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Research Article

Clarkia unguiculata: A New Plant Host of *Candidatus* Phytoplasma asteris (16SrI-B) Related Strain

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Abstract

Suspected phytoplasma symptoms of flat stem, witches'-broom, little leaf, and apical fasciation were observed on *Clarkia unguiculata* plants in March, 2022 at the Division of Plant Pathology, IARI. The plant samples (both symptomatic and asymptomatic) and leafhopper species were collected and subjected to nested PCR assays using universal primer pairs (P1/P7 followed by R16F2n/R2). All of the symptomatic samples yielded PCR amplicons of the expected 1.2 kb size for nested reaction. The only leafhopper species identified was *Hishimonus phycitis*, which likewise tested positive in PCR assays with the anticipated amplicon of 1.2 kb using the same set of phytoplasma specific primers as used for the plant samples. For further confirmation a multilocus gene, secA gene (480bp) was also amplified using universal primers. Nucleotide sequence identities of 100% with 16S rRNA and 100% with secA gene was found with strains of aster yellows group (16SrI-B). This is the first report of the phytoplasma association with *Clarkia unguiculata* in the world.

Keywords: Ornamental; Multilocus Gene; secA; 16S rRNA; North India

Introduction

Clarkia unguiculata, an annual flowering plant in the Onagraceae family, often known as "Elegant clarkia" or "mountain garland," is well-known for its beautiful flowering of thin, diamond-shaped blooms in the colours of crimson, purple, pink, and white. Californian woods are home to this unusual shrub.

Phytoplasmas are a group of obligate plant pathogenic bacteria that lack the cell wall and are found restricted to phloem sieve cells of plants and spread through vegetative propagation, insect vectors, and dodder in nature. The genetically diverse phytoplasmas have been classified into 37 groups and more than 150 subgroups and currently, 48 *Candidatus* Phytoplasma species have been named [1]. Among 37 groups of phytoplasmas reported so far, the 'Aster yellows' (AY) group of phytoplasmas affects the plants by causing a general reduction in quantity and quality of yield [2]. In many regions of the world, ornamental crops including aster, gladiolus, hydrangea, chrysanthemum, and purple coneflower are among the most severely impacted hosts by the AY group. Several leafhoppers species like *Macrosteles laevis, M. fascifrons*, *M. striiformis*, *M. quadripunctulatus*, *M. viridigriseus M. sexnotatus*, *Euscelis plebeja*, *E. incisus*, *E. lineolatus*, *Euscelidius variegatus*, *Aphrodes bicinctus*, *Colladonus montanus* and *C. geminatus*, *Hishimonoides sellatiformis*, *Dalbulus elimatus*, *Scaphytopius acutus* are reported to transmit AY group of phytoplasma leading to the disease incidence of varying range has been from 20 to 100% in different parts of the world in different crops [3-6]. *Neoaliturus haematoceps* and *Circulifer haematoceps* from Iran and Turkey [7,8]: *Orosius orientalis* from Iran, Turkey, and India [9,10]; and *O. albicinctus* (16SrII-D) from Iran and Pakistan [9,11]; *Hishimonus phycitis, Exitianus indicus*, *Orosius albicinctus* and *Amarasca biguttula* from India were also shown to be putative vectors and natural reservoirs of the 16SrI, 16SrII and 16SrVI groups of phytoplasma [12-14]. The current study was initiated to investigate the potential association between symptomatic plants and phytoplasma infection.

Materials and Methods

Survey, symptomatology, molecular diagnosis

During the month of March 2022, surveys of the garden, nurseries, and experimental fields at Indian Agricultural Research Institute, New Delhi, were conducted. Samples from five randomly chosen *C. unguiculata* plants showing flat stem symptoms were collected and named as flat stem New Delhi isolates (CuFND-1 to 5), another five plants showing witches'-broom symptoms and named as witches'-broom New Delhi isolates (CuWND-1 to 5) and collected five asymptomatic plants. The samples were stored at -20°C until further processed. The insect samples associated with *C. unguiculata* plants in and around the garden were collected using the sweep net, transferred to the polythene bags and stored for further identification and characterization. The insect samples (1 adult /sample) were subjected to morphological identification by an entomologist at division of entomology, IARI, New- Delhi and then used for DNA extraction using a DNeasy Blood and tissue kit (Qiagen, Germany). The method described by Ahrens and Seemuller in 1992 [15] was followed to extract DNA from the midrib portion of all the collected clarkia twigs.

All the DNA samples extracted were subjected to PCR assays using the universal phytoplasma primer pairs P1/P7 [16,17]. The second set of primer pairs R16F2n/ R16R2 were used to conduct further nested PCR reactions [18]. The previously characterized sesame phyllody phytoplasma strain [NCBI-Genbank accn. numbers OQ381047 (*16S rRNA*) and OQ420637 (*sec*A)] maintained on the *Catharanthus roseus* plants in the glasshouse at the Division of Plant Pathology, IARI, New Delhi was taken as a positive control for the PCR assays. The total volume of PCR (for amplifying the 16S rRNA gene fragment) mixture (25 µl) was made up of 1 µl of DNA template (100 ng/µl), 0.5 µl (20 pmol each of P1 and P7 primers (5'-AAGAGTTTGATCCTGGCTCAGGATT-3' / 5'-CGTCCTTCATCGGCTCTT-3), 1.0 unit of Taq DNA polymerase (GeneDireX, Inc.), 0.5 µl of dNTPs (0.2 mM), 3 µl of $MgCl_2$ (2.0 mM), and 1 µl of 1X PCR buffer. The first round of PCR was performed in a thermal cycler (Eppendorf, Germany), with an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 1 minute and extension at 72°C for 2 minutes, with a final extension for 10 minutes at 72 °C. The amplification products obtained from the direct PCR were diluted (1:30) as per standard protocol with nuclease-free water and 1 µl of the diluted product was used as a template in nested PCR assays using primer pair R16F2n/ R16R2 (5'-GAAACGACTGCTAAGACTGG-3' / 5'-TGACGGGCGGTGTGTACAAACCCCG-3), keeping all the PCR conditions same except for the annealing temperature that was raised by 1°C (56 °C). Ten microliters of direct and nested PCR products were analyzed by electrophoresis in 1.0% agarose gel and visualized by staining with GoodView™ Nucleic Acid stain (Beijing SBS Genetech Co., Ltd) under UV Gel Doc transilluminator (Azure Inc., USA). The 1.2 kb amplicons obtained from nested PCR were purified from gel using the Wizard® SV Gel and PCR Clean-up System (Promega, Madison, USA as per manufacturer's protocol).

For further confirmation of phytoplasma, protein translocase subunit A (*sec*A) gene universal primers *sec*Afor1/*sec*A rev3 (5'GARATGAAAACTGGRGAAGG3'/ 5'GTTTTRGCAGTTCCTGTCATCC3') followed by semi-nested PCR using *sec*Afor2/*sec*A rev3 (5'GATGAGGCTAGAACGCCT3'/ 5'GTTTTRGCAGTTCTGTCATCC3') were performed with the following PCR conditions: denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 1 min and extension at 72°C for 1 min. The last cycle was extended for an additional 10 min at 72°C [19]. The PCR conditions were the same for both the first round and semi-nested *sec*A primers.

Molecular cloning, sequencing, and sequence analysis

PCR products of both the genes (*16S rRNA* and *sec*A*)* were gel purified and cloned separately into pGEMT® easy cloning vector in *Escherichia coli* (DH5α). The cloned products consisting of both the gene fragments were sequenced at AgriGenome Labs Pvt ltd, Kerala, India. The acquired sequences of both (*16S rRNA* and *sec*A) the gene fragments were assembled separately using the Bio-Edit biological sequence alignment editor program (https://bioedit.software.informer.com/7.2) and aligned using the ClustalW tool [20] in comparison with the sequences of phytoplasma group/subgroup representatives retrieved from the NCBI GenBank database. The consensus sequences of both the genes obtained from the representative strains of this study were submitted to NCBI-GenBank. The sequences of the study and the representative sequences from the NCBI GenBank database were used to construct the phylogenetic tree with MEGA 11 software [21] through the neighbour-joining method with 1000 bootstrap replications. The phylogenetic tree for *16S rRNA* sequences was rooted using the *16S rRNA* gene sequence of *Acholeplasma laidlawii* (GenBank Accn. no. AB680603) and of the *sec*A gene sequences using corresponding sequences of *Acholeplasma oculi* (LK028559). Further, all the *16S rRNA* gene sequences were subjected to the *i*PhyClassifier online tool (22) and *in silico* virtual RFLP analysis and compared with the representative group and subgroup sequences of phytoplasma strains in the database.

Results

Survey, symptomatology and molecular diagnosis

Flat stem, witches'-broom along with little leaf and apical fasciation symptoms (Figure 1) were observed on 5% of *C. unguiculata* plants in the gardens of Division of Plant Pathology, IARI, New Delhi. Using the nested PCR (R16F2n/R16R2) and semi-nested (*sec*Afor2/ *sec*Arev3) technique, the PCR amplicons of expected size i.e. 1.2 kb and 480 bp respectively, were obtained in all the symptomatic samples. The leafhopper species associated with the clarkia plants was identified as *Hishimonus phycitis* (HPCuND-1) (Distant 1908) which also tested positive in the PCR assays with the expected amplicons of 1.2 kb and 480 bp with similar set of phytoplasma-specific primers as used for the plant samples.

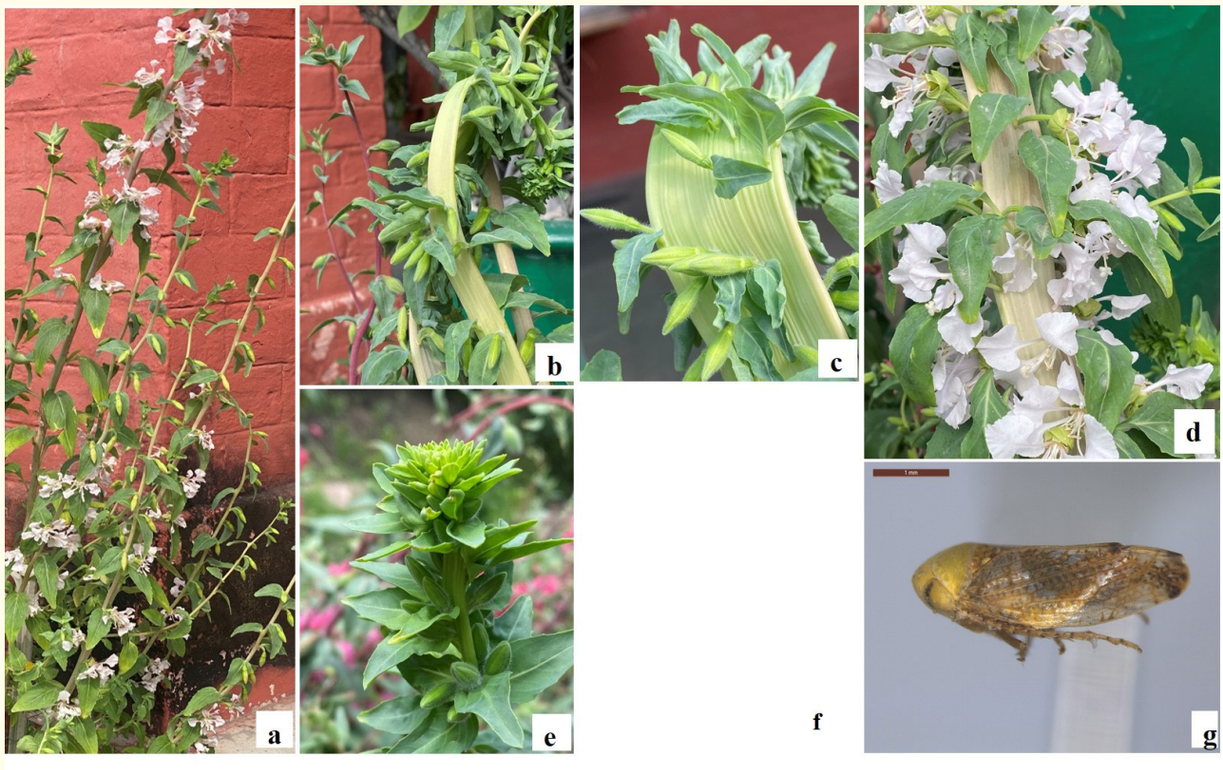

Figure 1: Different phytoplasma infection-like symptoms on clarkia plants. Healthy plant (a); flat stem and lodging (b-d); witches'-broom and little leaf (e); apical fasciation (f); and the adult individual of *Hishimonus phycitis* (g).

Molecular cloning, sequencing, and sequence analysis

The processed and aligned *16S rRNA* gene consensus sequences resulted in this study were published in the NCBI GenBank database under the accession numbers ON870385 (CuFND-1), ON870386 (CuWND-2) OP452924 (*H. phycitis* HpCuD-1). The *16S rRNA* gene sequences were compared with sequences of phytoplasma strains available in the NCBI GenBank database using the BLASTn program. The sequences of the clarkia and leafhopper phytoplasma strains shared 100% similarity within themselves and 100% sequence identity with the other isolates of *Candidatus* phytoplasma asteris (16SrI-B) strain from 99.72% (with Marigold witches'-broom phytoplasma isolate MK440290, Poland) to 100% (with North American grapevine yellows phytoplasma isolate KX236148, USA).

In phylogenetic sequence analysis of *16S rRNA* gene sequences of clarkia and leaf hopper isolate, they clustered with the strains related to 16SrI-B subgroup (Figure 2).

The pairwise sequence comparison of the partial *sec*A gene sequences resulted in this study (Acc. Nos. OP503628, OP503629, OP503630) showed a minimum of 99.13% sequence identity with the sequences of *Rosa hybrida* phytoplasma (MW362160) and a maximum of 100% identity with the sequences of *Elaeis guineensis* stunt phytoplasma from India (JF730880). In the phylogenetic analysis of *sec*A gene sequences of CuFND-1, CuWND-2 and HpCuD-1 (OP503628, OP503629, OP503630) phytoplasma strains clustered with the strains related with 16SrI phytoplasma group (Figure 3).

Further, the virtual RFLP analysis results produced similar virtual RFLP profiles identical to the reference strain of the 16SrI-B phytoplasma subgroup (aster yellows: Accn. No. M30790) with a similarity coefficient value of 1.0 (Figure 4) and indicated that all the samples tested were positive (clarkia and leafhopper) in the study.

Discussion

Symptoms suggestive of *Candidatus* Phytoplasma *asteris* on herbaceous plant hosts include reduced growth of the leaves, stunting of the plants, proliferation of auxiliary shoots resulting in a witches'-broom appearance, bunchy appearance of growth at the ends of stems (apical fasciation), flat stem, virescence of flowers with sterility and phyllody [23]. The ornamental plant under study represented few of the above-mentioned symptoms like flat stem, witches'-broom, and apical fasciation while yellowing, little

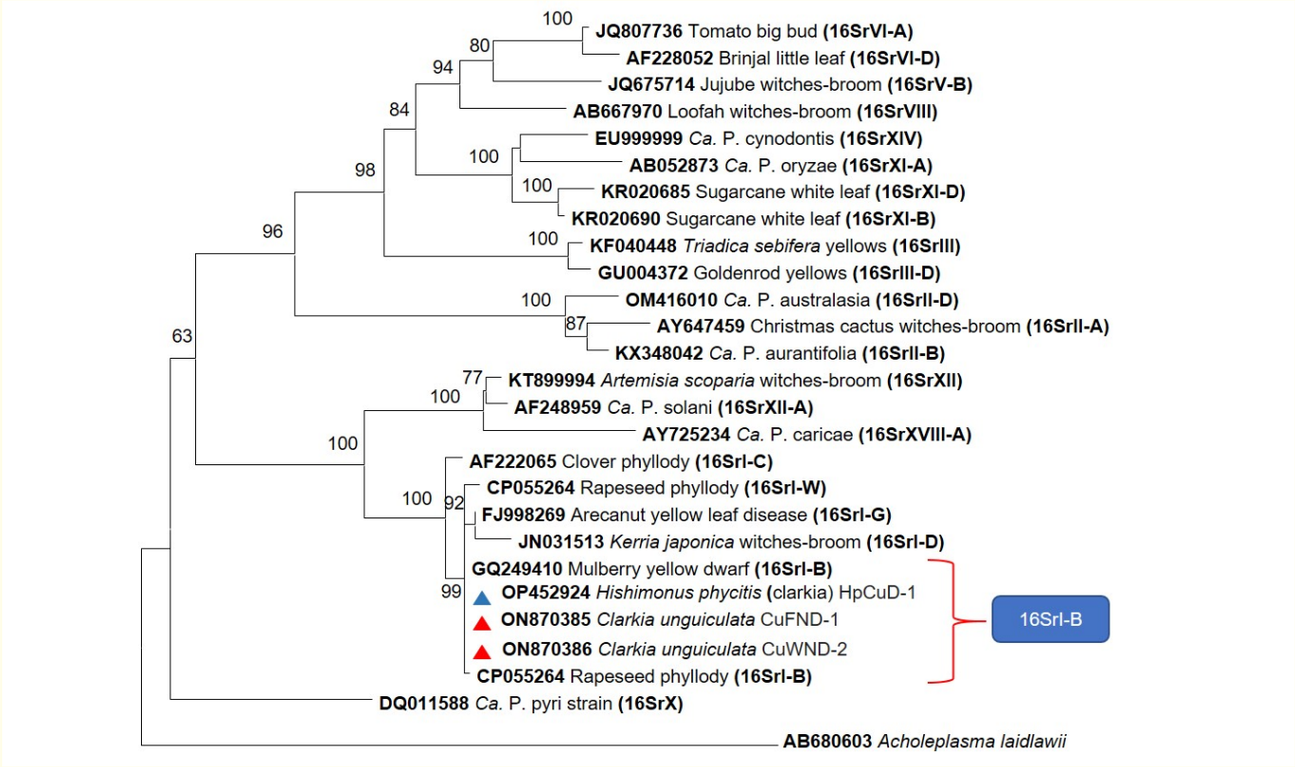

Figure 2: The 16Sr gene sequence-based phylogenetic tree constructed by the neighbor-joining method and bootstrap (1000 replications) model using MEGA 11 software, showing the evolutionary relationship among the identified *Clarkia unguiculata* phytoplasma isolates (red) and *Hishimonus phycitis* (blue) with reference to earlier reported phytoplasma strains of different groups. The tree is rooted with *Acholeplasma laidlawii* (AB680603).

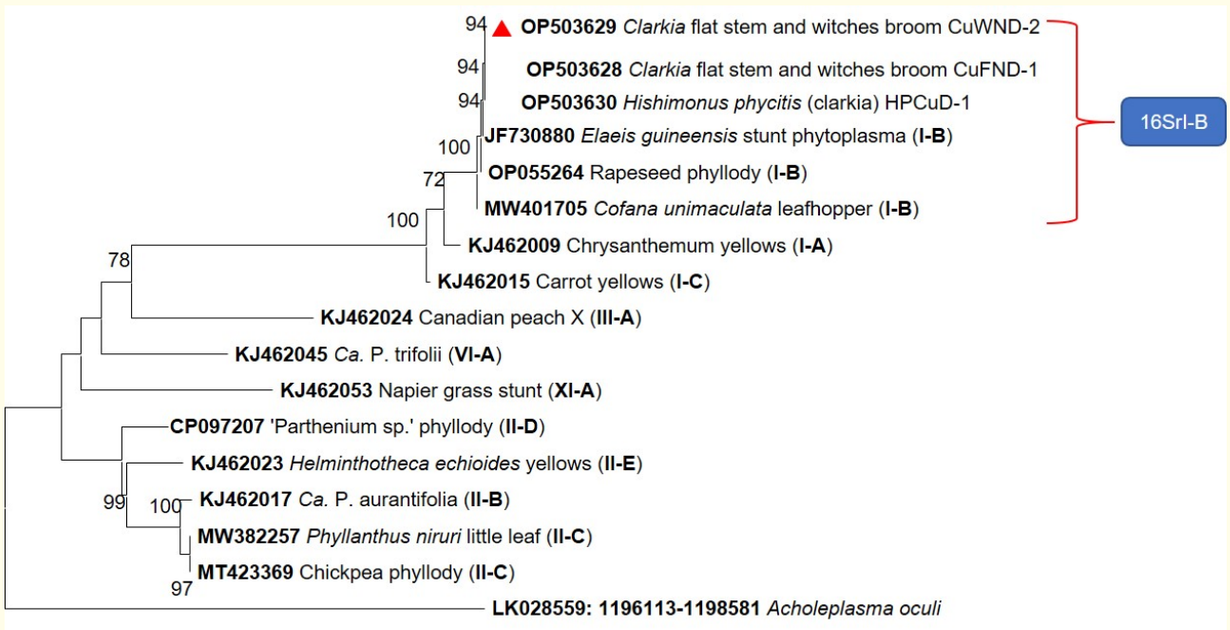

Figure 3: The secA gene sequence-based phylogenetic tree constructed by the neighbor-joining method with 1000 bootstrap replications using MEGA 11 software, showing the evolutionary relationship among the identified *Clarkia unguiculata* phytoplasma isolates (red) and *Hishimonus phycitis* (blue) with reference to earlier reported phytoplasma strains of different groups. The tree is rooted with *Acholeplasma oculi* (LK028559).

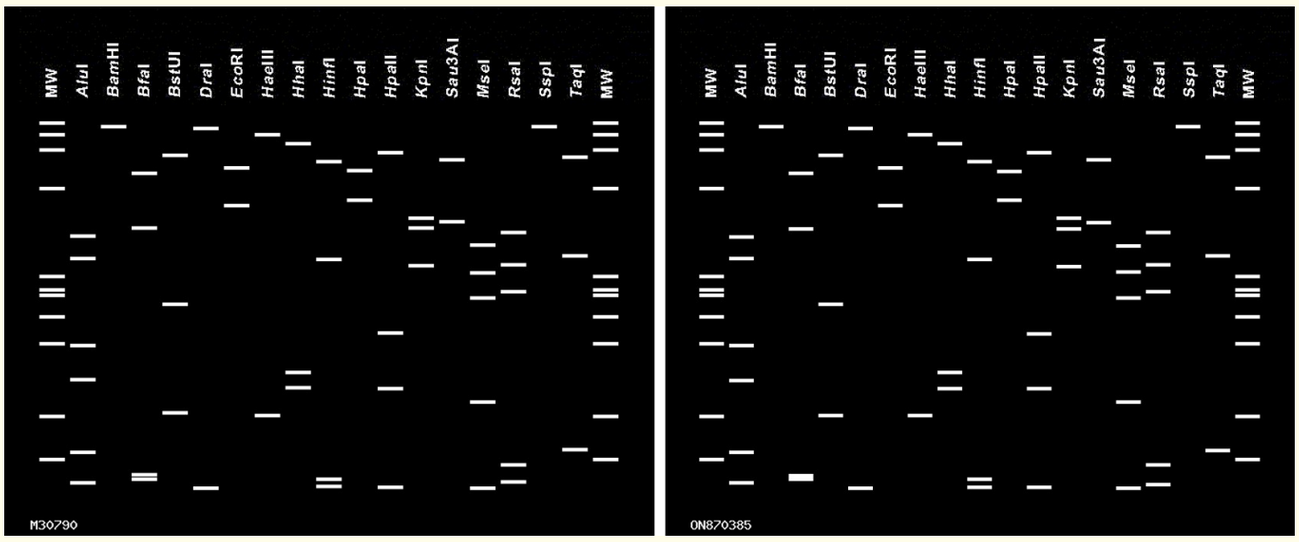

Figure 4: Comparison of virtual RFLP pattern derived from in silico digestion of 1.25 kb PCR product of 16S rDNA sequences of *C. unguiculata* flat stem and witches'-broom phytoplasma keeping the strain *Oenothera phytoplasma* (M30790) as the reference.

leaf, and witches'-broom were the common symptoms usually observed on the ornamental plants infected by the 16SrI group except for the *Petunia hybrida* and *Celosia argentea* which showed the flat stem along with the witches'-broom symptoms [24,25]. Aster yellows (AY) group is the largest and most diverse phytoplasma group globally has been delineated to seventeen subgroups, among which the subgroup 16SrI-B represents the largest strain cluster in the AY group [26,27]. Likewise, in India, the earlier reports showed 16SrI infections in various crops like cereals (rice and maize), fruits (mango, peach, pear and pineapple), vegetables (okra, pumpkin, and bell-pepper), industrial crops (sugarcane, sandal, and tobacco), legumes (red gram and cowpea), oil seeds (sesamum, soybean, and linseed), and ornamental crops (Celosia, Gladiolus, Hibiscus). The phytoplasma strains belonging to only five subgroups 16SrI (A, B, C, E, F) have been reported from India. Similar to the world scenario, the subgroup 16SrI-B is the most frequently observed and widely prevalent subgroup in the Indian subcontinent [2]. To discuss about the phytoplasma with special reference to ornamentals, the six phytoplasma groups *viz*, the aster yellows (16SrI), peanut witches'-broom (16SrII-D), clover proliferation (16SrVI), pigeon pea witches'-broom (16SrIX), rice yellow dwarf (16SrXI) and Bermuda grass white leaf (16SrXIV) are reported to be associated with ornamental plants in India [28]. Recently, the strains related to 16SrI have also been reported from the leaf yellowing of an ornamental plant, *Wrightia antidysenterica* [29]. However, to the best of our knowledge, the results of the present study establish the first report of a *'Ca*. P. asteris'- related strain causing flat stem phyllody disease of *C. unguiculata* in India or the world. The impact, distribution, and epidemiology of the disease in India are yet to be studied and are currently under investigation.

Conclusion

The study has verified the association of the 16SrI-B subgroup of '*Candidatus* Phytoplasma asteris' in *Clarkia unguiculata.* The nested PCR assays showed positive results for both plant DNA and leaf hopper for the *16S rRNA* gene and SecA gene. Subsequent molecular cloning, sequencing and sequence analyses proves the phytoplasma associated with *Clarkia unguiculata plants* was a strain of aster yellows sub-group B.

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Conflict of Interest

The authors hereby declare that they have no conflict of interest in the manuscript.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Hemavati Ranebennur] and [Kirti Rawat]. The first draft of the manuscript was written by [Hemavati Ranebennur] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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