based on the 16S rDNA sequences supported the BLAST sequence comparison results (Fig. 2). The chilli and brinjal phytoplasma was grouped in the same phytoplasma cluster of '*Ca. P. trifolii*' subgroup D. '*Candidatus P. trifolii*' has been reported to infect various vegetables, ornamentals and weeds in India and abroad (Bertaccini et al., 2014). Earlier, different phytoplasma strains were identified and characterized associated with chilli plants in different countries like India ('*Ca. P. asteris*', 16SrII-D; Khan and Raj 2006; Sharma et al. 2015) and Indonesia ('*Ca.* P. aurantifolia', Harling et al. 2009), China (16SrI-B; Li et al. 2013), New Mexico ('*Ca P. asteris*', '*Ca P. trifolii*'; Randall et al. 2009; Santos-Cervantes et al. 2008), Russia (16SrXII-A; Ember et al. 2011), Iran ('*Ca.* P. aurantifolia'; Faghihi et al. 2016), Australia (16SrII; Tran-Nguyen et al. 2003) and Spain ('*Ca. P. trifolii*'; Castro and Romero, 2002). In the present study, we confirmed association of '*Ca. P. trifolii*' subgroup D of phytoplasmas with chilli and brinjal plants at IARI, New Delhi, India. In India, '*Ca. P. trifolii*' subgroup D phytoplasmas have been reported to be associated with several diseases of plants, viz. sesame, *Hibiscus rosa-sinensis*, *Saponaria officinalis, Allamanda cathartica* and brinjal (Madhupriya et al. 2015; Khasa et al. 2016; Kumar et al. 2017). In addition, many weed species including *Datura stramonium*, *Portulaca oleracea* and *Cannabis sativa subsp. sativa* have also been reported to be alternate hosts of the 16SrVI-D subgroup of phytoplasmas in India (Rao and Kumar 2017). This new report of association of a 16SrVI-D subgroup of phytoplasma with witches' broom of chilli in India is important because of its epidemioloical significance. The results indicate that this phytoplasma strain might cause significant yield losses to a wide range of crops, and because it can be transmitted by leafhopper vectors it poses a serious socio-economic threat to commercially grown chilli and other important agricultural crops in India.

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