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Original article

Molecular identification of a '*Candidatus* Phytoplasma trifolii' (16SrVI-D) strain associated with Scurfy pea (*Cullen corylifolium*) phyllody and witches' broom in India

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Abstract

Scurfy pea (*Cullen corylifolium* (L.) Medik.) plants exhibiting symptoms of phyllody and witches' broom indicative of phytoplasma infection were observed in the research field of ICAR – Indian Agricultural Research Institute, Regional Station, Pune (Maharashtra, India). Phytoplasma presence in all symptomatic samples was confirmed through PCR and nested PCR assay using phytoplasma-specific primer pairs P1/P7 and R16F2n/R2. BLASTn analysis revealed that the amplicon sequence shares more than 99% identity with known strains of '*Candidatus* Phytoplasma trifolii' sequences. Phylogenetic analysis further clustered the sequence of the phytoplasma strain within the '*Ca*. Phytoplasma' species while virtual RFLP analysis assigned it to the 16SrVI-D subgroup. To the best of our knowledge, this is the first worldwide report of 16SrVI-D phytoplasma associated with phyllody and witches' broom in *C. corylifolium*.

Keywords: *Cullen corylifolium*, scurfy pea, phyllody, witches' broom, 16SrVI-D.

Introduction

Scurfy pea (*Cullen corylifolium* (L.) Medik.) is an herbaceous annual plant belonging to the family Fabaceae. It is known by several synonymous names, viz., *C. corylifolia*, *C. corylifolius*, *Psoralea corylifolia*, *P. patersoniae*, *Lotodes corylifolia*, *Trifolium unifolium* (Jafernik *et al.* 2020). It is popularly called 'babchi' in Hindi. *C. corylifolium* is found growing naturally in the plains of India, especially in the semiarid regions of Rajasthan, Punjab, and Uttar Pradesh. It is also found in other part of India such as the Himalayas, Dehra Dun, Bengal, Mumbai, Bundelkhand, Oudh, Deccan. and Karnataka (Sharma *et al.* 2001; Sah *et al.* 2006).

It is a widely recognized medicinal plant and is used in Ayurveda, Chinese traditional medicine and even in Western medicine particularly for managing dermatological disorders (Sah *et al.* 2006; Khushboo *et al.* 2010). Extracts derived from the whole plant have been used in traditional medicine to treat skin inflammations occurring in diseases such as psoriasis, vitiligo, and leprosy, and are also administered as a blood purifier following scorpion or snake bites (Wang *et al.* 1999). Bakuchiol, a meroterpenic phenol obtained from *C. corylifolium,* is increasingly being recognized in cosmetics as a safer, plant-derived alternative to retinol. It is used in dermatology and cosmetology for the prevention of wrinkles, skin hyperpigmentation, and acne lesions (Backhouse *et al.* 2001; Choi *et al.* 2010; Ohno *et al.* 2010). Bakuchiol also possesses valuable pharmacological properties, viz., hepatoprotective, anti-cancer, hypoglycemic, cardioprotective, antidepressant, hypolipemic, antioxidant, anti-inflammatory, and antimicrobial activities (Jafernik *et al.* 2020).

At present, the seeds of *C. corylifolium* are the main natural source for commercial-scale bakuchiol extraction (Khushboo *et al.* 2010; Uikey *et al.* 2010). Various pharmaceutical industries use the seeds of *C. corylifolium* as a raw material in the preparation of several formulations (Raghav *et al*. 2003). Seed extracts are diaphoretic and are also used as stimulants, aphrodisiacs, and in the treatment of asthma as well as chronic cough (Sharma *et al.* 2001; Liu *et al.* 2004).

Phytoplasmas have gained global significance as plant pathogens owing to their ability to cause considerable yield losses in important crops and their wide epidemiological prevalence. Phytoplasma associated diseases are known to occur in over 200 medicinal plants across the globe (Rao *et al.* 2018). Phytoplasmas linked to these diseases vary greatly in both their geographic range and the composition of their taxonomic groups and subgroups (Marcone *et al.* 2016). Medicinal and aromatic plants are an important source of secondary metabolites which find diverse applications in the pharmaceutical industry, cosmetics and in the control of

plant and human diseases (Pandey and Tripathi 2011). Notably, phytoplasma infections are known to severely reduce yield, quality, and longevity of medicinal and aromatic plants (Marcone *et al.* 2016).

In recent years, *C. corylifolium* is garnering significant interest, primarily owing to its high levels of bakuchiol content (1-7%) (Yao *et al.* 1995). However, scarce information is available pertaining to diseases of *C. corylifolium* with no records on infection of phytoplasma. In light of this, the present study was undertaken with the objective of detecting and characterizing the phytoplasma causing *C. corylifolium* phyllody and witches' broom using PCR amplification, sequencing, phylogenetic analysis, *in silico* RFLP and understanding the impact of the disease on bakuchiol production.

Materials and Methods

Sample collection and disease incidence

C. corylifolium plants exhibiting phyllody and witches' broom symptoms were observed in the research field ICAR – Indian Agricultural Research Institute, Regional Station, Pune (Maharashtra, India; 18°33'4"N, 73°48'27"E) in January 2024.

The disease incidence was calculated as the percentage of symptomatic plants over the total number of plants using the formula:

Disease incidence (%) = (Number of symptomatic plants/ Total number of plants) x 100.

Five symptomatic and five asymptomatic plants were randomly sampled and stored in a deep freezer (-80°C) for further identification and characterization of associated phytoplasma.

DNA extraction and PCR assays

Total genomic DNA was extracted from leaf petioles (100 mg) of both symptomatic and asymptomatic *C. corylifolium* plants with the DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. DNA from a 16SrII-C phytoplasma infected chickpea plant was used as the positive control (Verma *et al*. 2022a). The phytoplasma ribosomal DNA (rDNA) was amplified using the universal primer pair P1/P7 (Deng and Hiruki 1991; Schneider *et al*. 1995). Nested PCR was subsequently conducted with R16F2n/R2 primers (Gundersen and Lee 1996). Each PCR reaction (25µl) contained 12.5 µl DreamTaq PCR Master Mix (2X) (ThermoFisher Scientific, MA, USA), 0.5 µl of each primer (10 pmol/µl), 9.5 µl nuclease free

water and 2 µl of template DNA (0.5 µg). Reactions were carried out in a thermal cycler (Eppendorf, Germany). The cyclic conditions for P1/P7 amplification consisted of initial denaturation at 94ºC for 4 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 53ºC for 1 min, extension at 72ºC for 2 min and a final extension at 72ºC for 10 min. Nested PCR was performed using 0.2 µl of the first-round product as template, with conditions of initial denaturation at 94ºC for 5 min followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 59ºC for 2 min, extension at 72ºC for 3 min and a final extension at 72ºC for 10 min. Nested PCR products were resolved by electrophoresis on 1% (w/v) agarose gel, stained with Novel Juice nucleic acid stain (Bio-Helix, Taiwan) and visualized using a Gel Documentation system (Syngene, UK).

Sequencing and phylogenetic analysis

The amplified PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and outsourced to Eurofins, India for bidirectional sequencing with R16F2n/R2 primers. Sequence assembly and alignment was performed using Bio-Edit software. As the amplicon sequences from all symptomatic samples were identical, one representative sequence was deposited in NCBI GenBank under accession number PX149178. This sequence was subjected to BLASTn analysis against sequences available in GenBank. It was then aligned with a few previously reported *Candidatus* Phytoplasma trifolii phytoplasma sequences as well as phytoplasma sequences from different geographic regions belonging to other ribosomal group / subgroup using Clustal W (Thompson *et al*. 1994). Phylogenetic analysis was conducted using the Neighbor-Joining method with 1,000 replicates using MEGA X (Kumar *et al*. 2018). *Acholeplasma laidlawii* (GenBank accession number AB680603) was used as the outgroup to root the tree. Species-level assignment of '*Candidatus* Phytoplasma' and subgroup identification was performed using the *i*PhyClassifier online tool (Zhao *et al*. 2009).

Seed set observation

In order to assess the effect of the infection on reproductive performance, seed setting was recorded in both healthy and symptomatic *C. corylifolium* plants. Ten, both healthy and symptomatic, plants were selected randomly. Pod formation, filling and seed setting was examined by visual inspection. In symptomatic plants, special attention was given to the incidence of floral abnormalities such as phyllody and their impact on pod formation and seed setting was noted.

Results

The phytoplasma infected *C. corylifolium* plants exhibited phyllody and witches' broom symptoms (Figure 1). The diseased plants could be easily identified in the field due to their distinct morphology. The plants were growing as weeds alongside cucurbit, tomato, and papaya research plots. The disease incidence was recorded as 12% among 175 the plants examined.

Molecular detection assays confirmed the association of phytoplasma with the symptomatic plants. PCR using universal primer pair P1/P7 amplified fragments of expected size ~1.8 kb in all symptomatic samples and the positive control, whereas no amplification was observed in asymptomatic plants. Nested PCR with R16F2n/R2 primers produced ~1.2 kb amplicons in all symptomatic samples and the positive control but not in healthy plants, further confirming phytoplasma presence.

The nested PCR product was sequenced (PX149178) and BLASTn analysis revealed that the sequence shared over 99% identity with phytoplasma sequences reported from crops such as brinjal (PV040784; KP027513) from Tamil Nadu and Andhra Pradesh, respectively, and bottle gourd (MW672511) from Pune, Maharashtra. The sequence showed 86.69-99.76% identity with representatives of other ribosomal groups (Table 1).

Phylogenetic analysis placed the Pune strain with other 16SrVI-D sequences, clustering it closest to phytoplasma sequences reported in brinjal (PV040784) from Tamil Nadu and bottle gourd (MW672511) from Pune, Maharashtra (Figure 2). *In silico* RFLP analysis with *i*PhyClassifier confirmed its assignment to the 16SrVI-D subgroup with 98.80% similarity with '*Candidatus* Phytoplasma trifolii' reference strain (GenBank accession number AY390261) and a virtual RFLP similarity coefficient of 0.98 with 16SrVI-D reference pattern (Figure 3).

Observations on seed setting revealed clear differences between healthy and phytoplasma-affected *C. corylifolium* plants. Profuse flowering followed by normal pod development with well-formed seeds ensuring adequate reproductive success was observed in the healthy plants. In contrast, diseased plants exhibiting phyllody showed no seed set as the flowers were converted into leafy structures which failed to produce pods.

Discussion

In this study, phytoplasma infection in *C. corylifolium* plants was confirmed through molecular characterization, partial 16S rRNA gene sequencing and *in silico* RFLP analysis. To the best of our knowledge, this is the first report of 16SrVI-D phytoplasma associated with

phyllody and witches' broom in *C. corylifolium* worldwide, based on molecular and phylogenetic characterization.

The present study holds immense importance for the phytopharmaceutical and cosmeceutical industries engaged in extraction of bakuchiol from seeds of *C. corylifolium* plants and synthesis of various formulations for commercial use. The ability of the Pune strain to induce total phyllody along with impeding pod and seed formation could become a significant threat to bakuchiol production as it is primarily extracted from seeds. A related strain of *Candidatus* Phytoplasma trifolii has previously been reported to cause seed pod abortion in soybean which is also a member of the Fabaceae family (Zamharir and Aldaghi 2018). The 16SrVI-D strain is widely prevalent in India and is responsible for considerable yield losses, thereby posing a serious socio-economic threat to the commercial cultivation of several crops. Phytoplasmas belonging to the 16SrVI-D subgroup have previously been detected in several medicinal plants such as *Rauwolfia serpentina* (Kalita *et al.* 2019), *Solanum violaceum* (Dutta *et al.* 2022), *Withania somnifera* (Tiwari *et al.* 2022), *Nyctanthes arbor-tristis* (Dutta *et al.* 2024) and Nigella sativa (Gupta *et al.* 2024) in India. Phytoplasmas of this subgroup have also been reported to infect several vegetable crops such as carrot, brinjal, lettuce, cabbage, bitter gourd, and bottle gourd (Gopala *et al.* 2018; Verma *et al.* 2022b) in the country. The recent reports of the strain from the same area, Pune, Maharashtra (Verma *et al.* 2022b), are indicative of its growing presence in the region. *C. corylifolium* could potentially serve as an alternate host aiding the spread of 16SrVI-D phytoplasmas to nearby crops and weeds. Consequently, monitoring for phytoplasma infections in both plants and insect vectors, together with the application of integrated pest management practices is vital for restricting their spread. Furthermore, as phytoplasma infection is known to up- or downregulate secondary metabolite concentration in the affected plants (Favali *et al.* 2004), further studies need to be conducted to understand the effect of the infection on secondary metabolite synthesis in other plant parts of *C. corylifolium*. Additionally, studies employing multi-locus sequence analysis (MLSA) or multi-locus sequence typing (MLST) of several housekeeping genes such as *tuf*, *sec*A, *sec*Y, *rp, gro*EL, etc., would probably offer a higher level of genetic discrimination and support detailed molecular epidemiology studies aimed at understanding pathogen distribution and spread.

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Table 1. Phytoplasma sequences used in the bioinformatic analysis and their percentage identity with *Cullen corylifolium* phyllody and witches' broom phytoplasma (PX149178) from Pune (Maharashtra), India.

Phytoplasma associated disease (‘*Candidatus* Phytoplasma’ species)	**Host species**	**Country**	**% Identity**	**Ribosomal subgroup**	**GenBank Accession Number**
Sesame phyllody (*Ca*. P. asteris)	*Sesamum indicum*	Paraguay	90.01	16SrI-B	KY933669
Papaya phyllody	*Carica papaya*	Pune, Maharashtra, India	89.66	16SrII-C	MW404675
Chickpea phyllody	*C. arietinum*	India	89.93	16SrII-D	KX151120
Melia azedarach decline (*Ca*. P. pruni)	*Melia azedarach*	Brazil	93.62	16SrIII-B	FJ404775
Coconut lethal yellowing	*Cocos nucifera*	Jamaica	94.93	16SrIV-A	AF498307
Alder yellows	*Corchorus olitorius*	India	96.44	16SrV-C	KF501045
Clover proliferation (*Ca*. P. trifolii)	*Solanum tuberosum*	Turkey	98.47	16SrVI-A	MH683601
Clover proliferation (*Ca*. P. trifolii)	*Fragaria multicipita*	USA	98.25	16SrVI-B	AF190224
Clover proliferation (*Ca*. P. trifolii)	*Ulmus americana*	USA	98.56	16SrVI-C	AF409070
Ca. P. trifolii	*Solanum melongena*	India	99.76	16SrVI-D	PV040784
Bottle gourd phyllody (*Ca*. P. trifolii)	*Lagenaria siceraria*	Pune, Maharashtra, India	99.68	16SrVI-D	MW672511

Ca. P. trifolii	*Solanum melongena*	India	99.20	16SrVI-D	MW261866
Brinjal little leaf	*Solanum melongena*	India	99.20	16SrVI-D	KP027505
Clover proliferation (*Ca*. P. trifolii)	*Centaurea solstitialis*	Italy	98.72	16SrVI-E	AY270156
Clover proliferation (*Ca*. P. trifolii)	*Catharanthus roseus*	Sudan	98.47	16SrVI-F	EF186819
Clover proliferation (*Ca*. P. trifolii)	*Portulaca grandiflora*	India	97.98	16SrVI-H	EF651786
Clover proliferation (*Ca*. P. trifolii)	*Passiflora edulis f. flavicarpa*	Brazil	96.41	16SrVI-I	GU292081
Ash yellows (*Ca*. P. fraxini)	*Fraxinus sp.*	USA	96.33	16SrVII-A	AF092209
Luffa witches' broom (*Ca*. P. luffae)	*Luffa aegyptica*	Taiwan	95.65	16SrVIII-A	AF248956
Chicory bushy stunt (*Ca*. P. phoenicium)	*Cichorium intybus*	Saudi Arabia	93.15	16SrIX-J	KY986922
Apple proliferation (*Ca*. P. mali)	*Malus domestica*	Italy	90.31	16SrX-A	AJ542541
Strawberry yellows (*Ca*. P. fragariae)	*Fragaria x ananassa*	Lithuania	89.67	16SrXII-E	DQ086423
Papaya apical curl necrosis (*Ca*. P. meliae)	*Carica papaya*	Brazil	89.86	16SrXIII-E	JQ792171
Hibiscus witches' broom (*Ca*. P. brasiliense)	*Hibiscus rosa-sinensis*	Brazil	90.09	16SrXV-A	AF147708
Papaya bunchy top (*Ca*. P. caricae)	*Carica papaya*	Cuba	86.69	16SrXVII-A	AY725234

Figure 1. Phytoplasma infected *C. corylifolium* plant showing typical symptoms of phyllody and witches' broom (left) and healthy plant showing normal floral and vegetative development (right).

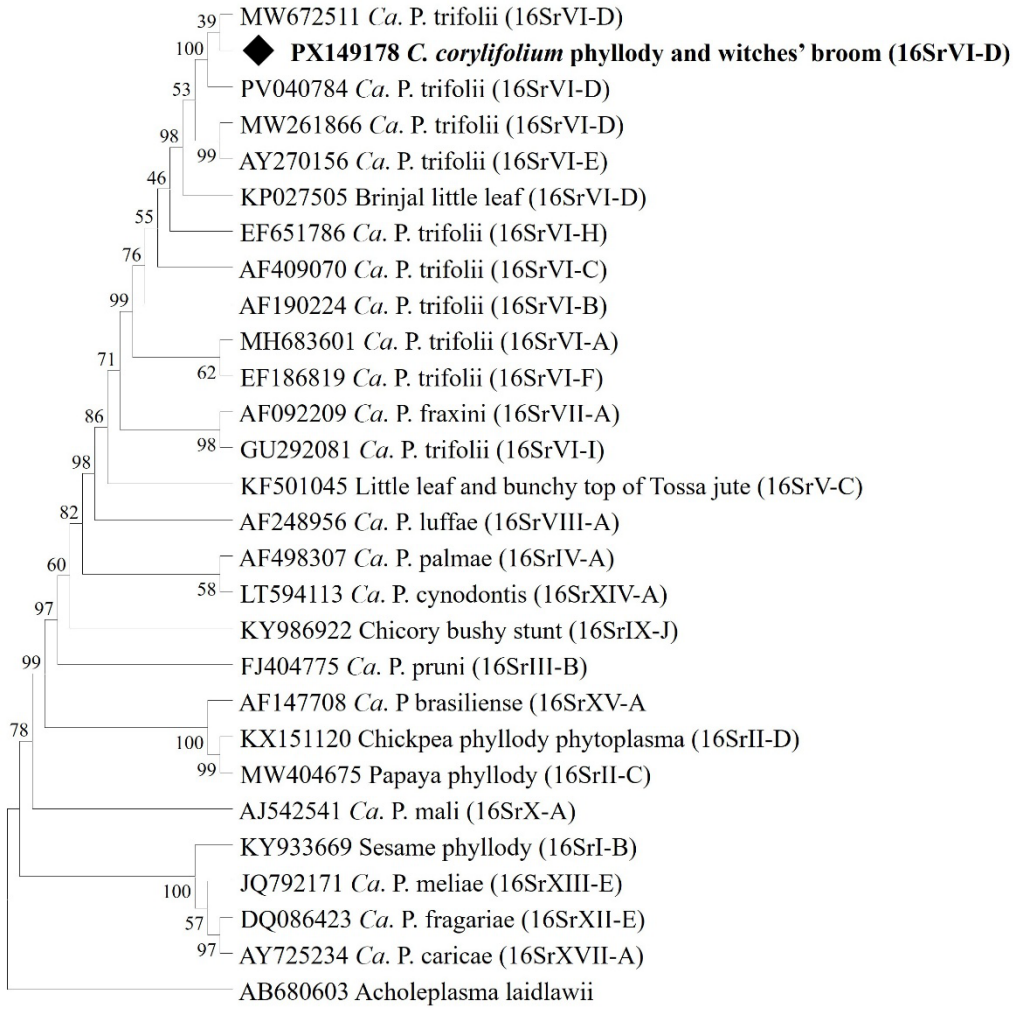

Figure 2. Phylogenetic tree showing the relationship between *C. corylifolium* phytoplasma sequence of the present study (PX149178; marked with solid diamond) with other phytoplasma sequences from diffirent 16Sr groups and subgroups. The tree was constructed using the Neighbor-Joining method with 1,000 bootstrap replicate values in MEGA X. *Acholeplasma laidlawii* (AB680603) was used as the outgroup.

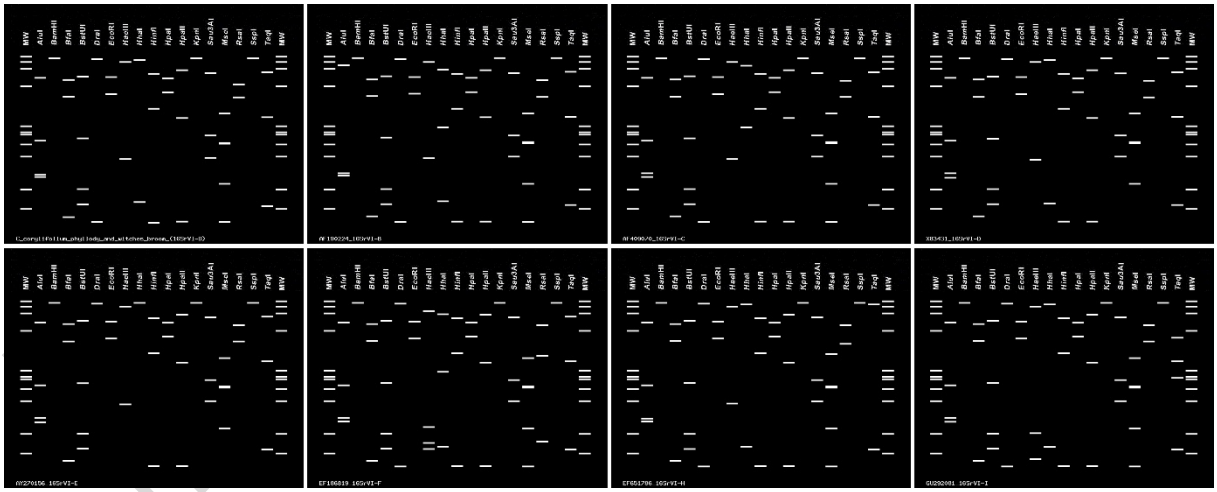

Figure 3.

Virtual RFLP patterns of 16S rRNA gene R16F2n/R2 fragment from (top: left to right) *C. corylifolium* phytoplasma (PX149178), 16Sr VI-B (AF190224), C (AF409070), D (X83431), (bottom: left to right) E (AY270156), F (EF186819), H (EF651786), I (GU292081) generated through in silico digestions using iPhyClassifier.