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'Candidatus Phytoplasma' belonging to the 16SrVI phytoplasma group, is associated with witches broom disease of *Azadirachta indica* in India

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Abstract

Five samples from neem trees exhibiting witches broom (NeWB) symptoms were collected from the Raichur district, Karnataka State, India. The identity of the phytoplasma associated with all five neem samples was confirmed through PCR using phytoplasma 16Sr RNA gene specific universal primers. The amplified products were cloned, sequenced and nucleotide (nt) sequence comparisons were made with published phytoplasmas 16S rRNA gene nt sequences available at NCBI database. The 16Sr RNA gene nt sequence of NeWB phytoplasma had 99 to 99.8% identity with '*Candidatus* Phytoplasma' group (16SrVI) isolates reported from different parts of the world. This was supported by the close clustering of NeWB phytoplasma in the current study with members of clover proliferation group-16SrVI in the phylogenetic analysis. The virtual RFLP pattern generated for the phytoplasma from neem was identical (similarity coefficient 1.00) to the reference pattern of 16Sr group VI and subgroup D (Brinjal little leaf-16VI-D, NCBI Ac.No.: X83431). The analysis further confirmed that the phytoplasma associated with NeWB disease of neem belongs to 16Sr group VI and subgroup 16Sr IV-D. This is the first report of '*Candidatus* Phytoplasma' belonging to the 16SrVI phytoplasma group associated with witches broom disease of *Azadirachta indica* from India.

Keywords Neem · 16SrIV · PCR · Phylogenetic analysis · Witches broom disease

Neem (*Azadiracta indica*) belongs to the family *Meliaceae* and is native to the Indian subcontinent. It is one of the most important versatile, evergreen, multipurpose plant species in the arid and semi-arid tropics and is ideal for reforestation programmes and for rehabilitating degraded, semiarid and arid lands (Stoney 1997). The bark yields tannin and gum, which are used in textiles and traditional medicines. Neem oil is used in making products such as soaps, shampoos and toothpaste. Twigs have long been used for cleaning teeth and treating skin infections. The use of neem as a medicinal herb dates back over to 5000 years and is extensively used in Ayurveda, Unani and Homoeopathic medicine. More than 140 biologically active compounds that are chemically diverse and structurally complex have been identified from different parts of neem tree, with antifungal, anti-bacterial, anti-viral, anti-malarial, anti-oxidant, anti-mutagenic, anti-carcinogenic, contraceptive and anti-ulcer activity (Subapriya and Nagini 2005). Neem extracts have anti-viral activity against poliovirus, HIV, coxackie B group virus, and dengue virus (Badam et al. 1999; SaiRam et al. 2000; Parida et al. 2002; Tiwari et al. 2010). In spite of its well-known properties, neem is not free from microbial attack and is reported to be infected by a number of fungal and bacterial pathogens (Girish and Shankara Bhat 2008). In the present study, we characterize '*Candidatus* Phytoplasma' belonging to the 16SrVI phytoplasma group, associated with witches broom disease of neem from India.

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Fig. 1 Neem plant showing partial (**a**) and complete (**b**) witches' broom/little leaf symptoms under natural condition

During August 2017, a leaf sample and a small twig from each of five neem trees exhibiting unusual symptoms of witches broom and non-symptomatic sample from a healthy neem tree were collected from Raichur district, Karnataka, India (Fig. 1). To confirm the association of phytoplasma with witches broom symptoms in neem trees, total genomic DNA was isolated from the leaf midribs and bark of twigs from both symptomatic and symptomless samples using CTAB method (Doyle and Doyle 1990). The DNA isolated from a known phytoplasma (16SrVI group) (Brinjal) was used as positive control. The genomic DNA samples were tested for phytoplasmas by PCR using universal primers P1/P7 (Deng and Hiruki 1991**)** followed by R16mF2/R16mR1 (Gundersen and Lee 1996) nested primers as previously described by Venkataravanappa et al. (2017). The resulting PCR amplicons of 1.8 kb and nested PCR amplicons of 1.4 kb corresponding to the phytoplasma 16S ribosomal RNA were obtained. There was no amplification in the sample collected from non-symptomatic neem trees. The amplified PCR products of P1/P7 primers derived from five neem samples were cloned into pTZ57R/T cloning vector according to the manufacturer's instructions (MBI Fermentas, Germany). The transformation was performed using *Escherichia coli* (DH5α) cells. Two positively confirmed clones from each sample (total 10 clones) containing 16S ribosomal DNA of phytoplasma were sequenced using the automated DNA sequencing facility at Eurofins Genomics India Pvt. Ltd., Bangalore, India. The 16S rRNA gene sequence analysis showed that all the 10 clones (two clones from each sample) shared 99.9% of nucleotide identity among themselves. Therefore, the sequence of one selected clone (NeWB -1) was deposited in GenBank (GenBank Accession number MF996472).

A sequence similarity search was performed by using BLASTn Program (http://www.ncbi.nlm.nih.gov/ BLAST) and the sequences which showed maximum scores were selected for further analysis. The sequence identity matrixes were generated using Bioedit Sequence Alignment Editor (version 5.0.9) **(**Hall 1999**).** *In-silico* RFLP patterns were generated from the phytoplasma 16S ribosomal sequence using the gel plotting program pDRAW32 (http://www.acaclone.com/) and a phylogenetic tree was constructed using the neighbour-joining method of MEGA 7 (Kumar et al. 2016) with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously.

The 16S rRNA gene sequence of NeWB phytoplasma (NeWB, GenBank Accession Number MF996472) from Karnataka obtained in the current study was compared with 13 16S rRNA gene sequences of phytoplasma belonging to the '*Candidatus* Phytoplasma' group (16SrVI) and 42 16S rRNA gene sequences of different phytoplasmas available in the database (Table S1). The 16S rRNA gene sequence of NeWB phytoplasma shared maximum nt identity of 92.5 to 99.8% with Brinjal little leaf-16VI-D (X83431, AF228052, EF186820), Periwinkle little leaf-16VI-D [AF228053], Potato witches-broom-16VI-A [AB076404], '*Ca.* Phytoplasma trifolii'-16VI-A [AB279597, EU573925], Clover proliferation-16VI-C [AF409069], *Fragaria multicipita*-16VI-B [AF036354], *Centaurea solstitialis* virescence-16VI-E [AY270156], *Catharanthus* phyllody, *Fragaria multicipita*-16VI-G [AF190225], and Passion fruit witches-broom-16SrVI-I [GU292081] (Table S1) all belonging to distinct subgroup lineages in the '*Candidatus* Phytoplasma' group (16SrVI) reported worldwide (Wei et al. 2008). Based on the classification of phytoplasma groups and subgroups, the 16S rRNA gene nt identity between two distinct groups of phytoplasma should be ranged from 88 to 94% (Lee et al. 1993; 2000**)**. Since the 16S rRNA gene nt sequence similarity of NeWB phytoplasma with members of clover proliferation (16SrVI group is above the threshold level of 94%), it is

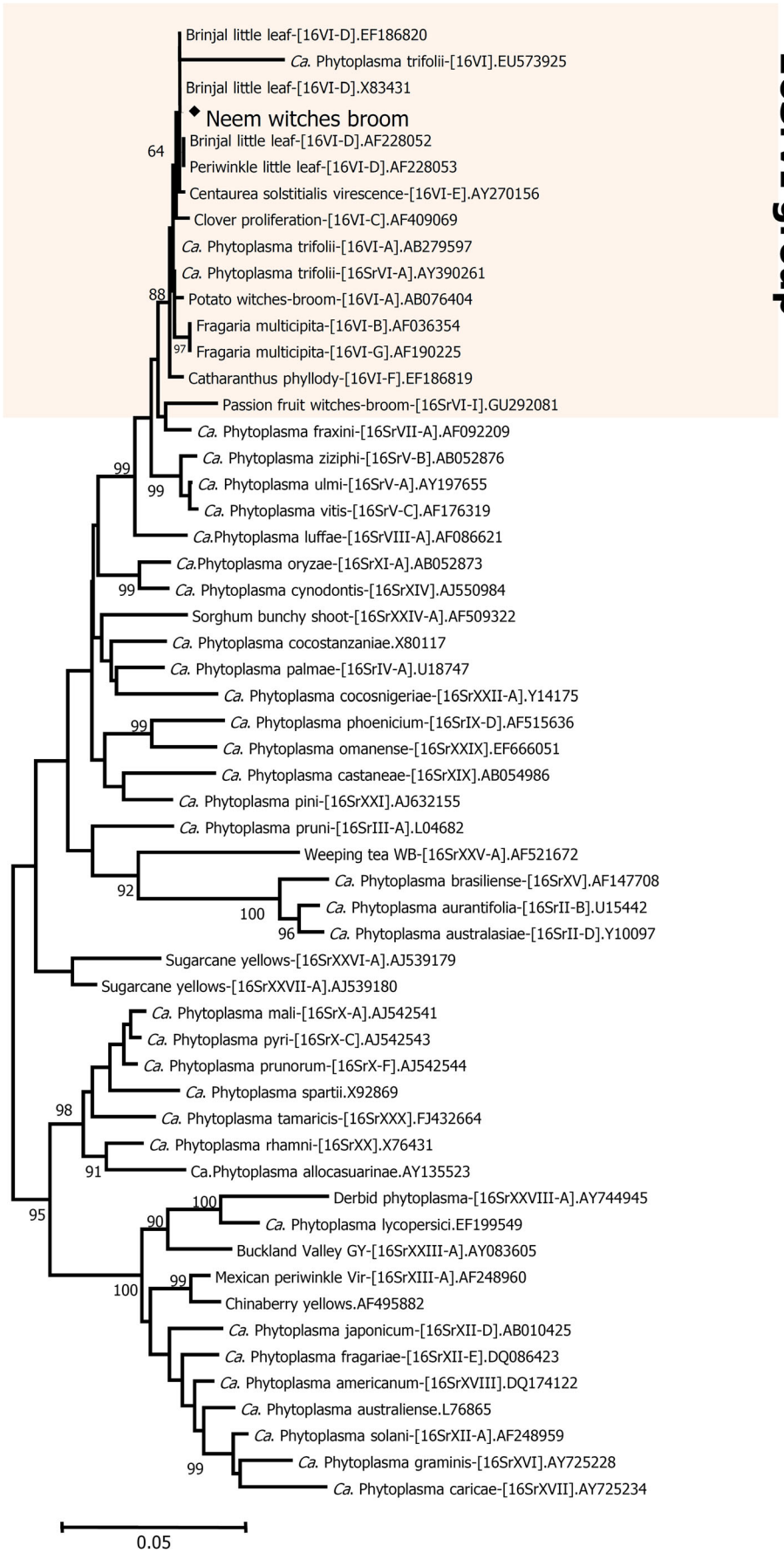

Fig. 2 Phylogenetic tree based on sequences of 16SrRNA gene of neem phytoplasma with other phytoplasma strains using Neighbor-joining algorithm. Horizontal distances are proportional to sequence distances, vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1000 replicates was performed and bootstrap percentage values more than 50 are numbered along the branches

proposed that the NeWB phytoplasma should be regarded as a member of the '*Candidatus* Phytoplasma' -16SrVI group.

The phylogenetic analysis showed that the phytoplasma associated with WB disease of neem in Karnataka (India) formed a new phylogenetic branch within the 16SrVI group cluster along with Brinjal little leaf-16VI-D (X83431, AF228052, EF186820), Periwinkle little leaf-16VI-D [AF228053], Potato witches-broom-16VI-A [AB076404], '*Ca.* Phytoplasma trifolii'-16VI-A [AB279597, EU573925], Clover proliferation-16VI-C [AF409069], *Fragaria multicipita*-16VI-B [AF036354], *Centaurea solstitialis* virescence-16VI-E [AY270156], *Catharanthus* phyllody, *Fragaria multicipita*-16VI-G [AF190225], and Passion fruit witches-broom-16SrVI-I [GU292081], i.e., belongs to the distinct subgroup lineages within the clover proliferation phytoplasma group (16SrVI) (Fig. 2).

In-silico RFLP analysis of the F2nR2 fragment of 16S rDNA sequence of NeWB phytoplasma using the online tool iPhyClassifier indicated that the virtual RFLP pattern derived from the query of the F2nR2 fragment of 16Sr RNA sequence of NeWB-1 phytoplasma was identical (similarity coefficient 1.00) to the reference pattern of 16Sr group VI and subgroup D (GenBank Accession number X83431) (Lee et al. 1998; Wei et al. 2007). Therefore, this NeWB phytoplasma belongs to the clover proliferation group 16SrVI and subgroup-D. This is the first report of a 16SrVI '*Candidatus* Phytoplasma' affecting neem from India.

In India, 16SrVI phytoplasmas have previously been associated with diseases in several other hosts and subgroup D has specifically been associated with brinjal little leaf, *Catharanthus roseus* little leaf, leaf yellowing and phyllody of *Hibiscus rosa-sinensis* and witches' broom of *Saponaria officinalis*, and *Allamanda cathartica* (Khasa et al. 2016). This is the first report of association of 16SrVI subgroup D phytoplasma with the witches broom disease on neem from India or worldwide. The detection is alarming given the medical and ecological importance of this native tree to India. The results also show that the host ranges of 16SrVI '*Candidatus* Phytoplasma', especially subgroup D, are expanding in India.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

Human and animal rights This article does not contain any studies with human or animal subjects performed by any of the authors.

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