ANALYSIS OF MOLECULAR CHARACTERIZATION OF PHYTOPLASMA ASSOCIATED DISEASES AND THEIR WEEDS BY USING NESTED PCR ASSAY

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ABSTRACT

Phytoplasma concomitant diseases acknowledged for a long time induce economic damage to a variety of cultivated, wild and various ornamental plants as well, dropping its quantum and quality gaining international importance for unspecific symptoms. In this study, the strains of phytoplasma i.e. 16SrXIV group were found an association with Cannabis diminutive leaf and witches'-broom disease. Further, actual and virtual RFLP analysis of the 16SrRNA gene of Cannabis phytoplasma segregates inveterate that it belonged to the 16SrXIV-A subgroup. BLAST examination of the sequence data of Parthenium was compared with sequences available in GenBank, which showed a maximum of 97% identity with 16SrRNA gene of members of the 1SrI 'Candidatus Phytoplasma asteris' (Ca. P. asteris) group. The utmost abundant phytoplasma species are Ca. P. cynodontis followed by Ca. P. asteris on weeds from India. Association of 16SrI, XI, XIV groups on weeds found positive in PCR/Nested PCR assays. An elevated genetic variability was observed with collected weeds. Collected maize plants were also found with the infection of 16SrIB subgroup phytoplasma.

Keywords: *Candidatus Phytoplasma asteris, Nested PCR, Phytoplasma*

INTRODUCTION

Phytoplasmas cause diseases in numerous plant species in many important foods, vegetables, fruit crops, ornamental plants, timber and shade trees. The list of diseases affected by phytoplasmas continuously growing, various newly emerging diseases, known diseases with an uncertain etiology and diseases with diverse geographic distribution have been identified in recent years with pigeon pea witches'-broom related phytoplasmas (16SrIX) in Brazil [1]. These conditions require molecular documentation of the pathogen to identify the respective phytoplasma and their vectors, in this way disease impact may be reduce in the different ecosystems. Many cultivated plants are subject to phytoplasma infection in both countries where agriculture is not advanced and in countries where agriculture is highly industrialized.

Geographically the occurrence of phytoplasmas is worldwide belonging to 98 families associated with various significant diseases in several hundred plant species reported in at least 85 nations.

Phytoplasmas are the most hazardous to herbaceous and woody plants [2] and are the initially limiting factors for various important crops all over the world. For example, the aster yellows phytoplasma causes major economic losses to vegetable crops (including lettuce, carrot and celery) and ornamental plants (including *Gladiolus*, *Hydrangea*, *China aster* and *Purple Coneflower*) in North America and Europe. Peach yellows and X-disease caused heavy losses during the 1990s in peach and cherry orchards in the United States. Phytoplasma diseases in various regions of the Middle East affect species of citrus, for example, lime witches'-broom has almost been eliminated in traditional lime production in the Sultanate of Oman and Iran [3] In many domains of south eastern Asia rice crops are highly damaged by Rice Yellow dwarf. Both phytoplasma diseases i.e. Potato witches'-broom and maize bushy stunt cause yield losses of potato and maize respectively in central and South America.

Phytoplasmas cause maladies in several weeds, which may act as alternative natural hosts facilitating the spread of phytoplasmas to other economically important plants and thereby increasing financial losses. The most peculiar symptoms observed on weeds include extensive chlorosis, the proliferation of axillary shoots, witches'-broom, yellowing and diminutive leaves. So far more than 43 weed species were reported to have phytoplasma infections from all over the world.

Nucleotide sequence studies have shown that weed-infecting phytoplasmas essentially belong to 16SrI, 16SrIV, 16SrVIII, 16SrXI and 16SrXIV groups. Among them, 16SrI and 16SrXIV phytoplasmas have a wider occurrence in nature all over the world. Even though the weeds identified as phytoplasma hosts often grow abundantly around field crops.

The molecular data of phytoplasma has considerable diversity among the various families and genetic nexus that are the main basis of several studies of phytoplasma phylogeny and taxonomy of phytoplasma [4].

A new plant pathogen which has a nature of pleomorphic, wall-less prokaryotes discovered by [5] *i.e.*known as a molecule-like organism (MLOs) also reported in the phloem of many plant species which was affected by yellow-type diseases. [6] reported that the application of mycoplasma (prokaryotes) was resolved and placed in a new taxon i.e. '*Candidatus phytoplasma*' [7].

The BGWL phytoplasma is a discrete taxon at the putative species level, for which the name *'Candidatus Phytoplasma cynodontis*' has been proposed [8, 9].[10] First reported on the occurrence of phytoplasmas in Bermuda grass in Cuba and observed that BGWL disease there is caused by a phytoplasma belonging to the 16SrXIV group. [11] first reported a 16SrII group phytoplasma in *Polygala mascatense*, a weed in Oman. Some *P. mascatense* plants showed stunting, small leaves, bushy growth and phyllody symptoms.

PCR assay confirmed the presence of phytoplasma infections causing witches'-broom in *Polygala*. RFLP results showed that the *Polygala*-infecting phytoplasma is similar to Lucerne phytoplasma (16SrII) group.

phytoplasmas can survive in many potential economical crops or because an insect vector is capable of transmitting phytoplasmas from other weeds to crops which are already known as phytoplasma hosts.

Phytoplasma is transmitted through an insect, which belongs to the members of various families such as Cicadellidae, Cixidae, Delphacidae, Psyllidae and Derbidae.

The possibilities of transmission of phytoplasmas and related diseases to important agricultural, economical and horticultural crops from weeds to the crops and vice-versa cannot be ignored. The chances of transmission seems high in the future. Therefore nested PCR assay is strongly used for the defection of Phytoplasmas so that infection can be control in the preliminary stage.

MATERIALS AND METHODS

Collection and maintenance of phytoplasma infected Maize and weed samples

In the year 2019-2020 various samples of infected maize and weed were collected. Collected samples were taken from two different fields location at Rampur U.P. Randomized Block Design (RBD) experiment was conducted at the experimental field. Control plants were treated by using chemical management, as per required and non control plants were treated by water only. In both the fields HM12 maize variety was grown.

In India, several places of different district of western and central Uttar Pradesh viz; Meerut, Bulandshahar, Gautam Buddha Nagar, Ghaziabad, Hapur, Saharanpur, Muzaffarnagar, Shamli, Moradabad, Bijnor, Rampur, Amroha, Sambhal, Bareilly, Badaun, Pilibhit, Shahjahanpur, Firozabad, Mainpuri, Mathura, Aligarh, Etah, Hathras, stray to 5% incidence of phytoplasma like disease were recorded on maize plants. Maize plants showed redness of the leaves and fruits are small in size and growth of the plants was also stunted. (Fig. 6) Only fewer places severity of incidence was observed. The symptoms indicated the possibility of phytoplasma association with the maize plants at surveyed locations. To confirm the etiology of phytoplasma on maize plant leaf samples were collected and brought to the laboratory for the further molecular analysis.

However weeds such as *Digitaria sanguinalis, Cynodon dactylon, Phragmites karka, Echinochloa crusgalli, Eleusine indica, Dinebra retroflexa, Acrachne racemosa, Dactyloctenium aegyptium, Green bristlegrass, Setaria glauca, Eragrostis tenuifolia, Trianthema portulacastrum, Solanum nigrum, Ageratum conyzoides, Dichanthium annulatum, Parthenium hysterophorus, Canabis sativa, Xanthium strumarium, Fumeria parviflora, Chenopodium album* were found near Maize fields (see Table -2). On the basis of symptomatology only *D. sanguinalis, C. dactylon, A. conyzoides, C. sativa, P. hysterophorus* were showed phytoplasma like symptoms. Symptoms are severe yellowing, little leaf while at some places white leaves are noticed, which suggested the possibility of phytoplasma disease. *Canabis* plants showed witches'-broom like appearance and little leaf; however *Ageratum* plants showed yellow leaf symptoms. The incidence of the disease was 1 to 30 % at different place of surveyed areas on weeds. Three leaf samples from *D. sanguinalis, C. dactylon, A. conyzoides, D. annulatum, C. sativa, P. hysterophorus* were collected along with non symptomatic leaf sample and brought to the laboratory for the further analysis. Detail analysis of weeds and maize phytoplasma are given below.

During survey of maize fields incidence of 2% percent leaf redness symptoms, suggestive of possible phytoplasma infection was observed on maize plants at Meerut place of Uttar Pradesh in 2019 (Fig-1 and Fig- 6). Total 5 symptomatic and 2 non symptomatic samples were collected for the characterization of associated phytoplasma D. sanguinalis plants were collected near maize grown fields at Shahjahanpur and Meerut Uttar Pradesh and the total DNA was isolated and subjected to PCR and nested PCR with universal primer pairs.

In 2020 three samples of Bermuda grass (c. dactylon) were collected from Muzaffarnagar of Uttar Pradesh. All the samples were stored at 4°C until processed for DNA extraction.
During a survey in 2020 in the Meerut district of Uttar Pradesh , many sample of *A. conyzoides* were collected from sugarcane fields with symptoms of Phytoplasma .

During the mid of 2020, *D.annulatum* Plants samples were collected at one location near Bareilly, Uttar Pradesh, diseased sample were growing along the roadsides in the Bareilly district During a survey of phytoplasma. diseases of weeds in and around agricultural fields of maize at Meerut, Uttar Pradesh, in 2020, Uttar Pradesh samples of cannabis sativa plants were collected.

The natural occurence of *P. virescence* and witches-broom was noticed on ~5-30% of *P. hysterophorus*. Plants growing widely along the roadside in Shahjahanpur districts of Uttar Pradesh in the mid of 2019. The infected plants showed excessive green, tiny narrow leaves shortening of internodes and witches-broom like symptoms. The samples were immediately used or kept in -20°C for further processing. *Catharanthus roseous* plants in the glasshouse were used as healthy plant (PCR negative for Phytoplasma) in all experiments.

Isolation of genomic DNA from symptomatic plants

The methodology [12] was used with Some modifications. 1g of plant material midrib was taken for total DNA isolation. The sample disinfected with ethanol and cut into small pieces. Grinded the plant tissue in liquid nitrogen and add 10ml DNA extraction buffer (DEB), Cetyl Trimethyl Ammonium Bromide buffer 10%, 5 M NACL, 0.5 M EDTA, 1M Tris-HCl, 100% Mercaptoethanol and was incubated at 65^{o}C for 30 min. In waterbath 10 ml of chloroform-isoamyl alcohol (24:1) was added to the mixture and centrifuged at 6000 rmp for 10 min at 4°C. After centrifugation, the aqueous layer was precipitated with two-third volume of cold, isopropanol was added and again centrifuged at 12000 rmp for 10 min at 4^{o}C. The supernatant was eliminated and the tube washed with 70% chilled ethanol, centrifuged at 10000 rmp for 20 min and dried at 37^{o}C for 30 min. Collected DNA was dissolved in 100µl of TE and stored at -20℃ for further use.

Isolation of genomic DNA from leaf hoppers

Ten leaf hoppers *Hishimonus phycitis* (same species of ten leaf hoppers) were trap on a sticky trap placed at the brinjal field, just above the plants (four traps per 100 m^2). Insects were recover from traps by spatula and were grounded in 500µl of Cetyl Trimethyl Ammonium Bromide (CTAB) buffer using sterile micropestle in a 1.5 ml eppendorf tube. (DEB-Cetyl Trimethyl Ammonium Bromide buffer 2%, 1.4M NaCl, 20Mm EDTA, 100Mm Tris-HCl, 0.2%. Mercaptoethanol). This suspension was vortexed and incubated for 30 min at 60℃ than centrifuged for 5 min at 12000 rpm at room temp. and transfer it to new 1.5 ml eppendorf tube, chloroform isoamyl alcohol (24:1) was added and inverted repeatedly.

The supernatant was transferred to new 1.5 ml eppendorf tube and added 1 volume of cold isopropanol (-20°C).The supernatant was recentrifuged at 12000 rpm 4 °C for 20 min. The pellet was dried at 37°C and re-suspend in 50µl of TE buffer.

PCR amplification

Universal phytoplasma primer pairs were used for amplification of 16SrRNA, 23SrRNA, in direct or nested PCR. (Table 1). Total PCR mix volume of PCR assay reaction is 50 µl which containing (5.0 µl of 10x PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 1.0 µl of 10.mM dNTPs, 1.0µl of Forward Primer (100 pmol. µl), 1.0 µl of reverse primer (100pmol µl), 0.5 µl of Taq polymerase (5 unit µl), 2.0 µl of Template DNA and 8.0 µl of sterile distilled water). Reactions were

performed in a Master cycler (Eppendorf Germany) and first round PCR for conserved region of the 16SrRNA gene, using P_1/P_6 primer pair at 94^{o}C for 5 min followed by 35 cycles of 94^{o}C for 45 sec, 55°C for 1 min, 72^{o}C for 2 min the last step at 72°C for 10 min. For nested PCR amplification of the 16SrRNA using R16F2n / R16R2 primer pair) at 94^{o}C for 5min followed by 35 cycle, 94^{o}C for 30 sec, 56^{o}C for 5 min, 72^{o}C for 1 min and final is 72^{o}C for 2 min. The PCR products were resolved on 2.8% agarose gel in Tris.EDTA (TAE) containing ethidium bromide [13]. 1 kb DNA ladder (Fermentas Life Sciences, Ontario,Canada) was used as molecular marker. Electrophoraris was carried out at 70-75 volts for 45 min to 1 hr, gel was observed under an UV. Transilluminator Gel-doc (Bio- Rad, USA) to observe the amplified bands. The PCR clean up system (Promega, USA) and Wizard^R SV gel were used to purify PCR products.

Purification of PCR products

The PCR clean up system (Promega, USA) and Wizard^R SV Gel were used to purify the gel slices were dissolved in membrane binding solution 10µl 10mg gel and incubated at 55°C until gel slice is completely dissolved than insert mini column in collection tube and transfer dissolved gel mixture to mine column assembly. It allow to centrifugate for 1min at 12000 rpm. Membrane wash solution 700µl (ethanol) added and again centrifugate for 1min at 12000 rpm. Mini column was transfered to clean 1.5 ml micro centrifuge tube and 50µl of nuclease free water was added to the minicolumn, after centrifugation discarded minicolumn and store DNA at 4 °C.

Sequencing, GenBank submission and BLAST analysis

Selected recombinant clones per isolate (2 to 3) and/or direct PCR products were sequenced directly in both directions using the same set of primers as for the PCR amplification at Xcelris Biotech (Bangalore, India). The sequences of PCR products were assembled using DNA baser V.4 program and were analyzed by Blast analysis (http:/www.ncbi.nlm.nih.gov/blast/). Sequences of other group and subgroup representative were obtained from GenBank. The multiple sequence alignment, pairwise alignment, sequence identity matrix and other basic analysis were carried out using BioEdit version 5.0.9 software [14]. CLUSTAL were used to generate multiple alignments sequence. [15] in sample of *D. sanguinalis*, total DNA was isolated and subjected to PCR and nested PCR with universal primer pairs. The nested PCR yielded ~ 1.2 kbp 3 products in all the symptomatic leaf samples, however, no amplicons were recorded in non-symptomatic samples. This confirmed the association of phytoplasma in symptomatic leaf. Further amplified products were, eluted and directly sequenced from both sides and used in the Blasts query.

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses of sequences were performed with MEGA 5 software programme [16] using the neighbour-joining method with default values and 1000 replications for bootstrap analysis for 16SrDNA. *Achleoplasma laidawii* sequence was used to root the tree.

Real and in silico RFLP analysis and virtual gel plotting

The 1.25 kb of 16SrDNA sequences (R16F2n/R16R2 primer primed) derived from phytoplasmas associated with maize and weeds were submitted to iPhyClassifier online tool (http://plantpathology.ba.ars.usda.gov/cgibin/resource/iphyclassifier.cgi) as described by [17]. *In silico* restriction analysis and virtual gel plotting with restriction endonucleases *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau3*AI (*Mbo*I), *Mse*I,

*Rsa*I, *Ssp*I and *Taq*I were obtained and compared with the virtual RFLP gel from16SrXI phytoplasma subgroups by the same restriction enzymes [17] (Fig. 5). Actual restriction fragment length polymorphism (RFLP) analysis of 1.2kb nested PCR product of maize & weeds phytoplasmas was performe using different restriction endonucleases viz. *Bam*HI, *Bfa*I, *and Taq*I (Fermentas,USA) (Lee et al. 1998). Restriction fragments were separated by electrophoresis through 2.8% agarose gel, stained with ethidium bromide and visualized under UV illumination.The obtained band patterns were compared with DNA standard markers, ØX174/*Hae*III marker (Fermentas) and with the reference phytoplasma isolate of 16SrXIgroup (Ac No. X76432) (NBRI, Lucknow U.P.)

RESULTS

Phytoplasma on maize

16SrDNA sequences showed the highest sequence identity with the several isolates belonging to '*Ca. P. asteris'* phytoplasma reported from different places. Based on the 16SrDNA sequence similarity and virtual RFLP pattern, the phytoplasma strain associated with maize leaf redness disease was identified as a strain of *Ca. P. asteris* subgroup B.

Digitaria sanguinalis

D. Sanguinalis plants showing chlorosis of leaves with 2-15% incidence. The study of *Digitaria sanguinalis* isolates, sequence shared the highest 99% sequence identity with several isolates of *Ca. P. asteris*. Phylogenetic analysis also supports the BLASTn results.

Cynodon dactylon

The collected samples of Bermuda grass (c. dactylon) showing whitening of the leaves and shortening of the stolons BLAST search analysis of all the three *C. dactylon* isolates revealed the highest 99-100% sequence identity with several members of the 16SrXIV group. During phylogenetic analysis, it showed a close relationship with members of the 16SrXIV group which confirmed that the present study isolates are a member of the 16SrXIV group of phytoplasma. the present study also reports the *Ca. P. cynodontis* with *C. dactylon* from Uttar Pradesh India.

Ageratum conyzoides

Many *A. conyzoides* plants growing near by sugarcane fields were found to be exhibiting little leaf symptoms accompanied by yellowing of leaf lamina, possibly characteristics of the Phytoplasma associated disease. (Fig. 7)

Cannabis sativa

Non specific yellowing, little leaf and witches- broom symptoms on *Cannabis sativa* plants followed by the death of plants were recorded BLASTn analysis of the sequence data was compared with sequences available in GenBank, showing a maximum of 97% identity with 16SrRNA gene of *Hibiscus yellows and little leaf* (FJ939287), *Plum little leaf* (GU289674), *Oilseed rape virescence* (HM590625), *Gladiolus witches'- broom* (HM590619), *Diplotaxis virescence* (HM590618), *American aster yellows* (HM590617), and *Sesame phyllody* (AB558132); all members of the 1Sr I *'Candidatus Phytoplasma asteris'* group. In phylogenetic and molecular evolutionary analyses conducted using MEGA version 5.0, the *A. conyzoides* phytoplasma isolate clustered in a clade with the members of the 16SrI group and showed only distinct relationships with phytoplasmas belonging to other 16SrRNA groups (Fig. 2).

D. annulatum

White leaf disease of *D.annulatum* was observed (Fig. 8) [18] 2-10 % disease incidence observed and the most striking symptoms of the white leaf disease affecting *D. annulatum* were excessive chlorosis, bushy growth, small leaves and stunting of the plants.

Nucleotide sequence analysis revealed that the phytoplasma detected in diseased *D. annulatum* in India is closely related to strains of the Bermuda grass white leaf (BGWL) agent (‘*Candidatus Phytoplasma cynodontis’*) whose 16SrDNA sequences are available in GenBank database, sharing with them a sequence similarity which varied from 98.1 (Malaysian strain, GenBank accession no. EU294011) to 99.1% (Chinese strain, GenBank accession no. EU999999). The name *Dichanthium* white leaf (DicWL) phytoplasma is proposed for this BGWL phytoplasma-related agent.

The phylogenetic relatedness of DicWL phytoplasma to BGWL strains and other closely related strains as well as to selected reference phytoplasmas is depicted in (Fig. 3). However, despite the close relationship, further data based on other molecular markers, cross inoculation experiments and vector transmission specificity are needed to regard DicWL phytoplasma and ‘*Candidatus Phytoplasma cynodontis’* as the same taxonomic entity.

BLASTn search analysis of the 16S rRNA sequences of *C. Sativa* sub spp. phytoplasmas shared 99% identity with phytoplasma strains of the 16SrXIV group (*‘Candidatus Phytoplasma cynodontis*’). Phylogenetic analysis of *C. Sativa* isolates revealed (Fig. 4) further their closest relationship with members of the 16SrXIV group. This confirms the association of strains of 16SrXIV group phytoplasma with *Cannabis* little leaf and witches'-broom disease (Fig - 9). Further, actual and virtual RFLP analysis of the 16SrRNA gene of *Cannabis* phytoplasma isolates confirmed that it belonged to the 16SrXIV-A subgroup.

BLASTn analysis of the sequence data was compared with sequences available in GenBank, showing a maximum of 97% identity with 16SrRNA gene of *Hibiscus yellows* and *little leaf* (FJ939287), *Plum little leaf* (GU289674), *Oilseed rape virescence* (HM590625), *Gladiolus witches'-broom* (HM590619), *Diplotaxis virescence* (HM590618), *American aster yellows* (HM590617) and *Sesame phyllody* (AB558132); all members of the 1 SrI ‘*Candidatus Phytoplasma asteris*’ group.

The PCR/nested PCR results confirmed the association of phytoplasma on *D. sanguinalis, C. dactylon, A. conyzoides, C. Sativa, P. hysterophorus, and D. annulatum* plants collected during the survey.

No amplified DNA was observed in reaction mixture containing nucleic acid from ten stored insects of same species.

We could confirm that phytoplasma is the most important disease in western & central U.P. and needs immediate attention for management and require further studies on the epidemiology of the disease. Furthermore, studies are also required to confirm the phytoplasma on weeds and maize plants at the genetic level by using PCR assays. This would clear the picture that whether the phytoplasma on maize and weeds are the same at the genetic level or different.

In our study, we reported the association of 16SrI, XI, and XIV groups on weeds found positive in PCR/Nested PCR assays. High genetic variability was observed in the present study with collected weeds. Maize plants collected in the study were also found with the infection of 16SrIB subgroup phytoplasma.

DISCUSSION

The phytoplasmas infecting the mentioned weeds, belonging mainly to 16SrI, 16SrII and 16SrVI groups are also known to induce yellows diseases of considerable economic importance in vegetable, ornamental, medicinal, forage and fruit crop plants in India [18].

The species is affected by a yellow, destructive disease named Bermuda grass white leaf (BGWL) that is associated with '*Ca. Phytoplasmas cynodontis'*, member of the BGWL phytoplasma group or 16SrXIV group, subgroups 16SrXIV-A and 16SrXIV [19]. Bermuda grass caused by the phytoplasma group (16SrXIV) and characterized by whitening of the leaves and shortening of the stolons [20, 21].

[22] Bermuda grass (*C. dactylon*) showing whitening and little leaves, bushy growth and shortened internodes, and whitening of leaves. In the present investigation are already reported [23, 24]. The association of 16SrXIV group phytoplasma with all the three collected isolates in the present investigation agrees with the earlier reports from India [23].

A. conyzoides has been reported as a host of pathogens associated with important crop diseases [25, 26], demonstrating its potential to harbour different pathogens under natural conditions.

D. annulatum is regarded as a highly esteemed fodder grass, especially in India. [18] Because the symptoms observed in India were similar to those previously described for white leaf diseases affecting other gramineous plants [27, 19, 18] This phytoplasma is, thus, a member of the same subclade, the BGWL group or 16SrXIV group.

Cannabis sativa L. has two main sub-species, Both sub-species of *Cannabis* grow quite common as weeds in India. A witches'-broom disease of hemp has been recorded in India and Iran. Main symptoms included shoot proliferation, shortened internodes, little leaves and witches'- brooms. [23] 16SrI phytoplasmas were found to be associated with the mentioned disease [28, 29] witches'-broom disease on *Cannabis* species has been associated with several phytoplasma groups including the elm yellows group (16SrV) in China [30], stolbur group (16SrXII) in Iran [31] and aster yellows (16SrI) group in India [32, 33]. [28],We are reporting the association of a member of a different phytoplasma subgroup (16SrXIV-A) with *C. Sativa* plants showing excessive apical branch proliferation, leaf yellowing and witches' broom symptoms.

*Parthenium hysterophorus*L. (family Asteraceae) is one of the 10 worst weeds in the world.

[34] Phytoplasma on weeds has already been reported by several workers in India, especially from Uttar Pradesh [35, 36]. [37] found rice yellow dwarf symptoms caused by phytoplasma in the weed, *Echinochloa colonum*. They also successfully transmitted the phytoplasma from rice to this weed. [19, 38] observed phytoplasma in *C. dactylon* and yellowing disease of *Urocha panicoides* in South India. Pleomorphic phytoplasma bodies were present in phloem tissue of infected plants [39]. They found that the application of oxytetracycline hydrochloride suppressed the symptoms temporarily. [39] Transmission of the associated phytoplasma from diseased to healthy plants using the insect vector *Hishimonus phycitis* was also accomplished. [40] reported on a white leaf disease of *Imperata arundinacea* caused by phytoplasma. *Cannabis sativa* (Cannabinaceae) and *Achyranthes Aspera* (Amaranthaceae) showing witches'-broom and yellowing symptoms in India were also found to be associated with 16SrI group phytoplasma infections [28, 35]. Many weeds with phytoplasma diseases from India, especially from eastern Uttar Pradesh in the last decades have been reported such as leaf phyllody of *Phyllanthus fraternus, the* grassy and bunchy shoot of *Cenchrus ciliaris, the* bunchy shoot of *Dactyloctenium aegyptium,* white leaf of *Imperata arundianacea*, white leaf of *Cynodon*

dactylon, Oplismenus burmannii & *Dichanthium annulatum*, veinal chlorosis of *Digitaria sanguinalis*, little leaf of *Datura inoxia*, leaf yellowing of *Amrantheus sp*, yellow leaf of *Achyranthes aspera* and little leaf, leaf yellowing of *Ageratum conyzoides* [41, 23, 18, 42, 43, 44, 36].

Datura stramonium L., which is known as Jimsonweed (Family: Solanaceae) is an annual invasive weed species found widely grown in India and has been found with little leaf and witches'-broom disease at the Gorakhpur location. The phytoplasma of clover proliferation group (16SrVI) was reported by [45] for the first time worldwide. Later, at a similar location (Gorakhpur), *Ranunculus sceleratus* L., family Ranunculaceae, was found to be associated with little leaf disease and the associated pathogen was identified as *Ca. P. cynodontis* (16SrXIV) group [45], which was also first time identified from any part of the world.

[46] found *Phyllanthus niruri* plants growing wild showed symptoms of little leaf and leaf chlorosis. Phylogenetic analysis further confirmed the clustering of *P. niruri* phytoplasma with strains from the 16SrI group. However, it was earlier reported [47] simply based on an electron microscopy examination from Lucknow.

A very recent, [48], reported the symptoms of yellowing, little leaf and witches'-broom on *Cannabis sativa* L. ssp. *Sativa* and *C. sativa* L. ssp. indica plants at Shahjahanpur, location in UP. [46] Phylogenetic analysis of the 16SrDNA sequences of both phytoplasma isolates revealed their closest relationship with members of the 16SrXIV group. Actual and virtual RFLP analysis of the 16SrDNA sequences confirmed that they belonged to the 16SrXIV-A subgroup. [23] This was considered the first world report of a 16SrXIV-A subgroup phytoplasma infection in *C. Sativa* sp. *Sativa* and *C. Sativa* sp. Indica plants. However, earlier, the association of aster yellows (16SrI) group was reported from Lucknow and Gorakhpur locations on *Cannabis* plants [28, 32].

[49], reported the strong leaf chlorosis, shortened stolons rhizomes, stunting and witches'-broom symptoms on *C. dactylon* and established the relation between *C dactylon* phytoplasma and *E indicus* insect found near the field. The 16SrDNA sequences of phytoplasma from symptomatic *C. dactylon* and *E. indicus* were compared and found 99% similar. The identification of *'Ca. P. cynodontis'* and 99% 16SrDNA sequence identity between *C. dactylon* and *E. indicus* phytoplasmas, suggested that the latter may be a putative vector for 16SrXIV group phytoplasmas and may play a role in the spread of '*Ca. P. cynodontis'* strains in nature.

More than 20 weeds have been reported with different phytoplasma species from India to date. The most abundant phytoplasma species are *Ca. P. cynodontis* followed by *Ca. P. asteris* on weeds from India.

In our study, we reported widespread occurrence of phytoplasma disease on several weeds growing near maize fields and also on maize plants in the present investigation in western Uttar Pradesh based on symptomatology. The symptom was so prevalent and affected plant could produce small fruit in maize. [50] revealed phytoplasma bodies in phloem cells of diseased *C. dactylon* plants collected near Varanasi, U.P., India and proved the causative role of the phytoplasma bodies with the disease.

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Authors' contributions
A. Saeed Contributed the topic selection, Preparation of materials, collection of data from various points S.A.H. Andrabi, R.A. Mir Contributed in the adopted methodology and V. Kumar Contributed to read, analysis of data and review process of manuscript.

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Table 1. Phytoplasma universal/group-specific primers are used for conventional PCR assays

Primer pair	Sequence	Target gene	Amplicon size (bp)	Reference
P1/P6	5'-AAGAGTTTGATCCTGGCTCAGGATT-3' 5'-TGGTAGGGATACCTTGTTACGACTTA-3'	16S rRNA	~ 1500	[51]
P1/P7	5'-AAGAGTTTGATCCTGGCTCAGGATT-3' 5' -CGTCCTTCATCGGCTCTT-3'	16SrRNAand 16S-23S rRNAISR	~ 1800	[52]
R16F2n /RI6R2n	5'-GAAACGACTGCTAAGACTGG-3' 5'-TGACGGGCGGTGTGTACAAACCCCG-3'	16S rRNA	~ 1250	[53]

Table 2. *Details of weeds sample collected, their symptoms, PCR results and group/sub-group classification*

Samples name (Place)	PCR	Nested PCR	incidence	Symptoms	16Sr-Group
Digitaria sanguinalis	-	+	2-15%	Chlorosis of leaves	16SrI
Chenopodium album	-	-	-	-	-
Fumeria parviflora	-	-	-	-	-
Xanthium strumarium	-	-	-	-	-
Canabis sativa	-	+	1-30%	Witches – broom like appearance and little leaf	16SrXIV-A
Parthenium hysterophorus	+	+	1-24%	Excessive Green, tiny narrow leaves, Shortening of internodes and wiches-	16SrI

				broom	
Dichanthium annulatum	-	+	1-10%	White leaf disease	16SrXI
Ageratum conyzoides	-	+	1-20%	Yellow leaf lamina	16SrI-B
Solanum nigrum	-	-	-	-	-
Trianthema portulacastrum	-	-	-	-	-
Eragrostis tenuifolia	-	-	-	-	-
Setaria glauca	-	-	-	-	-
Setaria viridis	-	-	-	-	-
Dactyloctenium aegyptium	-	-	-	-	-
Acrachne racemosa	-	-	-	-	-
Dinebra retroflexa	-	-	-	-	-
Eleusine indica	-	-	-	-	-
Cynodon dactylon	-	+	1-15%	Whitening of leaf and shorting of the stolons	16SrXI V
Phragmites karka	-	-	-	-	-
Echinochloa crusgalli	-	-	-	-	-

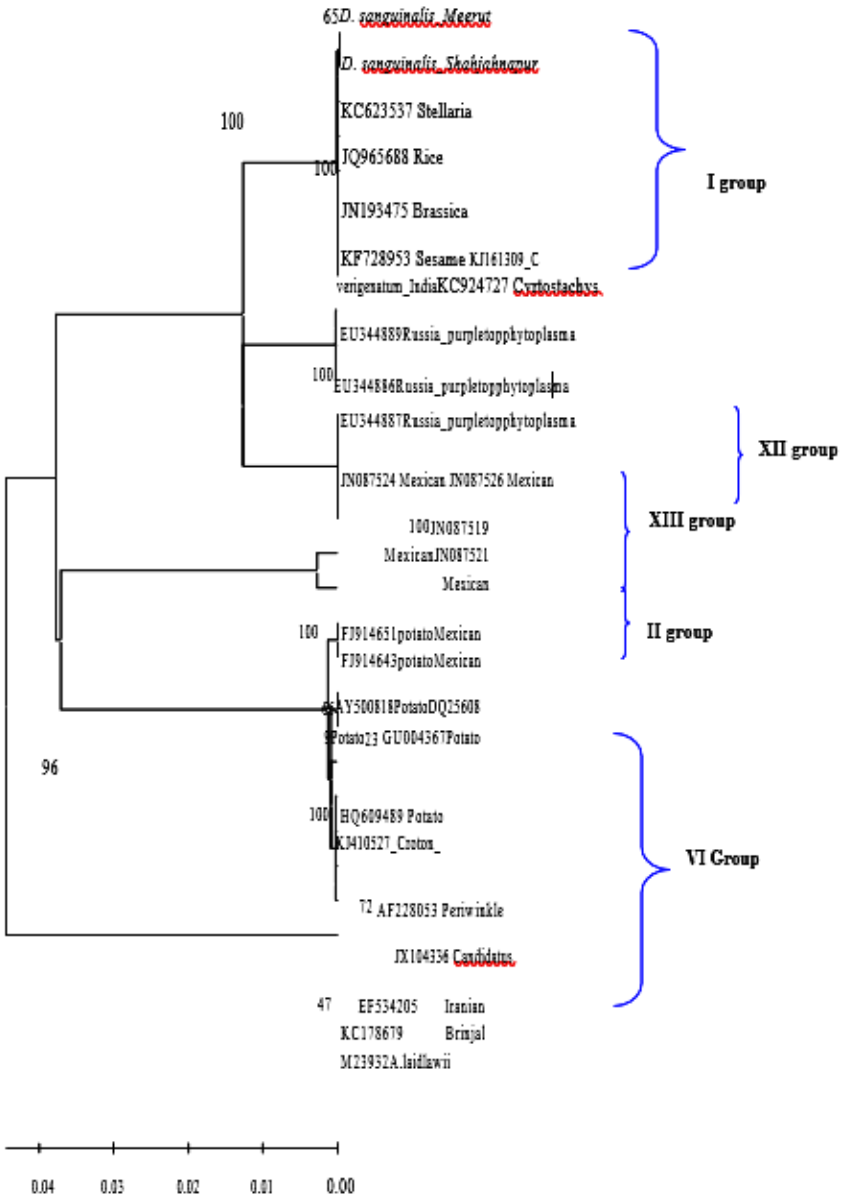

Fig. I Phylogenetic analysis of Shahjahanpur and Meerut isolates showing close relationship with 16SrI group of Phytoplasma.

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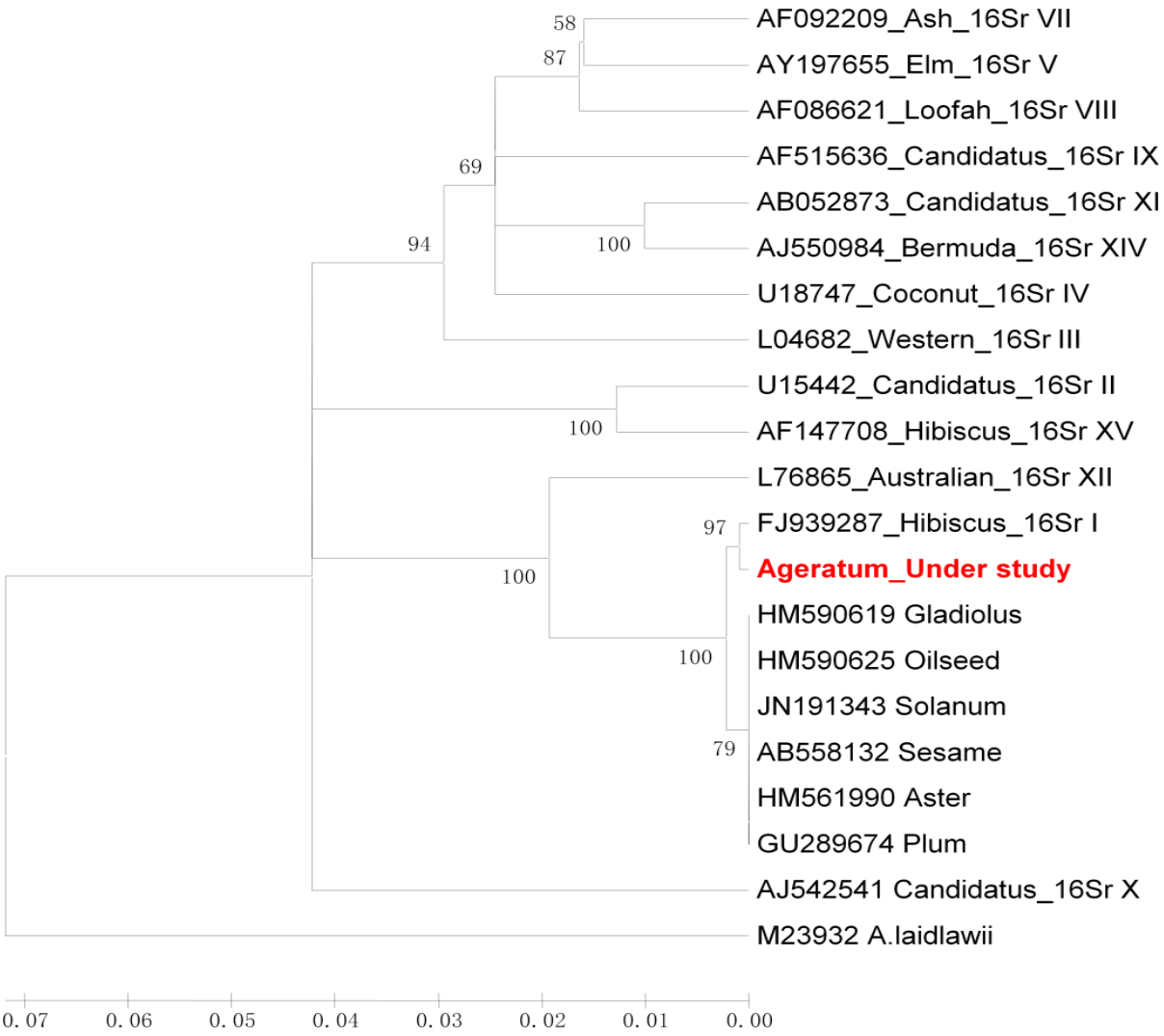

Fig. 2 Phylogenetic analysis of *Ageratum* isolate showing a close relationship with different reported isolates of 16Sr I group of phytoplasma.

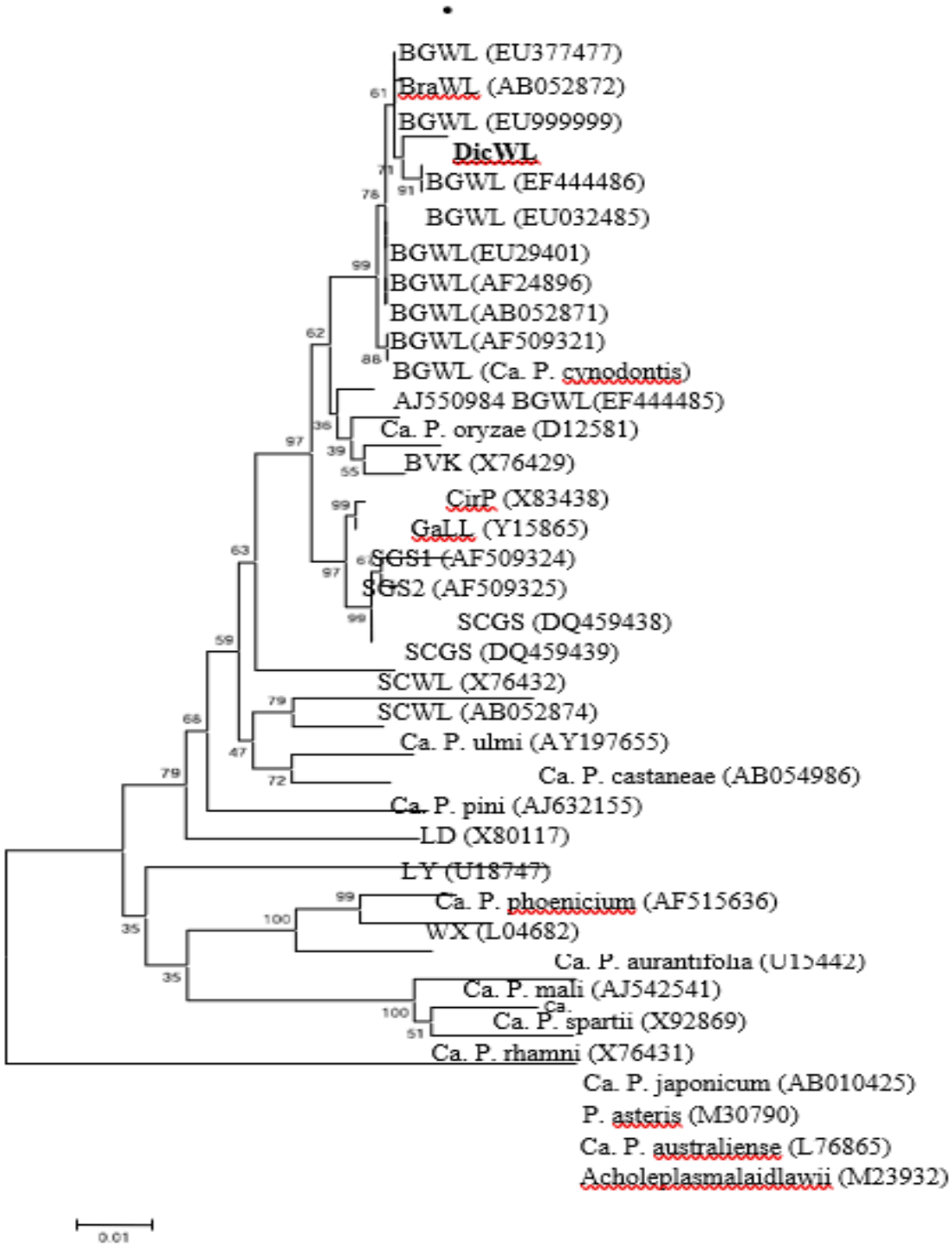

Fig. III Phylogenetic analysis of *D. ammulatum* isolate showing close relation to BGWL reported from different parts of the world

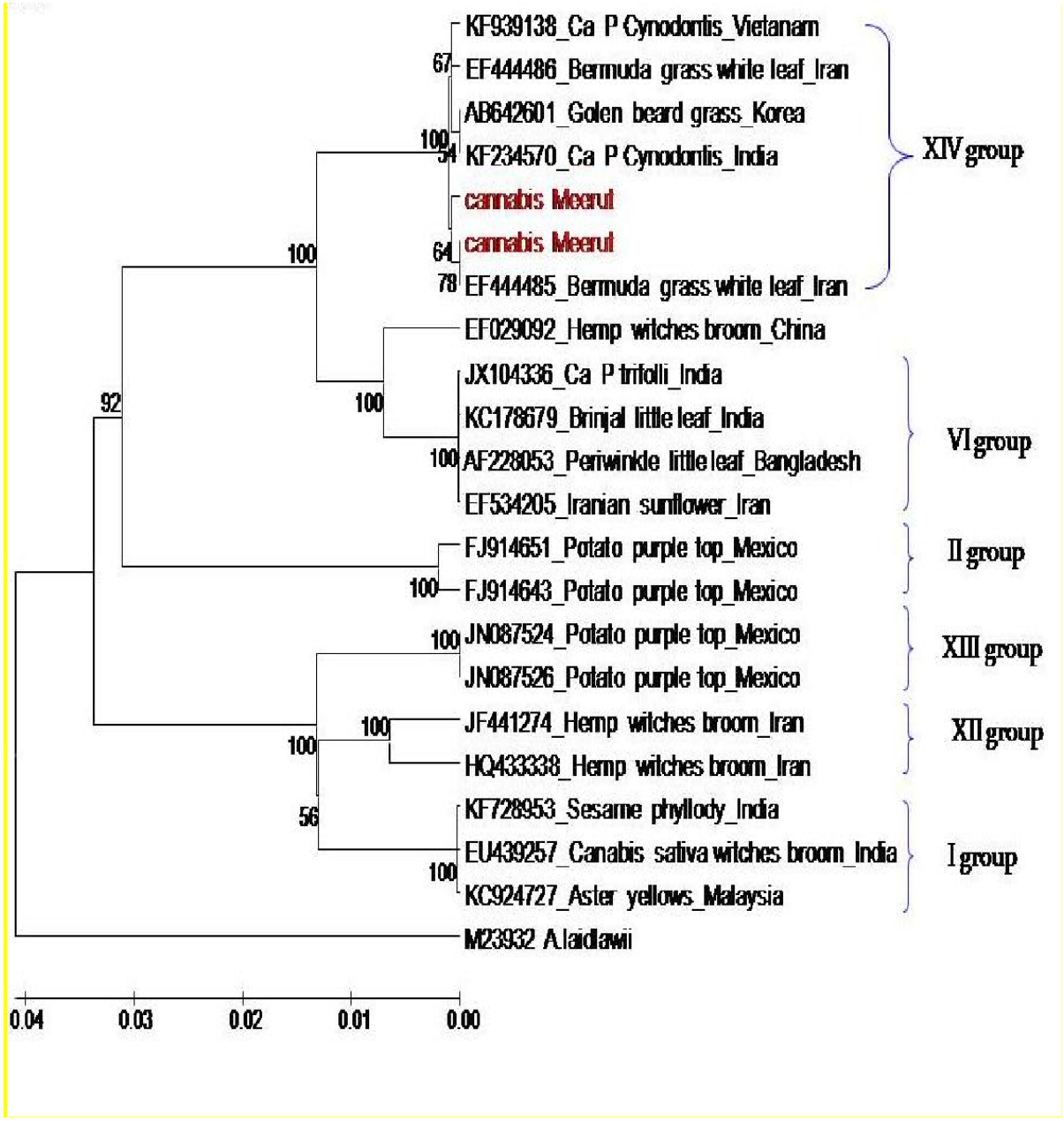

Fig. 4 Phylogenetic tree constructed by the neighbour-joining method showing the relationships of *Cannabis* phytoplasma isolates (India) and reference phytoplasma strains (Accession numbers are specified on the tree nodes. Acholeplasma laidlawii was used as an outgroup. The tree was Constructed by using Mega 5.0 software. Numbers on branches are bootstrap values obtained for 1000 replicates).

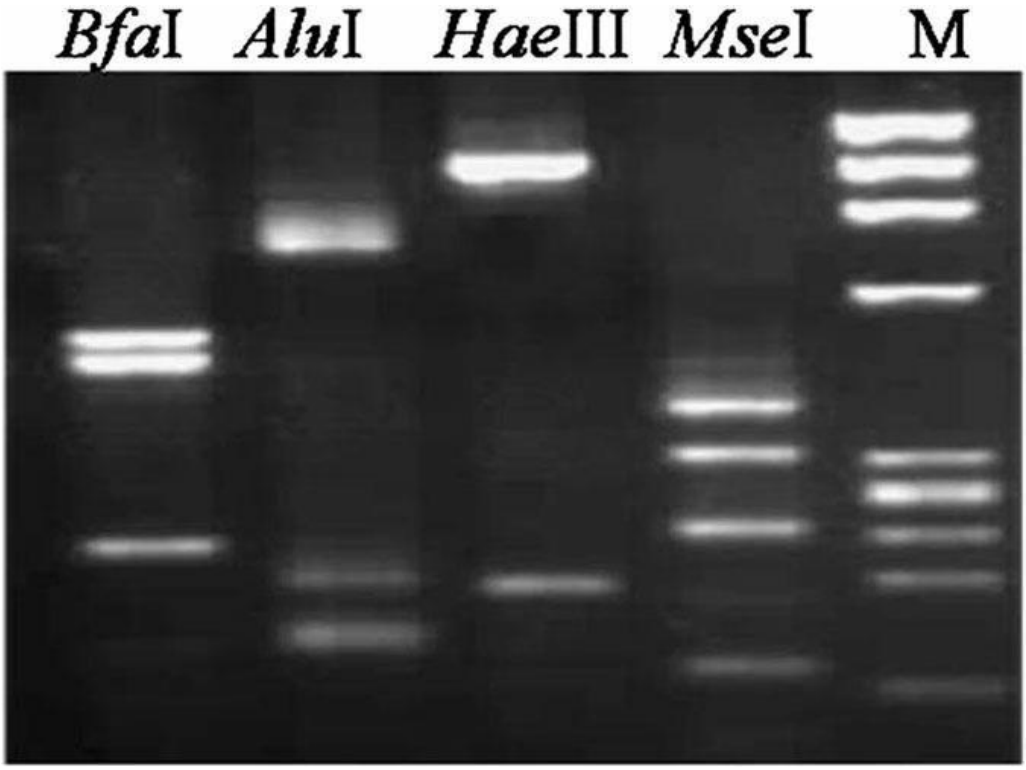

Fig. 5 RFLP analysis of 1.2 Kb PCR products from symptomatic *Cannabis sativa* with restriction enzymes *BfaI, AluI, HaeIII and MseI, M: HaeIII-digested øX174* marker

Fig. 6 Maize leaf redness symptoms

Fig. 7 Ageratum plants showing leaf yellows and little leaf symptoms

Fig. 8 *D. annulatum* were excessive chlorosis, bushy growth, small leaves and stunting of the plants

Fig. 9 (a-d).*Cannabis sativa* plant showing (a) excessive apical branching giving a witches' broom appearance to the affected plants ; (b) typical yellowing of leaves with stunting and little leaf symptoms; (c) stunting and shortening of internodes of affected plants with little leaves ; (d) typical witches' broom and dying symptoms