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ORIGINAL ARTICLE

Genetic diversity of phytoplasmas associated with several bamboo species in India

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Abstract

Symptoms of phytoplasma diseases, including witches' brooms, shoot proliferation, little leaf, yellowing and decline were observed in eleven species of bamboos in eight states of India. Symptomatic bamboo samples were indexed for phytoplasma presence using universal phytoplasma-specific 16S rRNA gene primer pairs. Sequence comparison analysis and virtual RFLP analysis of 16S rRNA sequences indicated that the symptomatic bamboo samples of *Dendrocalamus asper*, *Dendrocalamus strictus* and *Pseudoxytenanthera stocksii* from Tripura, Uttar Pradesh, Uttarakhand and New Delhi and *Bambusa bambos*, *Bambusa nutans*, *Bambusa pallida*, *Bambusa tulda*, *Dendrocalamus hamiltonia* and *D. strictus* from Karnataka were infected with '*Candidatus* Phytoplasma australasia'-related strains (16SrII-C or 16SrII-D); *Bambusa vulgaris*, *D. asper* and *P. stocksii* from Karnataka were positive for '*Ca.* P. asteris' strains (16SrI-B), and one phytoplasma in *B. nutans* from Sikkim was identified as '*Ca.* P. cynodontis' strain (16SrXIV-A). Weeds and leafhoppers from the vicinity of bamboo plantations were also tested, and 16SrII group of phytoplasmas was identified in eight symptomatic weed species (*Ageratum conyzoides*, *Cannabis sativa*, *Cleome viscosa*, *Datura stramonium*, *Parthenium hysterophorus*, *Ocimum canum*, *Phyllanthus niruri* and *Tephrosia purpurea*) from five different states and one leafhopper species (*Mukaria spledida*) from three states. These results suggest that wide genetic diversity of phytoplasma exists in various bamboo species in different states of India. The report of 16SrI-B, 16SrII-D and 16SrXIV-A subgroups of phytoplasmas infecting eight bamboo species except *D. strictus* are the first records in the world.

KEYWORDS

'*Ca.* P. asteris', '*Ca.* P. australasia', '*Ca.* P. cynodontis', bamboo species, natural reservoirs

1 | INTRODUCTION

Bamboos are perennial grasses with multifaceted utilities, varying from industrial to household purposes (Liese et al., 2015). India has a huge diversity of bamboos covering approximately 149.4 million hectares (ISFR, 2021); the genera *Bambusa*, *Dendrocalamus*, *Melocanna*, *Ochlandra* and *Thyrsostachys* are commercially important (Anonymous, 2021). *D. strictus*, found commonly across the country is used in many forms. Under the aegis of the National Bamboo Mission, the cultivated area under bamboo is increasing every year (Anonymous, 2019). Bamboos are affected by a variety of pests and pathogens, which decrease the value of the culms. While the effect of pests on bamboos is studied in detail, bamboo diseases are often overlooked (Shu & Wang, 2015).

Phytoplasmas are cell wall-free, pleomorphic, Gram-positive bacteria confined to the phloem tissues of vascular plants and also

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colonize insect vectors. These systemic pathogens affect the physiology of plants often rendering infected plants sterile. Symptoms include phyllody, witches' broom, virescence and shoot proliferation, among others (Namba, 2019). Phytoplasmas are known from at least 1000 plant species around the world encompassing various agricultural crops and weeds (Rao et al., 2018).

The 16S rRNA gene, which is highly conserved in bacterial species, is used in the classification of phytoplasmas for which ribosomal groups and subgroups have been identified and reported using the gene from around the world (Bertaccini, 2019). Classification has also been refined by employing multiple house-keeping genes, such as *sec*A, *sec*Y or *tuf*, to present a higher resolution (Martini et al., 2019).

Recent surveys in various commercially important bamboo species revealed the presence of phytoplasma-like symptoms, including witches' broom, little leaf, shoot proliferation, yellowing and decline of culms, in eight states of India. A study, to detect and characterize the phytoplasmas associated with symptomatic bamboo species, and in associated weeds and leafhoppers found in bamboo ecosystems, was performed to verify the presence of phytoplasmas and to understand correlations between the ecological factors sustaining these infections.

2 | MATERIALS AND METHODS

2.1 | Survey and collection of plant samples

Eight bamboo-growing (commercial or natural) states (Bihar, Karnataka, Sikkim, Tamil Nadu, Tripura, Uttar Pradesh, Uttarakhand, Delhi) were surveyed for the presence of phytoplasma symptoms on eleven bamboo species. The presence of weeds exhibiting phytoplasma-like symptoms in the bamboo plantations was also recorded and the plants sampled. Samples were stored at −20°C until processed for molecular assays.

2.2 | Collection of leafhoppers and identification

Insects at the bamboo plantations of Delhi, Uttarakhand and Tripura were collected using sweep nets and yellow sticky traps. Any leafhoppers caught were identified using taxonomic keys with the assistance of the Division of Entomology, IARI, New Delhi (Viraktamath & Meshram, 2019) and stored in 70% ethanol until processed for DNA extraction and other molecular assays.

2.3 | Nucleic acid extraction and detection of phytoplasma by PCR assays

Total nucleic acid was extracted from all the symptomatic bamboo and weed samples using a modified version of the CTAB method (Ahrens & Seemüller, 1992). DNA from the insects was obtained from the insect body using the CTAB method (Marzachì et al., 1998). Extracted DNA was checked for quality and used at 100ng/µl for PCR. Conditions for PCR were: 1X PCR buffer, 1 mM $MgCl_2$, 200 µM dNTPs each, 0.2 µM each of forward and reverse primers, 0.5 U/µl of *Taq* polymerase and 100 ng template DNA made up to the final volume of 25 µl using nuclease-free water. Amplification of the 16S rRNA gene was achieved using phytoplasma-specific universal primer pairs P1/P7, R16mF2/R16mR1 and R16F2n/R16R2 in various direct and nested PCR cycle combinations with the reaction conditions: initial denaturation for 4 min at 94°C, followed by 35 cycles of 94°C, 45 s; 55°C, 1 min; 72°C, 2 min, and terminated after a final extension at 72°C for 10 min (Deng & Hiruki, 1991; Gundersen & Lee, 1996). DNA extracted from chickpea phyllody (Acc. No. MN551492) was the positive control and DNA from asymptomatic plants and nuclease-free water were used as negative controls. Five microlitres of each of the direct or nested PCR products along with 5µl of 1kb DNA ladder RTU (GeneDireX®, Inc.) were subject to gel electrophoresis on 1% (w/v) agarose gels prepared in 1X TAE buffer and amended with GoodView Nucleic Acid Stain (SBS Genetech Co., Ltd., Beijing), and observed under a UV transilluminator.

2.4 | Nucleotide sequencing and sequence analyses

Amplicons were purified by using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) and the purified products were sequenced directly using the Sanger method by AgriGenome Labs Pvt. Ltd., Cochin, India, in both directions using the primers utilized in the PCR. Local pairwise alignment of the sequences was done through the NCBI-BLASTn tool. Forward and reverse sequences obtained for each amplicon were aligned, edited and assembled using BioEdit (version 7.2.5). Contigs were utilized in multiple alignment analysis using the ClustalW program (Thompson et al., 1994) and representative sequences submitted to GenBank (NCBI).

2.5 | Phylogenetic and in silico RFLP analyses

Gene sequences were subjected to phylogenetic analysis along with representative sequences retrieved from the NCBI database to determine the '*Candidatus* Phytoplasma' species present in the bamboo samples. Phylograms were constructed using the neighbour-joining method utilizing MEGA 7.0 (Kumar et al., 2016) with 1000 bootstrap replications. *Acholeplasma laidlawii* (Acc. No. NR074448) was the outgroup used to root the tree. The 16S rRNA gene sequences corresponding to the R16F2n/R2 region were submitted to the online tool *i*PhyClassifier to carry out in silico RFLP analyses, and the similarity coefficient of RFLPs with the reference sequences estimated, enabling the attribution of the detected phytoplasmas to ribosomal groups and subgroups (Zhao et al., 2009).

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TABLE 1 List of different bamboo isolates collected from 8 states of India during 2019–21, symptoms recorded and phytoplasma found to be associated with different symptomatic bamboo species

S. No	Bamboo species	Locality	Symptoms	Strains	Accession number	Group/ Subgroup
1	*Dendrocalamus strictus* (Roxb.) Nees	Bihar/Bankatwa	Leaf yellowing, little leaf	–	–	–
2	*Dendrocalamus strictus* (Roxb.) Nees	Bihar/Dhanha	Leaf yellowing, little leaf	–	–	–
3	*Bambusa vulgaris* Schrad	Karnataka/Bengaluru Rural	Little leaf, leaf yellowing, drying	BWB-K8	MZ297958	16SrI-B
4	*Pseudoxytenanthera stocksii* (Munro) T. Q. Nguyen		Nodal witches' broom, shoot proliferation	BWB-K9, BWB-K10	MZ503614, MZ503615	
5	*Dendrocalamus asper* (Schult.) Backer		Leaf yellowing, culm discoloration, weaker declining culms	BWB-K5	MZ297957	
6	*Bambusa bambos* (L.) Voss		Witches' broom, leaf yellowing, drying	BWB-K6, BWB-K12	MZ295216, MZ509502	16SrII-D
7	*Bambusa nutans* Wall		Little leaf, shoot proliferation	BWB-K14	MZ509500	
8	*Bambusa pallida* Munro		Leaf yellowing	BWB-K11	MZ509501	
9	*Bambusa tulda* Roxb.		Leaf yellowing, shoot proliferation	BWB-K7	MZ295215	
10	*Dendrocalamus hamiltonia* Gamble		Leaf yellowing, shoot proliferation	BWB-K13	MZ509503	
11	*Dendrocalamus strictus* (Roxb.) Nees		Nodal witches' broom, little leaf, drying and decline	BWB-K4	MZ509508	
12	*Bambusa balcooa* Roxb.		Leaf yellowing, little leaf	–	–	–
13	*Thyrsostachys oliveri* Gamble		Leaf yellowing, culm discoloration	–	–	–
14	*Dendrocalamus strictus* (Roxb.) Nees	Karnataka/Mysuru	Witches' broom, little leaf, drying	BWB-K2, BWB-K3	MZ295208, MZ295209	16SrII-D
15	*Dendrocalamus strictus* (Roxb.) Nees	Karnataka/Sirsi	Witches' broom, shoot proliferation, little leaf	BWB-K1	MZ303748	
16	*Dendrocalamus asper* (Schult.) Backer	Delhi/New Delhi	Witches' broom, little leaf, proliferation	BWB-ND2	MZ295212	
17	*Dendrocalamus strictus* (Roxb.) Nees		Witches' broom, little leaf, proliferation	BWB-ND1	MZ295211	
18	*Bambusa nutans* Wall	Sikkim	Witches' broom, little leaf, leaf yellowing	BWB-S1	MZ292984	16SrXIV-A
19	*Dendrocalamus strictus* (Roxb.) Nees	Tamil Nadu/Coimbatore	Leaf yellowing, little leaf	–	–	–
20	*Dendrocalamus strictus* (Roxb.) Nees	Tamil Nadu/Salem	Leaf yellowing, little leaf	–	–	–
21	*Dendrocalamus strictus* (Roxb.) Nees	Tripura/Kamalghat	Witches' broom, nodal proliferation	BWB-T3	MZ295210	16SrII-D
22	*Pseudoxytenanthera stocksii* (Munro) T.Q. Nguyen	Tripura/Lembucherra	Witches' broom, leaf yellowing	BWB-T2	MZ295217	
23	*Dendrocalamus strictus* (Roxb.) Nees	Uttar Pradesh/ Kushinagar	Witches' broom, little leaf, nodal proliferation	BWB-UP2	MZ295213	
24	*Dendrocalamus strictus* (Roxb.) Nees	Uttar Pradesh/ Shahjahanpur	Leaf yellowing, terminal witches' broom	BLY-UP1	MZ295214	16SrII-C
25	*Dendrocalamus strictus* (Roxb.) Nees	Uttarakhand/Pantnagar	Witches' broom, decline	BWB-Uk1	MZ295218	16SrII-D

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3 | RESULTS

3.1 | Survey and symptomatology on bamboo and weed species

Phytoplasma like symptoms of witches' broom, little leaf, leaf chlorosis, shoot proliferation, discoloration of culms, drying and decline of clumps was noticed on eleven different species of bamboos: *Bambusa balcooa*, *Bambusa bambos*, *Bambusa nutans*, *Bambusa pallida*, *Bambusa tulda*, *Bambusa vulgaris*, *Dendrocalamus asper*, *Dendrocalamus hamiltonia*, *Dendrocalamus strictus*, *Pseudoxytenanthera stocksii* and *Thyrsostachys oliveri* from the eight states of India where surveys were conducted (Table 1; Figure 1; Figure 2A–H). The incidence of symptoms on *D. strictus* at Gottipara (Bengaluru Rural) was approximately 24%, with symptoms recorded mostly on the periphery of the plantations. Most bamboo clumps observed showed bunchy growth at the terminal portion of the culms.

Phytoplasma symptoms including witches' brooms, yellowing, virescence, flat stem, little leaf and shoot proliferation were also observed on eight weed species near the bamboo plantations, including on *Ageratum conyzoides*, *Cannabis sativa*, *Cleome viscosa*, *Datura stramonium*, *Ocimum canum*, *Parthenium hysterophorus*, *Phyllanthus niruri* and *Tephrosia purpurea* from Karnataka, Uttarakhand, Uttar Pradesh, New Delhi and Tripura (Table 2; Figure 3A–H).

3.2 | Identification of Leafhoppers

Leafhoppers collected from in and around bamboo plantations with symptomatic plants, in Uttarakhand, Tripura and New Delhi, were identified as *Mukaria splendida* using morphological characteristics (Figure 4).

3.3 | Phytoplasma detection using PCR and sequence analyses

Dendrocalamus strictus samples from Sirsi produced amplicons of ~1.8 kb with primer pair P1/P7; six bamboo samples of *D. strictus* from Mysuru, New Delhi, Kamalghat, Kushinagar and Shahjahanpur and one *D. asper* sample from New Delhi yielded amplicons of ~1.4 kb using the nested R16mF2/mR1 primer pair;

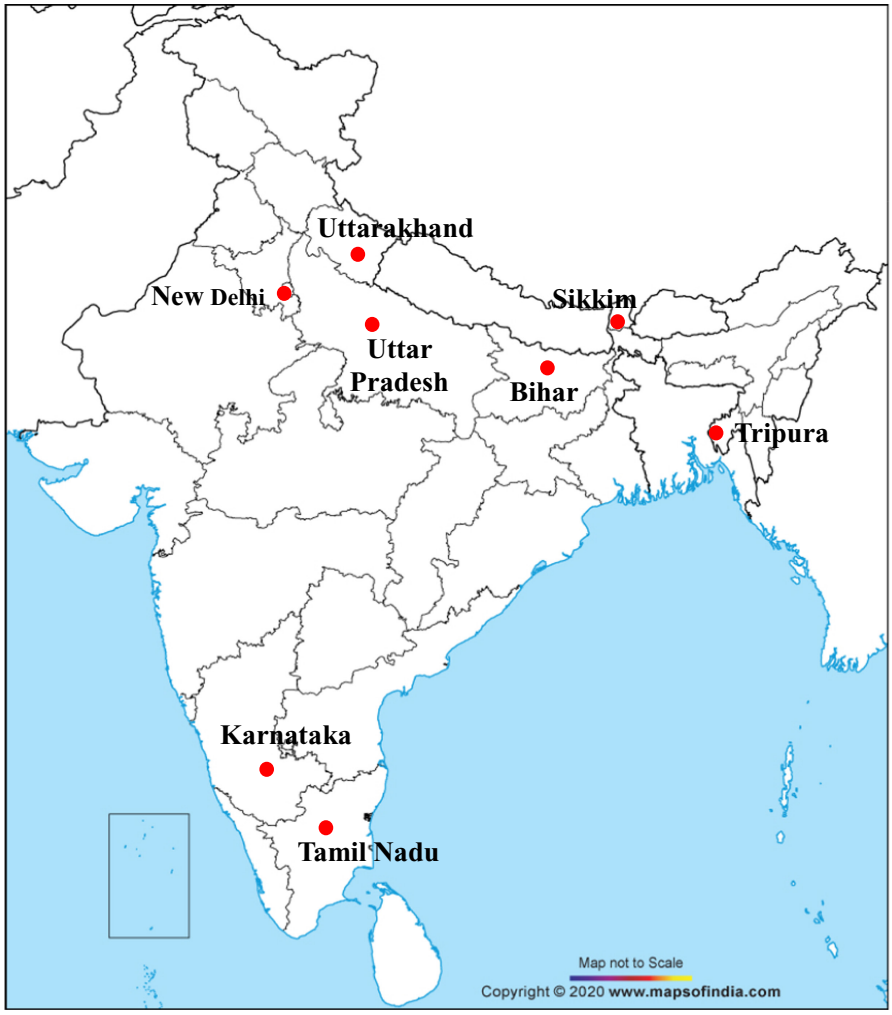

FIGURE 1 Map showing different states (red dots) in India surveyed for phytoplasma-like symptoms on bamboo species

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FIGURE 2 Various phytoplasma symptoms observed on bamboos from different regions of India. (a) Witches' broom on *Dendrocalamus strictus* (Lembucherra, Tripura); (b) discoloration of culms on *Dendrocalamus asper* (Bengaluru Rural, Karnataka); (c) witches' broom and little leaf on *D. strictus* (Sirsi, Karnataka); (d) witches' broom on *D. strictus* (Pantnagar, Uttarakhand); (e) desiccation and decline of *D. strictus* (Bengaluru Rural, Karnataka); (f) nodal witches' broom on *Pseudoxytenanthera stocksii* (Bengaluru Rural, Karnataka); (g) chlorosis and witches' broom on *Bambusa nutans* (Sikkim); (h) desiccation and decline of *Bambusa bambos* (Bengaluru Rural, Karnataka)

TABLE 2 Identification and distribution of phytoplasmas associated with weeds from five different states along with symptomatology, primer used and GenBank accession number

S. No	Weed species	Family	Locality	Symptoms observed	Strains	Accession number	Group/ subgroup
1	*Phyllanthus niruri* L.	Phyllanthaceae	Karnataka/ Bengaluru Rural	Little leaf, leaf yellowing	PnK–1	MZ424219	16SrII-D
2	*Parthenium hysterophorus* L.	Asteraceae	Delhi/New Delhi	Witches' broom, virescence	PhND–1	MZ424214	16SrII-D
3	*Tephrosia purpurea* (L.) Pers.	Fabaceae		Little leaf, leaf yellowing	TpND–1	MZ424213	16SrII-D
4	*Ageratum conyzoides* L.	Asteraceae	Tripura/ Lembucherra	Leaf yellowing, vein clearing	AcT–1	MZ424212	16SrII-D
5	*Parthenium hysterophorus* L.	Asteraceae		Stalk curling, flat stem	PhT–1	MZ424211	16SrII-D
6	*Cleome viscosa* L.	Cleomaceae	Uttar Pradesh/ Kushinagar	Witches' broom, stunting	CvUP–1	MZ424217	16SrII-C
7	*Datura stramonium* L.	Solanaceae		Witches' broom, shoot proliferation	DsUP–1	MZ424218	16SrII-D
8	*Cannabis sativa* L.	Cannabinaceae	Uttar Pradesh/ Shahjahanpur	Witches' broom, little leaf	CsUP–1	MZ424216	16SrII-C
9	*Parthenium hysterophorus* L.	Asteraceae		Witches' broom, little leaf	PhUP–1	MZ424215	16SrII-C
10	*Ocimum canum* Sims	Lamiaceae	Uttarakhand/ Pantnagar	Little leaf, shoot proliferation	OcUk–1	MZ424220	16SrII-D

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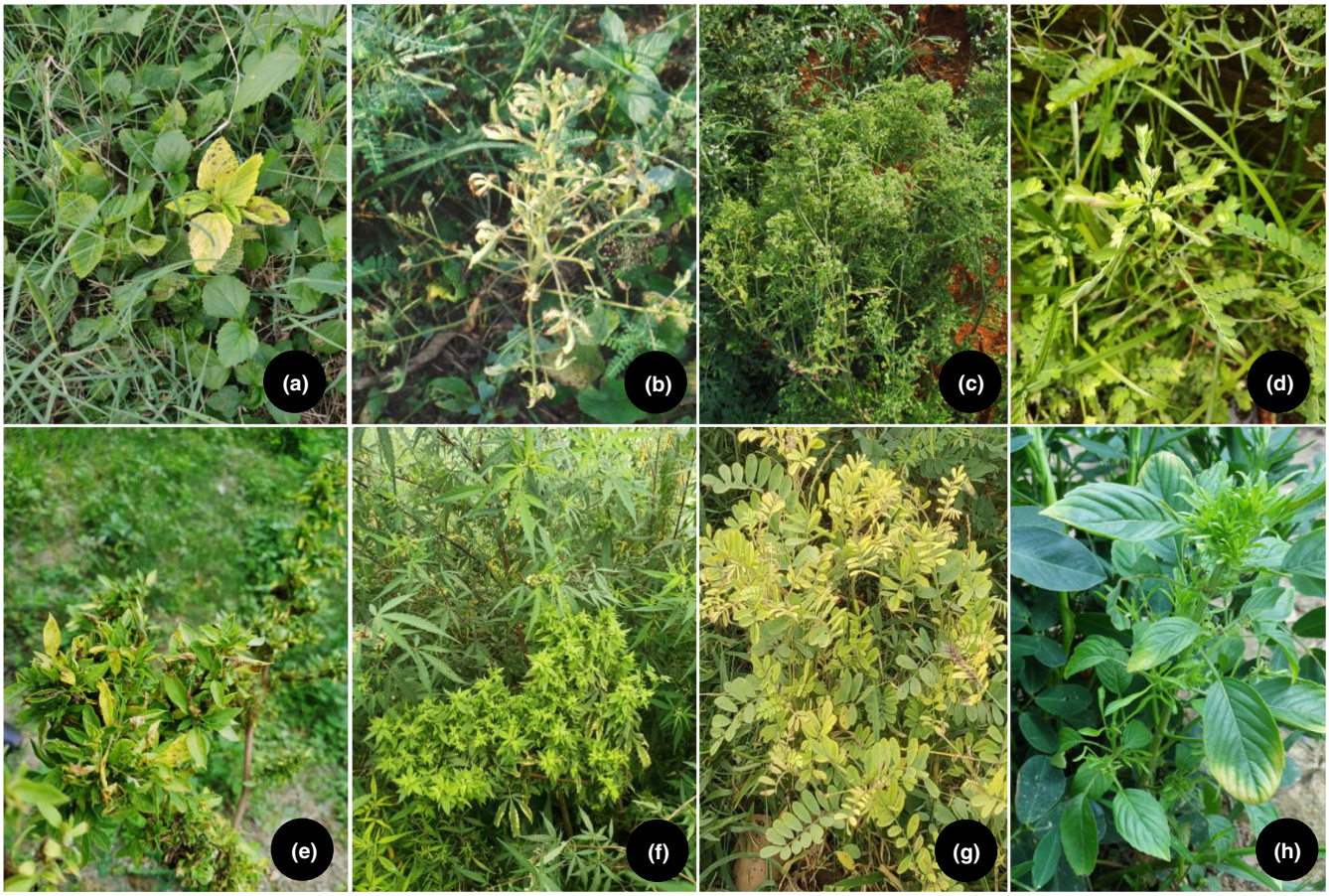

FIGURE 3 Phytoplasma symptoms observed on weed species from different regions of India. (a) Chlorosis on *Ageratum conyzoides* (Lembucherra, Tripura); (b) little leaf and stunting in *Cleome viscosa* (Kushinagar, Uttar Pradesh); (c) witches' broom on *Parthenium hysterophorus* (Shahjahanpur, Uttar Pradesh); (d) little leaf on *Phyllanthus niruri* (Bengaluru Rural, Karnataka); (e) witches' broom on *Datura stramonium* (Kushinagar, Uttar Pradesh); (f) witches' broom and little leaf on *Cannabis sativa* (Shahjahanpur, Uttar Pradesh); (g) little leaf and shoot proliferation on *Ocimum canum* (Pantnagar, Uttarakhand); (h) little leaf and chlorosis on *Tephrosia purpurea* (New Delhi, Delhi)

fourteen bamboo samples (*B. bambos*, *B. nutans*, *B. pallida*, *B. tulda*, *B. vulgaris*, *D. asper* and *D. hamiltonia*) from Bengaluru rural, *B. nutans* from Sikkim, *D. strictus* from Bengaluru Rural and Pantnagar, *P. stocksii* from Bengaluru Rural and Lembucherra produced amplicons of ~1.25 kb using the nested R16F2n/R2 primer pair and the positive controls (data not shown). No amplification was obtained in any asymptomatic bamboo samples and the negative controls (Table 1).

All eight symptomatic weed species and the leafhopper specimens collected from the different localities produced amplicons of 1.25 kb using PCR with P1/P7 followed by a nested PCR cycle with R16F2n/R2 primer pairs (Table 2). No amplification was detected in any of the asymptomatic bamboo and weed samples, or the negative controls. Amplified PCR products were sequenced, and the assembled 16S rRNA gene sequences were deposited in GenBank.

Bamboo phytoplasma strains from *B. vulgaris*, *D. asper* and *P. stocksii* from Bengaluru Rural region shared 99.92% sequence identity with strains of coconut lethal yellowing phytoplasma (Acc. No. MK617534) and *Santalum album* phytoplasma (Acc. No. MT745881) belonging to '*Ca.* P. asteris' (16SrI).

The bamboo phytoplasma strains obtained from *D. strictus* growing in Karnataka, New Delhi, Uttar Pradesh, Uttarakhand and Tripura, *D. hamiltonia*, *B. bambos*, *B. nutans*, *B. pallida* and *B. tulda* from Bengaluru Rural (Karnataka) and *P. stocksii* from Tripura exhibited 100% sequence identity with *Helianthus annuus* phyllody (Acc. No. MK421430), faba bean phyllody (Acc. No. KP869129) and sesame phyllody (Acc. No. KT005454) and members of the '*Ca.* P. australasia' (16SrII)-related strains of phytoplasma. The 16S rRNA gene sequence of the *B. nutans* phytoplasma strain from Sikkim exhibited 99.92% sequence identity with palm grass phytoplasma (Acc. No. MH551478) and members of '*Ca.* P. cynodontis' (16SrXIV).

Phytoplasma strains infecting the ten weed species (*P. niruri* from Karnataka, *T. purpurea* and *P. hysterophorus* from New Delhi, *C. viscosa*, *C. sativa*, *D. stramonium* and *P. hysterophorus* from Uttar Pradesh, *O. canum* from Uttarakhand, *A. conyzoides* and *P. hysterophorus* from Tripura; Table 2) had 99.3% similarity analysis in the 16S rRNA gene sequences with *Helianthus annuus* phyllody (Acc. No. MK421430), brinjal little leaf (Acc. No. MZ425931) and *Catharanthus roseus* chlorosis (Acc. No. MW622022) and other '*Ca.* P. australasia' (16SrII)-related strains.

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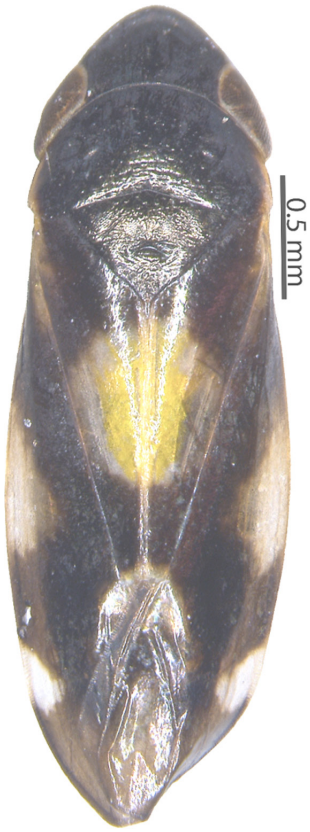

FIGURE 4 *Mukaria splendida* (Tribe: Mukarinii; Family: Cicadellidae) collected from bamboo groves in Pantnagar, Uttarakhand

The 16S rRNA gene sequences of the phytoplasmas from *M. splendida* from Uttarakhand, New Delhi and Tripura (Acc. Nos. MZ295219–21) exhibited 100% sequence identity with faba bean phyllody (Acc. No. KP869129), sesame phyllody (Acc. No. KT005454), brinjal little leaf (Acc. No. MZ429532) and other members of '*Ca.* P. australasia' (16SrII)-related strains.

3.4 | Phylogenetic analyses

A phylogram was constructed with the 16S rRNA gene sequences of various phytoplasma from bamboos, weeds and leafhoppers, from different localities in addition to sequences retrieved from GenBank. The phytoplasma was clustered in three groups. Four bamboo phytoplasma from Karnataka clustered with '*Ca.* P. asteris' (16SrI-B). One bamboo and three weed strains from Uttar Pradesh were related to phytoplasma in 16SrII-C subgroup. Sixteen phytoplasma from bamboos and seven from weeds from Karnataka, New Delhi, Uttar Pradesh, Tripura and Uttarakhand in addition to three leafhoppers from Uttarakhand, Tripura and New Delhi clustered with '*Ca.* P. australasia'-related strains (16SrII-D subgroup). Only one bamboo phytoplasma from Uttarakhand clustered with the '*Ca.* P. cynodontis' (16SrXIV-A subgroup; Figure 5).

3.5 | Virtual RFLP analyses

Virtual RFLP comparison analysis of the ~1.25 kb 16S rRNA gene sequences of phytoplasma strains from bamboo, weed and leafhopper species showed that they had similar restriction profiles to representative strains of four ribosomal subgroups (16SrI, 16SrII, 16SrVI and 16SrXIV) displaying a similarity coefficient values of 0.99–1.00 (Table 1; Figure 6A–B,E–G,F–K). However, two of the phytoplasma strains, *B. nutans* witches' broom from Sikkim (Acc. No. MZ292984) and *P. hysterophorus* stalk curling from Tripura (Acc. No. MZ424211) showed variation in the RFLP profiles when using *Dra*I and *Hha*I enzymes (Figure 6D,H), indicating that these phytoplasma strains are molecular variants.

4 | DISCUSSION

Witches' broom symptoms on bamboos have previously been attributed to a fungus, *Balansia linearis* (Mohanan, 2004). However, later the association of phytoplasmas was reported in a few bamboo species, including *Phyllostachys nigra* (Henon bamboo) infected with 16SrI group of phytoplasmas in Korea (Jung et al., 2006); *D. strictus* (Calcutta bamboo) with 16SrII group in India (Yadav et al., 2016) and *D. asper* (Betung Bamboo) and *Gigantochloa apus* (Apus Bamboo) with 16SrXIV group in Indonesia (Prasetya et al., 2020). Recently severe witches' broom symptoms suspected of phytoplasma association on *Bambusa bambos*, *Dendrocalamus asper*, *D. strictus* and *Pseudoxytenanthera stocksii* (Syn. *D. stocksii*) were recorded in Kerala and Karnataka states of India, but the cause of the diseases was not associated with any phytoplasma at the time (Mondal et al., 2019). Though India possesses a vast diversity of bamboo species, reports of phytoplasmas in bamboos are rare. Only, witches' broom disease of *D. strictus* was identified to be associated with 16SrII phytoplasma group in India (Yadav et al., 2016).

The current study confirmed the association of four phytoplasma subgroups (16SrI-B, 16SrII-C, 16Sr II-D, 16SrXIV-A) in eleven different bamboo species in India. However, samples collected from two of the states in the survey, Bihar and Tamil Nadu did not test positive for phytoplasmas. The absence of phytoplasmas from these species could be attributed to the symptoms being caused by other abiotic and/or biotic factors, which require further investigation. Finding phytoplasmas in the positive bamboo species (except for *D. strictus*) is the first report of its kind internationally.

Weeds and wild plants as hosts of phytoplasmas play an important role in the epidemiology and emergence of phytoplasma diseases of economically important crops. Phytoplasmas have been reported from several species of weeds, which could serve as inoculum reservoirs for nearby crop plants, whether annual or perennial (Rao, 2021). In the present study, eight different species of weeds were identified as hosts of 16SrII-C and 16SrII-D subgroups of phytoplasmas. The identification of 16SrII-C subgroup in *C. sativa* and *C. viscosa* and of 16SrII-D subgroup in *A. conyzoides*, *D. stramonium*,

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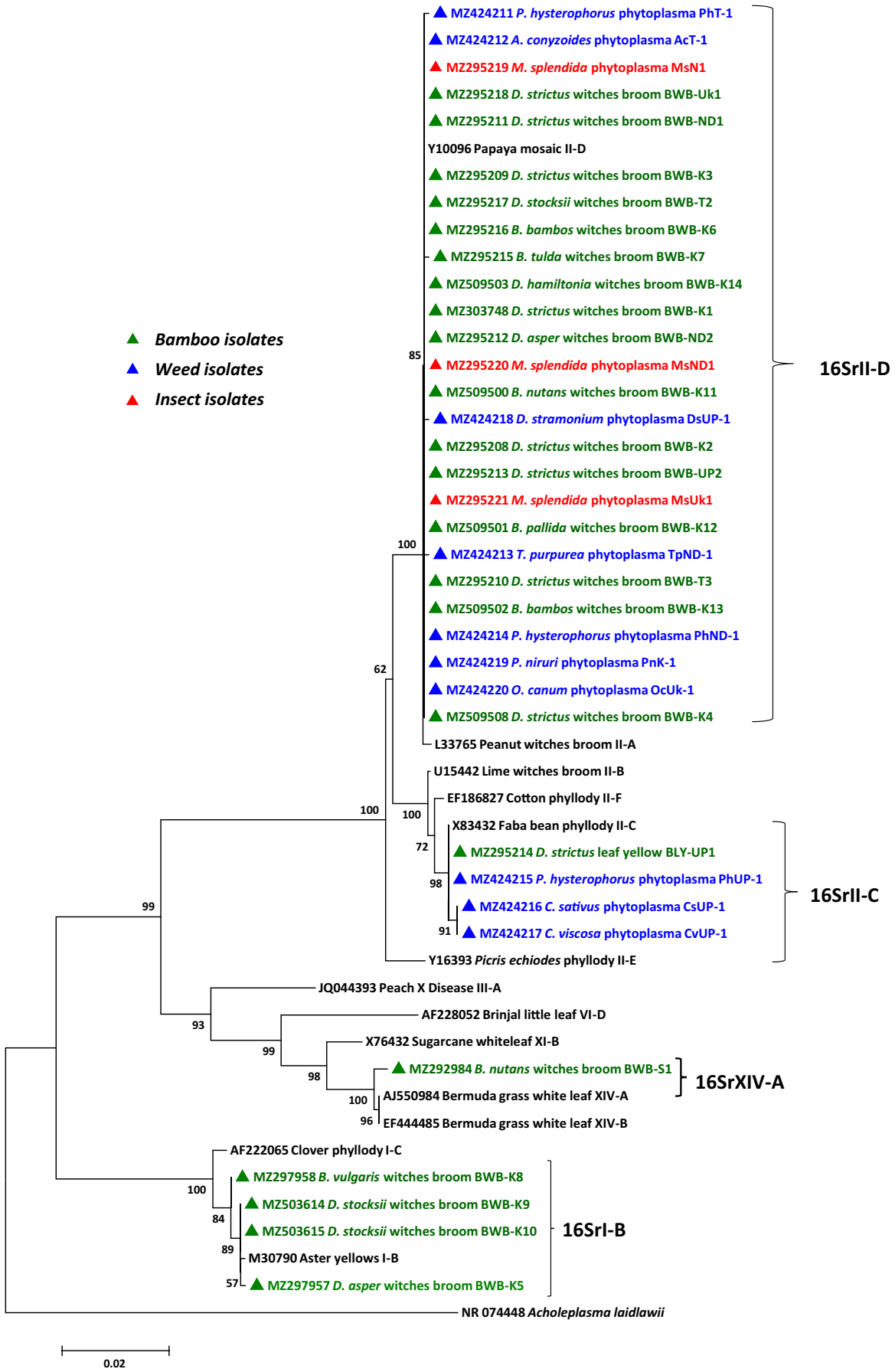

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FIGURE 5 Phylogenetic relationship of phytoplasma inciting bamboo witches' broom and leaf yellowing disease in bamboos collected from 6 different states of India compared with various GenBank submitted sequences analyzed by the neighbour-joining method using MEGA 7.0 software based on ClustalW alignment of the 16S ribosomal RNA encoding region. Bootstrap values (1000 replications) are shown as percentages at the branch points. GenBank accession number, host and isolates are listed (green colour: bamboo phytoplasma strains; blue colour: weed phytoplasma strains; red colour: leafhopper phytoplasma strains)

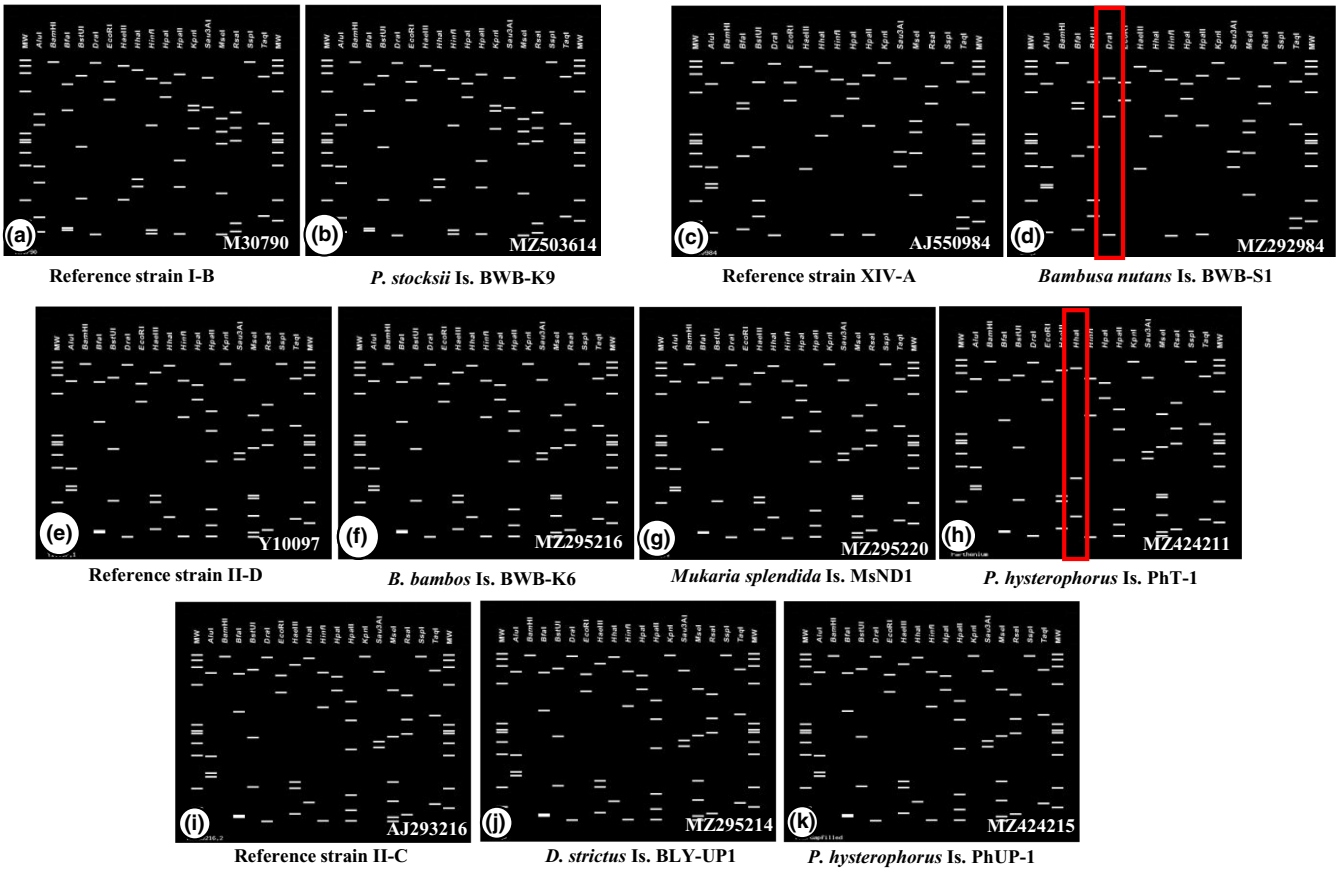

FIGURE 6 Comparison of virtual RFLP patterns derived from in silico digestion of ~1.25kb 16S rRNA sequences from reference phytoplasma subgroup with 17 different restriction endonucleases using *i*PhyClassifier tool; (a) 16SrI-B reference strain (*Oenothera* phytoplasma; Acc. No. M30790); (b) *Pseudoxytenanthera stocksii* strain BWB-K9 from Bengaluru Rural, Karnataka (Acc. No. MZ503614); (c) 16SrXIV-A reference strain (Bermuda grass white leaf phytoplasma; Acc. No. AJ550984); (d) *Bambusa nutans* strain BWB-S1 from Sikkim (Acc. No. MZ292984): The *Dra*I lane showed different restriction pattern; (e) 16SrII-D reference strain (Papaya yellow crinkle phytoplasma; Acc. No. Y10097); (f) *B. bambos* strain BWB-K6 from Bengaluru Rural, Karnataka (Acc. No. MZ295216); G: *Mukaria splendida* strain MsND1 from New Delhi (Acc. No. MZ295220); (h) *Parthenium hysterophorus* strain PhT-1 from Lembucherra, Tripura (Acc. No. MZ424211); (i) the *Hha*I lane shows a different restriction pattern; I: 16SrII-C reference strain (Cactus witches' broom phytoplasma; Acc. No. AJ293216); (j) *Dendrocalamus strictus* strain BWB-UP1 from Shahjahanpur, Uttar Pradesh (Acc. No. MZ295214); (k) *P. hysterophorus* strain PhUP-1 from Shahjahanpur, Uttar Pradesh (Acc. No. MZ424215)

O. canum and *P. niruri* are the first reports worldwide; however other groups of phytoplasmas have been identified in these weed species in India (Rao, 2021).

Leafhoppers function as vectors in the transmission of phytoplasmas (Alma et al., 2019). One leafhopper species, *M. splendida* of the bamboo-feeding leafhopper tribe, Mukarinii was collected from bamboo plantations in three states, New Delhi, Uttarakhand and Tripura, tested positive for phytoplasmas of the 16SrII-D subgroup. The detection of similar groups of phytoplasmas in weed species and leafhoppers from bamboo plantations in these states indicates the possibility of their participation in the perpetuation of the pathogen in the ecosystem.

The confirmed association of *Ca.* P. asteris and *Ca.* P. australasia-related phytoplasma strains with multiple bamboo species along with several weeds and a leafhopper species in India, suggest their widespread occurrence in various ecoregions of India. These phytoplasma strains could also inflict serious losses to other economically important agricultural crops raised in different parts of India and other potential leafhopper/planthopper species are known to transmit these phytoplasma strains (Rao, 2021). The current report of phytoplasmas of 16SrI and 16SrII groups with various novel bamboo species hosts has great epidemiological significance in India. The widespread occurrence of phytoplasma disease has been confirmed in several bamboo species, which warrants further indexing and characterization of phytoplasma

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in plantations and natural forests of similar or other bamboo species in other parts of India to plan strategic management approaches so that losses due to phytoplasma in bamboos can be minimized.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

M. Ravi involved in survey, detection and identification, sequence analysis and manuscript preparation. Govind Pratap Rao involved in survey and manuscript correction and formatting. N. M. Meshram involved in collection and identification of leafhoppers, and manuscript correction. R. Sundararaj involved in survey for bamboo samples and manuscript correction.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/nucleotide/.

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