Arch Biol Sci. 2017;69(4):707-714 https://doi.org/10.2298/ABS170223017V

Detection and characterization of '*Candidatus* Phytoplasma asteris' associated with littleleaf disease of bitter gourd from India by 16S rRNA phylogenetic and RFLP (*in vitro* and virtual) analysis

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Received: February 23, 2017; **Revised**: May 7, 2017; **Accepted**: May 10, 2017; **Published online**: June 12; 2017

Abstract: Bitter gourd plants showing symptoms of little leaf disease are prevalent in farmers' fields in the Bangalore rural district, Karnataka state, India. Twenty leaf samples from different locations were collected to determine the etiology of the disease. Using PCR and phytoplasma 16S rRNA gene-specific universal primers, we observed positive amplification for the phytoplasma specific primers in five out of twenty samples. The amplified products were cloned, sequenced and nucleotide (NT) sequence comparisons were made with the available phytoplasmas' 16S rRNA gene NT sequences in the NCBI database. The 16S rRNA gene NT sequence of bitter gourd phytoplasma shared highest identity of 81.7-96.0% with '*Candidatus* Phytoplasma asteris' (*Ca.* P. asteris) 16Sr I group isolates from different parts of the world. This was supported by close clustering of phytoplasma of the current study with the *Ca*. P. asteris 16Sr I subgroup by phylogenetic analysis. The virtual restriction fragment length polymorphism (RFLP) pattern generated for the Phytoplasma from bitter gourd was in congruence with the *in vitro* RFLP pattern for the six enzymes. This was typical to *Ca*. P. asteris from the 16Sr I group. Further, virtual RFLP analysis with 11 more enzymes used for RFLP pattern prediction revealed differences only in the Mse I RFLP pattern, with a similarity coefficient of 0.91, which is less than the threshold similarity coefficient for a new subgroup. We propose that the phytoplasma detected in the present study that infects bitter gourd and causes littleleaf disease should be considered as a new subgroup of group 16Sr I (*Ca*. P. asteris). This is the first report of phytoplasma associated with littleleaf disease of bitter gourd from India.

Key words: bitter gourd, phytoplasma, group 16SrI, PCR, *in silico* analysis

INTRODUCTION

Bitter gourd (*Momordica charantia* L.) belongs to the family Cucurbitaceae. This plant is one of the most important vegetables grown in tropical and subtropical regions of Southeast Asia, Africa and Caribbean countries [1]. It is extensively cultivated in all states of India [2]. The fruits are consumed as vegetables that are very low in calories and provide an excellent source of vitamins, minerals and high dietary fiber. The fruits contain an insulin-like polypeptide that has been suggested as an insulin replacement in diabetic patients [3]. The leaves, fruits and roots are traditionally used among Indian people to treat fever [4], in reproductive health as an abortifacient birth control agent, to treat painful menstruation and to facilitate child birth [5,6]. The bitter gourd has antiviral [7] and antimalarial activities [8]. Because of its considerable medicinal properties, the demand for bitter gourd has increased [9].

How to cite this article: Venkataravanappa V, Narasimha Reddy LRC, Polam S, Subbanna SK, Manem Krishna R. Detection and characterization of '*Candidatus* Phytoplasma asteris' associated with littleleaf disease of bitter gourd from India by 16S rRNA phylogenetic and RFLP (*in vitro* and virtual) analysis. Arch Biol Sci. 2017;69(4):707-14.

Areas under cultivation of this crop are decreasing worldwide due to many abiotic and biotic stresses. Among the different biotic factors, major constraints resulting in crop loss are due to viral [10] and phytoplasma diseases [11, 12]. Different phytoplasma groups associated with bitter gourd disease include witches' broom phytoplasma, described for the first time by electron microscopy [13], X disease caused by '*Candidatus* Phytoplasma pruni' and *Ca*. P. asteris in Taiwan [11], phyllody disease caused by aster yellows phytoplasma (AYP) identified by RFLP analysis in Thailand [14], and littleleaf phytoplasma in Myanmar caused by *Ca.* P. asteris and identified based on 16S rRNA, ribosomal protein (RP), protein translocase subunit (secY) gene and RFLP analysis [12].

Phytoplasma have an uneven distribution in a plant's system, normally inhabiting the phloem at low concentrations. This characteristic makes their detection and identification difficult [15], and was a major concern until the use of PCR assays with universal primers derived from conserved 16S rRNA gene sequences [16-18,38]. PCR has proven to be a more sensitive and convenient method for the diagnosis of phytoplasma than other methods, such as electron microscopy, serology and nucleic acid hybridization assays. An effective approach to detect and classify phytoplasma was by targeting a highly conserved region of 16S rRNA and the spacer region between the 16S and 23S rRNA genes [19,20-22]. Computer-simulated RFLP analysis of the 16S rRNA gene was used as a marker for the classification of phytoplasma into different groups and subgroups [23,24]. Based on the analysis of 16S rRNA gene sequences, 31 groups and 100 subgroups of diverse phytoplasmas were identified [25-27]. From India, 16SrI, 16SrII, 16SrV, 16SrVI, 16SrIX, 16SrXI and 16SrXIV groups were reported. The aster yellow group (16SrI) was reported in northeastern parts of India, either alone or associated with more than 31 diseases of different crop plants [28]. However, there are no reports of phytoplasma associated with bitter gourd in India. Here we report on the symptoms, describe molecular detection and characterize the 16Sr I *Ca.* P. asteris group associated with bitter gourd littleleaf (BitLL) disease in India.

MATERIALS AND METHODS

Disease incidence and plant material

The systematic roving survey was conducted during 2014-2015 in ten different farmers' fields in the Bangalore rural district, Karnataka state, to estimate the incidence of littleleaf phytoplasma disease on bitter gourd. The number of infected plants (plants showing littleleaf, mosaic, leaf curl, vein thickening, shorter internal length, perfuse flowering and bushy appearance) and total plants were counted by moving diagonally across rows in the fields in order to obtain the disease incidence. Twenty infected leaf samples were collected; one leaf sample from each location was without any symptoms and served as the negative control.

Phytoplasma detection

PCR

Total nucleic acid was isolated from infected (20) and healthy leaf samples by the cetyl trimethylammonium bromide (CTAB) method [29]. The DNA isolated from known phytoplasma (sesame and brinjal) was used as a positive control, and from samples without symptoms it served as a negative control. The DNA pellets were dried under vacuum, resuspended in 50 µL of Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0) and maintained at -20°C until use. The total DNA isolated from bitter gourd samples was amplified by PCR using phytoplasma 16S rRNA gene-specific universal primer pairs (P1/P7) [19,30] and nested primer pairs (R16F2n/R2) [22,20]. The first round of PCR and nested PCR were carried out sequentially for phytoplasma detection. DNA amplification was performed with 35 cycles of denaturation for 1 min at 94°C, primer annealing for 45 s at 55°C, and primer extension for 1 min at 72°C, with an initial denaturation at 94°C for 3 min and a final extension for 10 min at 72°C. The PCR reactions were carried out in a Gene Amp PCR system 9700 (PE Applied Biosystems, Foster City, CA) thermocycler. All amplifications were performed in 25 µL that contained the PCR mix containing 2 µL DNA template, 1.5 U Taq DNA polymerase, 25 mM $MgCl_2$, 2 mM dNTPs and 20 pmol of each primer. PCR products

were separated by electrophoresis (1 h at 80 V) in 0.8% agarose gels in Tris-borate-EDTA buffer, pH 8. Gels were stained with ethidium bromide (10 mg/mL) and viewed in a gel documentation system (Alpha Innotech, USA). The amplified nested PCR product (1.2 kbp) was purified from agarose gels using the Qiaquick Gel Extraction kit (Qiagen, Hilder, Germany) and cloned into a pTZ57R/T cloning vector according to the manufacturer's instructions (MBI Fermentas, Germany). The transformation was performed using *Escherichia coli* (DH5α) cells. The transformed plasmid was isolated using the Qiagen Plasmid Miniprep kit (Qiagen, Hilder, Germany) and sequenced using the automated DNA sequencing facility at Eurofins Genomics India Pvt. Ltd, Bangalore, India.

RFLP analysis

The 16Sr RNA gene products of five BitLL phytoplasma samples amplified with R16F2n/R2 primer pairs (~1.2 kbp) were digested with different restriction enzymes (*Alu*I, *Eco*RI, *Hha*I, *Hae*III, *Rsa*I and *Taq*I) separately, according to the manufacturer's instructions (Fermentas, Germany). The digested DNA products were analyzed by electrophoresis in a 2% agarose gel with 0.5xTBE as running buffer. DNA bands were visualized with a UV transilluminator after gels were stained with ethidium bromide.

Sequence analysis

To assess the taxonomic position of BitLL phytoplasma, the ~1.2 kbp PCR product of 16S rRNA gene NT sequence (the conserved motifs) that correspond to the annealing sites of universal 16S rRNA primer pair R16F2n/R16R2 derived from BitLL phytoplasma was subjected to the iPhyClassifier online tool (http://www.plantpathology.ba.ars.usda.gov/cgibin/resource/iphyclassifier.cgi) [31,32]. Further, the sequence was subjected to a BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn) NCBI search for similar sequences in the database (January 10, 2017). The related sequences obtained from the database were aligned using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) method implemented in the SEAVIEW program [33]. The NT sequence identity matrices for the bitter gourd phytoplasma were generated using Bioedit Sequence Alignment Editor (version 5.0.9) [34]. The phylogenetic tree was constructed by the neighbor-joining method using MEGA ver. 6.01 software [35] with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously.

Computational virtual RFLP analysis

In silico restriction analysis and virtual RFLP plotting of the R16F2n/R2 fragment of BitLL phytoplasma were carried out using pDRAW32 [36] and iPhyClassifier software [31]. The sequence of the R16F2n/R2 fragment of BitLL phytoplasma was digested with 17 different restriction enzymes (AluI, BamHI, BfaI, BstUI (ThaI), DraI, EcoRI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, Sau3AI (MboI), MseI, RsaI, SspI and TaqI) used for the classification of phytoplasma and were plotted in a virtual 3.0% agarose gel. The phytoplasmas were differentiated based on the 16S rRNA gene by RFLP analysis using endonuclease restriction enzymes [36] as the RFLP pattern of each phytoplasma is conserved. The virtual RFLP patterns with the enzymes that can be distinguished from previously recognized group/subgroup patterns were made in iPhyClassifier (http://www.ba.ars.usda.gov/data/mppl/).

RESULTS

Detection of phytoplasma

By visual inspection, the most commonly observed symptoms at the flowering stage are mosaic and leaf curl, with a few plants exclusively showing littleleaf disease symptoms. The leaves were reduced in size and closely arranged on the top of the stem with very short intermodal length giving the appearance of a broom (Fig. 1). All floral parts were converted into dark green leaf-like structures, floral proliferation, floral virescence and shortened petioles. A disease incidence of 10-15% was recorded.

Five of the twenty bitter gourd samples (BG1, BG2, BG3, BG4, BG5) showing littleleaf disease were confirmed for phytoplasma infection by PCR; no amplification was observed in any of the nonsymptomatic leaf samples. The ~1.2 kb PCR amplified products

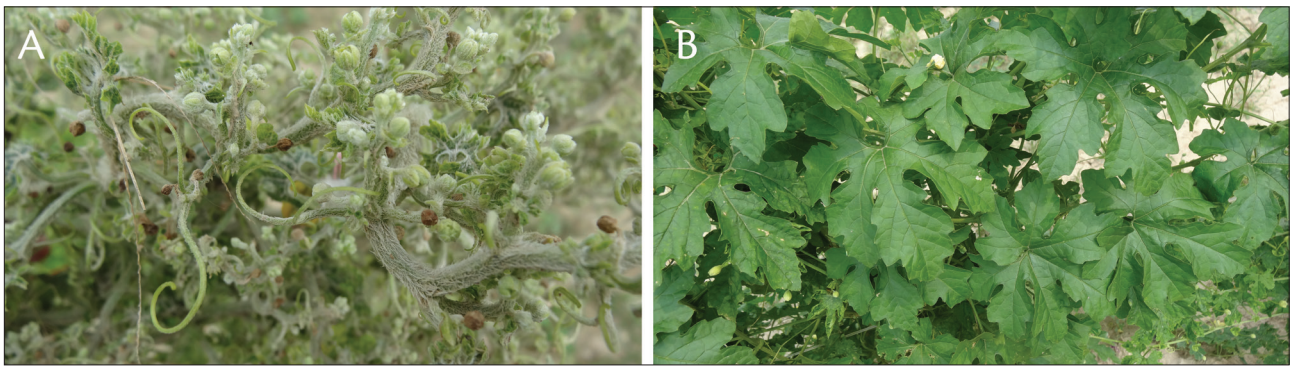


Fig. 1. Bitter gourd plant; **a** – littleleaf symptoms under natural conditions, **b** – healthy plant.

from five phytoplasma infected bitter gourd samples were cloned and sequenced. All five sequences (BG1, BG2, BG3, BG4, BG5) of the 16S rRNA gene were identical. One representative sequence (BG1) was deposited in the GenBank (Accession No. KX179474).

16S rRNA gene sequence analysis

Sequence alignment

The 16S rRNA gene sequence of BitLL phytoplasma from Karnataka (BG1) obtained in the study was compared with 74 16S rRNA gene sequences of *Ca.* P. asteris (16SrI) and 38 16S rRNA gene sequences of different phytoplasmas available in the database on the date of search (January 10, 2017). The 16S rRNA gene sequence of BitLL-1 phytoplasma shared a maximum NT identity ranging from 81.8% (*Periwinkle virescence*, EU371934) to 96.0% (periwinkle proliferation, FJ008869), with phytoplasma belonging the *Ca.* P. asteris group (16SrI). Within the 16SrI group, the 16S rRNA gene of BitLL phytoplasma shared NT identity of 94.4-95.8% with the following phytoplasmas: Brinjal littleleaf (JQ518317, JQ518318), sandal spike (EF198362) and *Withania somnifera* (DQ151998), all identified in India. Based on the classification of phytoplasma groups and subgroups, the 16S rRNA gene NT identity between two distinct groups of phytoplasma should range from 88 to 94% [20,37]. Since the 16S rRNA gene NT sequence similarity of BitLL phytoplasma with members of 16SrI (*Ca.* P. asteris) was above the 94% threshold level, we propose that BitLL phytoplasma is regarded as a member of *Ca.* P. asteris (16Sr I group).

Phylogeny

The phytoplasma 16S rRNA gene sequence identified from bitter gourd was compared with 74 sequences of phytoplasma that belong to the *Ca.* P. asteris (16SrI) group. The analysis showed that bitter gourd littleleaf (BitLL) phytoplasma was closely grouped with black-eyed Susan phyllody 16SrI-A (DQ855286) and black pepper phyllody 16Sr I (AY823413) of *Ca.* P. asteris (16SrI) (Fig 2). The Indian bitter gourd infecting phytoplasma form a monophyletic cluster with phytoplasmas of Asian-Iran origin and established a close relationship with 16Sr I-A.

RFLP analysis of 16Sr RNA gene

The PCR amplified F2n/R2 fragments of five BitLL phytoplasma samples were digested with restriction endonucleases used previously in the classification of phytoplasma and differentiation of the *Ca.* P. asteris 16Sr I group and subgroup (AluI, EcoRI, HhaI, HaeIII, RsaI and TaqI) [23,32,36]. The restriction patterns of the five BitLL phytoplasma samples were the same for all six enzymes (Fig. 3) and were typical for the *Ca.* P. asteris 16Sr I group, indicating that the phytoplasma associated with bitter gourd belongs to this group

Virtual RFLP analysis

The virtual RFLP pattern with 17 enzymes was obtained for the BitLL phytoplasma and compared with the earlier reported virtual RFLP patterns for phytoplasma. The result revealed that 16 enzymes produced

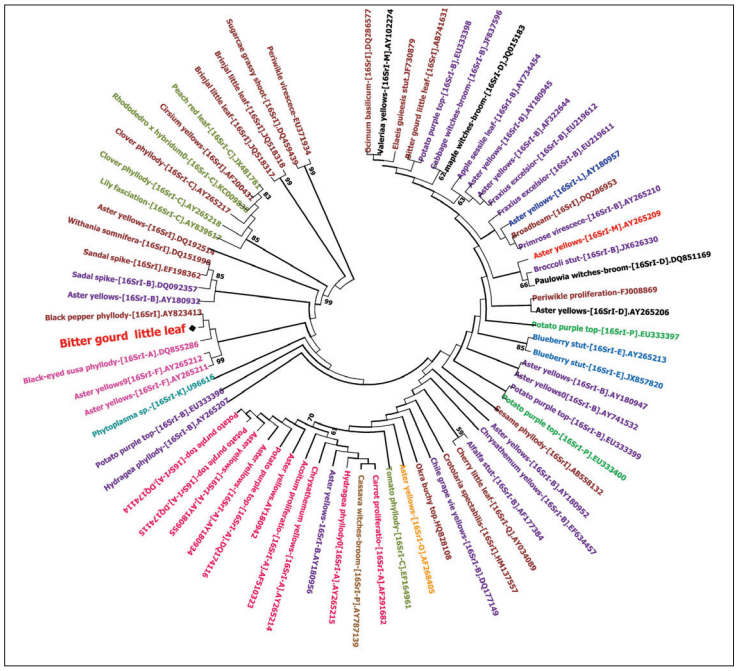
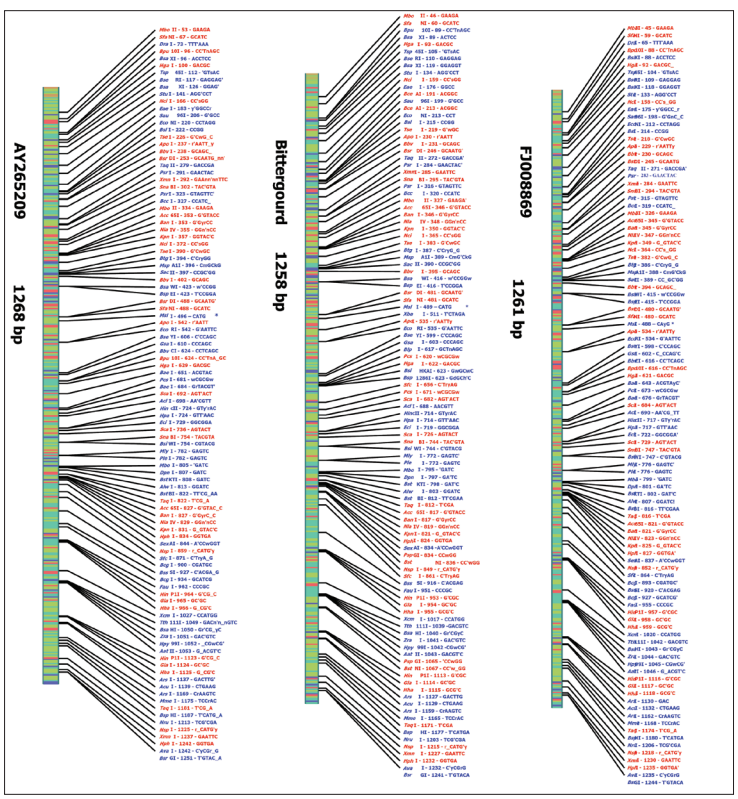


Fig 2. Phylogenetic tree based on nucleotide sequences of the 16S rRNA gene belonging to the phytoplasma 16SrI group with other phytoplasma by the neighbor-joining algorithm. Horizontal distances are proportional to sequence distances, vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1000 replicates was performed and the bootstrap percentage values for more than 50 are numbered along the branches.



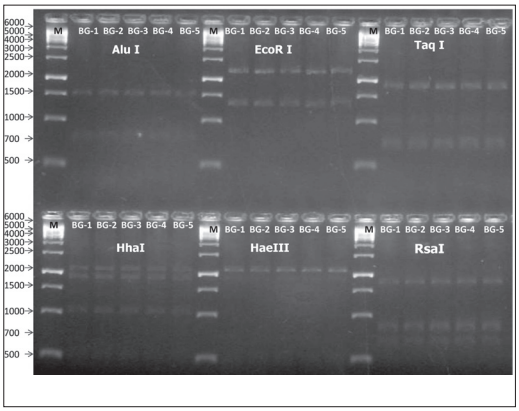


Fig 3. RFLP patterns of the 16S rRNA gene from Indian bitter gourd littleleaf phytoplasma amplified by PCR using R16F2/R16R2 primer pair. DNA products were digested with restriction endonucleases AluI, EcoRI, Taq1, HhaI, HaeIII and RsaI. Lane M – molecular marker 1kb ladder; lanes 1-5 – BG1-5 (samples from bitter gourd plants infected with phytoplasma by phytoplasma-specific PCR.

a virtual RFLP pattern that was similar to the *Ca*. P. asteris 16Sr I group of phytoplasma. The six enzymes, AluI, EcoRI, HhaI, HaeIII, RsaI and TaqI, commonly used in *in vitro* and virtual RFLP analysis, also produced the same pattern. Although BitLL phytoplasma showed maximum NT identity (96%) with periwinkle proliferation (FJ008869) of the *Ca*. P. asteris (16SrI) group, the results of *in silico* restriction digestion and gel plotting separated this group with respect to MseI. This was further supported by the restriction map of BitLL phytoplasma obtained from pDRAW32 (AcaClone Software; http://www.acaclone.com) analysis that showed significant differences in MSeI with periwinkle proliferation (FJ008869) and other 9 representatives of subgroups (16Sr I – A, B, C, D, E, F, M, P, Q, R, T) (Fig. 4).

Fig 4. Comparative analysis of virtual restriction sites in the 16S rRNA gene. 16S rRNA gene sequences of phytoplasma from bitter gourd (Accession No. KX179474), Aster yellow phytoplasma (AY265209), periwinkle proliferation phytoplasma (FJ008869). * highlights the important difference in the restriction site.

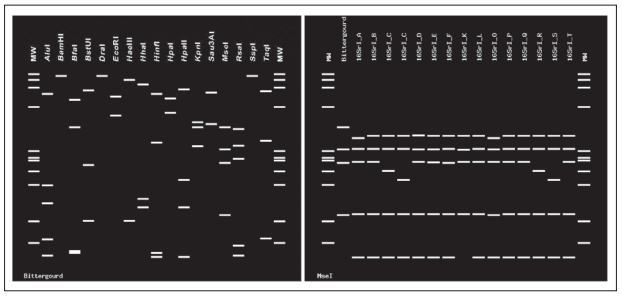


Fig 5. Virtual RFLP patterns obtained by *in silico* digestion using iPhyClassifier for the R16F2/R16R2 fragment of the 16S rRNA gene from bitter gourd littleleaf phytoplasma (BitLL). Virtual RFLP patterns of BitLL; MseI was used to distinguish between Indian phytoplasmas and other strains in group 16SrI. The restriction fragments were resolved by *in silico* electrophoresis in a 3% agarose gel. M – virtual molecular ladder from the WX174 DNA HaeIII digest (1353, 1078, 872, 603, 310, 271, 281, 234, 194 and 118 bp).

The BitLL phytoplasma yielded a distinct RFLP profile with MseI (similarity coefficient was 0.91) when compared with closely related (on the basis of NT) phytoplasmas, periwinkle proliferation and aster yellow of group 16Sr I (Fig 5). The similarity coefficients of 16S rRNA gene RFLP patterns were used to classify phytoplasma into groups and subgroups, with the threshold similarity coefficient for new subgroup delineation set at 0.97. A strain is treated as new if its virtual 16S rRNA gene RFLP pattern similarity coefficient is less than or equal to 0.97 with all existing representative strains of the given group [23, 36]. Since the strain under investigation showed a similarity coefficient of 0.91, this BitLL phytoplasma can be considered as a new subgroup within the 16Sr group I. This is the first report of a 16Sr I phytoplasma affecting bitter gourd in India.

DISCUSSION

The diseases caused by phytoplasma are emerging as a major constraint for the production of several crops across the globe [44]. Phytoplasma belonging to the 16S rRNA that infects bitter gourd has been well documented in Asia [11,12,14]. However, from India there were no reports about its prevalence on bitter gourd. A survey for littleleaf disease of bitter gourd based on the visual symptoms revealed its presence in Karnataka. Investigations based on PCR detection using universal primers specific to phytoplasma confirmed its association in 25% of analyzed plants that showed disease symptoms, indicating expansion of the host range of *Ca*. P. asteris, in addition to the already reported expansion to host plants, Luffa and cotton [39], brinjal [40], sandalwood and *Withania somnifera* in India, *Cucumis sativus* L. in Taiwan, carrot, cabbage, beetroot and tomato in Cuba [1,41,42] and *Sechium edule* (Jacquin) Swartz in Costa Rica [43].

The 16S rRNA gene sequence and virtual RFLP analysis suggests that BitLL phytoplasma from Karnataka that was associated with *Ca*. P. asteris belongs to the 16Sr I group, based on existing classification criteria [17]. This was well supported by phylogenetic analysis that showed a close clustering of phytoplasma with the *Ca*. P. asteris 16Sr I group. The NT sequences of unique regions of the 16S rRNA gene, 5'-GGGAGGA-3' (positions 226-232), 5'-CTGACGGTACC-3' (positions 476-485) and 5'-CACAGTGGAGGTTATCAG TTG-3' (positions 1008- 1028) of BitLL phytoplasma are identical to *Ca.* P. asteris 16Sr I group [17].

The RFLP patterns of each phytoplasma are conserved and unknown phytoplasmas are identified by comparing the patterns of unknown with available RFLP patterns of known phytoplasmas, without coanalyses of all reference representative phytoplasmas [24,36]. This provides a reliable means for the differentiation of a broad array of phytoplasmas and has become the most comprehensive and widely accepted phytoplasma classification system [44].

In vitro restriction analysis of five BitLL phytoplasma samples collected from different farmers' fields obtained by digesting the F2n/R2 fragment using six restriction enzymes that are used to classify phytoplasma into groups and subgroups [36] produced a similar pattern, typical to *Ca*. P. asteris, which belongs to the 16Sr I group. The virtual RFLP pattern generated for the phytoplasma from bitter gourd was also congruent with the *in vitro* RFLP patterns obtained by the six enzymes. This suggests that computer-simulated RFLP analysis of the 16S rRNA gene and the calculation of similarity coefficients can serve as a tool for the delineation and finer differentiation of members of *Ca*. P. asteris from the 16Sr I group into subgroups [24]. In addition, virtual RFLP analysis was conducted for 11 more enzymes. It revealed differences only for the

MseI RFLP pattern (a similarity coefficient of 0.91) and 16Sr I phytoplasma associated with brinjal little-leaf, sandal spike and *Withania somnifera* phytoplasma from India [1,41,42].

This study provides the first evidence that BitLL disease from Karnataka is related to *Ca*. P. asteris belonging to 16Sr I. The outcome of the study with respect to symptomatology, detection, diversity analysis, *in vitro* and virtual restriction analysis will provide invaluable basic knowledge and improved understanding of the diseases of various crops caused by phytoplasmas. This should pave the way for a better understanding of disease resistance, epidemiology and host-vector interactions, and lead to the design of novel disease management strategies.

Acknowledgments: The research was supported by the project "Consortium platform on Vaccines and diagnostics", funded by Indian Council of Agricultural Research, Government of India, New Delhi, India.

Authors' contribution: We declare that all authors participated in the research and article preparation. VV design of the experiments and drafted the article. CNLR designed the study, participated in the acquisition and analysis of the data and revision of the manuscript, PS participated in the collection of data. KSS revised the manuscript. MKR guided and designed the study and revised the article. All authors approved the submitted version of the article.

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

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