Characterization of a novel strain of Candidatus Phytoplasma aurantifolia infecting cowpea (Vigna unguiculata) based on 16S rDNA sequence analysis

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Research Article

Keywords: Cowpea, phytoplasma, bud proliferation

Posted Date: January 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1278395/v1

Abstract

An attempt was made to characterize the Phytoplasma in suspected cowpea (*Vigna unguiculata* L.) samples showing symptoms consisting of bud proliferation and stunting at Experimental Farm, ICAR-NBPGR, New Delhi during *Kharif* 2020. Associated phytoplasma was characterized based on sequence analysis of target 16S rDNA fragments of 1247 bp amplified from symptomatic samples of cowpea by nested-polymerase chain reaction assay using universal phytoplasma specific primers pairs (P1/P7 and R16F2n/ R16R2). The 16S rDNA sequences understudy shared 98.6% similarity with that of the *Candidatus* Phytoplasma aurantifolia (GenBank accession: U15442). In phylogenetic analysis, the target 16S rDNA fragment from two samples (Cow-1 and Cow-2) clustered with strains of the 16SrII group of *Ca.* Phytoplasma aurantifolia. The virtual RFLP pattern showed the highest similarity with the reference pattern of the 16SrII-D subgroup (GenBank accession: Y10097) with a coefficient of 0.97. Since obtained coefficient value is equal to the threshold (0.97) for designating a different strain. Therefore, this phytoplasma infecting cowpea is proposed to be a new strain of *Ca.* P. aurantifolia as 16SrII-Ysubgroup.

Introduction

Cowpea (*Vigna unguiculata* L.) is one of the most important legumes across the semi-arid tropics valued for its pods and dried seeds (Kumar et al. 2012). Pests and diseases are the major biotic stresses that decrease yield, raise production costs, and limit the storability and marketability of food and feed. Among biotic stresses, phytoplasma diseases have been reported to infect a large number of plants that includes ornamentals, fruits trees, vegetables, cereals, legumes and grapevines worldwide. Phytoplasma is prokaryote without cell-wall restricted to sieve tubes in plants and transmitted by phloem-feeding insects. They have also been reported to infect many leguminous crops such as greengram (Hameed et al. 2017), chickpea (Latha et al. 2021), French bean (Arocha et al. 2008), cowpea, pigeonpea and lentil (Rao et al. 2017a).

Association of phytoplasma caused by *Candidatus* Phytoplasma asteris, a 16SrI-B subgroup in cowpea showing bud proliferation on the main shoot and stunting was first reported from India by Kumar et al (2012). Subsequently, Thorat et al (2016) and Rao et al (2017b) reported the association of respectively, *Ca*. P. aurantifolia, 16SrII-D subgruop and *Ca.* P. cynodontis, 16SrXIV-A subgroup from cowpea plants showing little leaf, witches' brooms and flat stem symptoms. Phytoplasma 16SrVI-A subgroups have also been reported infecting cowpea from Iran (Kardani and Jamshidi 2018) and 16SrXIV- A subgroup from Iraq (Al-Kuwaiti et al. 2019). Besides these reports, some sequences of phytoplasma obtained from cowpea have also been submitted at NCBI database indicating the presence of phytoplasma16SrII-peanut WB group from China (KC953009 to KC953019) and Taiwan (KU170534 and KU170535).

Symptoms of bud proliferation and stunting suspected to be caused by phytoplasma were noticed in cowpea (accession number IC16966) growing at Experimental Farm, New Area, ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR), New Delhi. Since there is meagre information available on the association of phytoplasma in cowpea from India, the present investigation was carried out to identify

the phytoplasma in symptomatic cowpea and to study diversity in cowpea infecting phytoplasma, if any in India.

Materials And Methods

This study was undertaken jointly at ICAR-NBPGR, New Delhi and ICAR-IIPR, Kanpur, India during the year 2020-21.

Collection of samples and DNA isolation: Five samples (Cow-1, Cow-2, Cow-3, Cow-4 and Cow-5) of cowpea genotype (accession no. IC016966) showing bud proliferation and stunting along with two healthy samples (Cow-6 and Cow-7) collected from Experimental Farm, New Area, ICAR-NBPGR, New Delhi (**Fig.** 1a & b) were brought to the laboratory. Total DNA was extracted from 100 mg of symptomatic as well as healthy plant parts using DNeasy Plant Mini Kit (QIAGEN GmbH, Hilton) following the manufacturer's instructions. The DNA extracted from the phytoplasma affected chickpea sample (Akram et al. 2016) was used as a positive control.

Amplification and cloning of 16Sr DNA fragments: Phytoplasma-specific primers P1 (5' AAGAGTTT GATCCTGGCTCAGGATT 3') and P7 (5' CGTCCTTCATCGGCTCTT 3') (Deng and Hiruki, 1991; Smart et al., 1996) amplifying ~1,800 bp fragment of 16Sr DNA were used to detect phytoplasma in first-round PCR. Another primer pair (R16F2n-5'GAAACGACTGCTAAGACTGG3'/ R16R2-5'TGACGGGCGGTGTGTACAAAACCCC3') which amplify a DNA fragment of ~1,250 bp from a portion of the 16S rDNA was used in nested-PCR (Lee et al. 1998). The first round-PCR and nested-PCR were conducted simultaneously in a total volume of 50 µl PCR reaction mix prepared using Dream Taq Green Master Mix 2X (Fermentas) containing 25 µl 2x master mix, 25 pmol each primer, 2 µl template DNA and nuclease-free H_2O. The PCR conditions involved an initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 2 min for P1/P7 and 56°C for 1 min for R16F2n/R16R2, primer extension at 72°C for 2 min and final extension at 72°C for 10 min. PCR products were separated by electrophoresis in 1% (w/v) agarose gel prepared in a 1xTAE buffer. The DNA was stained with ethidium bromide added to the gel. The DNA bands were visualized on a UV trans-illuminator and photographed using a mobile digital camera. The DNA extracted from two healthy plants and double-distilled water (negative control) was used as an experimental control. The DNA extracted from the phytoplasma affected chickpea plant (Akram et al. 2016) was used as a positive control.

The amplicons of the expected size ~1250bp were excised from the gel and purified using Nucleopore Gel/ PCR clean up kit. The concentration of purified fragments was measured using Nanodrop 2000 spectrophotometer (Thermo Scientific) and the 50 ng DNA was ligated into CloneJET/1.2 blunt vector and transformed in *E. coli* (DH5á) cells using CloneJET and Bacterial Transformed Aid Kits (Fermentas) following the manufacturer's instructions. Initially, the transformed bacterial cells were confirmed by colony-PCR using primers (pJETF/pJETR) and finally by using restriction digestion of the plasmids. The cloned 16S rDNA fragments were sequenced from both sides through the sequence service provider (Genotypic Technology). For each sample, two clones were sequenced.

Sequence analysis: The sequence data obtained were blasted and trimmed using Bioedit (v.7.2) to remove the vector sequences. Since the sequences obtained from five samples were identical, only two representative 16S rDNA sequences were submitted to GenBank under accession no. MW827058 and MW827059. These sequences were subjected to the program iPhyclassifier online tool (http:// plantpathology.ba.ars.usda.gov/ cgi-bin/ resource/ iphyclassifier. cgi) for further analysis. The computer-simulated restriction analysis of the subjected sequences (MW827058 and MW827059) generated the restriction profile with 17 restriction enzymes (AluI, BamHI, BfaI, BstUI, DraI, EcoRI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, Sau3AI, MseI, RsaI, SspI and TaqI) which gave output in the form of a virtual gel. The 16Sr DNA sequences obtained from the present study and phytoplasma sequences consisting of one representative sequence of each known phytoplasma group, subgroups of 16SrII and those infecting cowpea (Table 1) retrieved from NCBI database were used to generate phylogeny using MEGA X (Kumar et al. 2018).

Results

Field observations: During *Kharif* 2020, symptoms of bud proliferation and stunting were observed in cowpea genotype (IC016966) (**Fig.** 1a & b) with 4% incidence. The symptoms like bud proliferation on the main shoot and stunting (Kumar et al. 2012) and little leaf, witches' broom, leaf yellowing and stem fasciations (Thorat et al. 2016; Rao et al. 2017b, Al-Kuwaiti et al. 2019) have been reported to be caused by phytoplasma in cowpea. Similarly, symptoms of little leaf and thickened leaves, phyllody, the proliferation of shoot, wrinkled and malformed leaves, stem fasciations and stunting associated with phytoplasma infection in cowpea have also been reported from Iran (Kardani and Jamshidi 2018).

Molecular characterization: In the present study, the nested-PCR using universal primer pairs, P1/P7 and R16F2n/R16R2 gave positive results (**Fig.** 1d) with all the five cowpea samples (Cow-1 to Cow-5) showing symptoms of bud proliferation and stunting symptoms typically associated with phytoplasma infection in plants, whereas negative results were obtained with the healthy samples (Cow-6 and Cow-7). The presence of the DNA fragments of expected size ~1247 bp in the PCR products of all the five samples (amplified by R16F2n/R16R2 primers pair) in the gel confirmed the association of the phytoplasma in symptomatic cowpea plants. These amplicons were successfully purified from the gel and cloned into pJET/1.2 cloning vector. The positive clones identified by colony-PCR were used to isolate plasmids. The plasmid DNA was subjected to restriction digestion released desired DNA fragments confirming them to be the correct clones (**Fig.** 1e). Two such clones of each sample were sequenced. Both the sequences obtained were found 100% similar. Only two sequences were, however, submitted at NCBI database under the accession numbers MW827058 and MW827059 and analyzed by *i*PhyClassifier (Zhao et al. 2009).

Sequence analysis: The sequence analysis of the sample Cow-1 and Cow-2 confirmed the presence of phytoplasma infection in cowpea samples tested as these sequences have a phytoplasma-specific partial rRNA operon and a partial 16S–23S rRNA intergenic spacer. Sequences of 16S rRNA gene of these isolates subjected to *i*PhyClassifier for species/subspecies identification. Both of the sequences obtained in this study showed 98.6% identity with that of the reference strain of *Ca*. P. aurantifolia (GenBank

accession: U15442). This indicated that the phytoplasma understudy is closely related to *Ca*. Phytoplasma aurantifolia.

The virtual RFLP pattern derived from the query 16S rDNA F2nR2 fragment (MW827058) indicated most similarity with a reference pattern of the 16Sr group II, subgroup D (GenBank accession: Y10097) with a similarity coefficient of 0.97, a threshold to designate/consider a different strain (Zhao et al. 2009). Among 17 restriction enzymes used to generate virtual RFLP, *Hae*I restriction enzyme gave different RFLP pattern from reference strain (Y10097-16SrII-D-Australia-Papaya). The enzyme (*Hae*I) released four DNA fragments in reference strain (Y10097), whereas in understudy (MW827058) phytoplasma, it produced five DNA fragments (Fig. 1c & f). Thus, the phytoplasma found associated with cowpea samples in the present study is a new subgroup of *Ca*. Phytoplasma aurantifolia under the 16SrII group.

Discussion

In this study while compiling this manuscript, 24 subgroups designated after English alphabets A to X (Yang et al. 2017; Al-Subhi et al. 2017; Omar et al. 2020) have been reported. The 16S rDNA sequence generated in the present study and 61 sequences of different phytoplasma sequences representative of each known groups and 16SrII subgroups retrieved from GenBank were used to construct phylogeny by neighbour-joining method with MEGA X (Kumar et al. 2018). Phylogenetic analysis revealed that all the strains of phytoplasma belong to 16SrII group formed a major cluster. Further this cluster (16SrII group) was subdivided into clad for each subgroup. The present strain of Ca. Phytoplasma aurantifolia formed separate clad (**Fig.** 2). It is therefore proposed to name the phytoplasma associated with cowpea disease with symptoms of bud proliferation and stunting at Delhi as *Ca*. Phytoplasma aurantifolia, 16SrII-Y subgroup.

Phytoplasma 16SrI-B (*Ca.* Phytoplasma asteris) and 16SrXIV-A (*Ca*. P. cynodontis) from India (Kumar et al. 2012; Rao et al. 2017a & b; Rao 2021), 16SrVI-A subgroup (Clover Proliferation Group) with similarity coefficient 1.00 from Iran (Kardani and Jamshidi, 2018) and 16SrXIV-A subgroup (Bermuda White Leaf Group) from Iraq (Al-Kuwaiti et al. 2019) infecting cowpea have been reported. The 16SrXII-B strain of '*Ca*. Phytoplasma australiense' associated with witches'-broom and small leaves of *V. unguiculata* var. *sesquipedalis* (snake bean) has been reported from Australia and shown to be infecting cowpea upon transmission (Saqib et al. 2006). Based on the sequences available at NCBI database (**Table** 1), 16SrII (peanut WB group) has been reported to infect cowpea from China (KC953009 to KC953019) and Taiwan (KU170534 and KU170535).

Phytoplasmas have been found associated with several diseases in plant species including crops and causes significant economic losses. Cowpea is one of the most important legumes used as pods, dried seeds and fodder in many places of the world. Various phytoplasma groups and subgroups are reported infecting cowpea crops from Iraq, Iran, India, China, Taiwan and Australia. However, in the present study, we reported that the phytoplasma strain is different from all the previously established 16SrII subgroups, which appears to represent a new subgroup within the 16SrII from India. Reports of association of

phytoplasma with different plant species including agri-horticultural crops including legume crops are increasing. It is therefore imperative to conduct a countrywide survey to understand the current status of phytoplasma diseases per se and to decipher the diversity of this pathogen in the changing scenario of climatic conditions.

Declarations

Funding: Not applicable

Conflicts of interest/Competing interests: The authors declare that they have no conflict of interests.

Availability of the data and material (data transparency): Not applicable

Code availability (software application or custom code): Not applicable

Ethics approval: The research does not involve any human participation and/or animals. The materials in the article have not been published in whole or in part elsewhere and not currently being considered for publication in another journal

Consent to participate (include appropriate statement): Yes

Consent for publication (include appropriate statement): Yes

Acknowledgements

The authors wish to express sincere thanks to ICAR, New Delhi, Director, ICAR-NBPGR, New Delhi and Director, ICAR-Indian Institute of Pulses Research, Kanpur for providing laboratory facilities.

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Table

Table 1. Details of the phytoplasma sequences used and some selected sequences retrieved from NCBI database.

.	Host	Associated Disease	Group	Sub Group	Accession number	Country	Reference
	Cowpea	Cowpea bud proliferation	*Indian cowpea bud proliferation*	16SrI	HM449952	India	Kumar et al. 2012
	Cowpea	Cowpea bud proliferation	*Vigna unguiculata bud proliferation phytoplasma*	16SrI	OK586501	India	Unpublished
	Cowpea	Cowpea bud proliferation	*Vigna unguiculata bud proliferation phytoplasma*	16SrI	OK586500	India	
	Soybean		*Ca. P. costaricanum*	16SrII	HQ225630	Costa Rica	Lee et al. 2011
	Cowpea	Peanut Witches broom	*Ca. P. aurantifolia*	16SrII	KP677497	India	Unpublished
	Cowpea	Peanut Witches broom group	*Vigna unguiculata* subsp. *Sesquipedalis phytoplasma*	16SrII	KU170535	Taiwan	Unpublished
	Cowpea	Peanut Witches broom group	*Vigna unguiculata* subsp. *Sesquipedalis phytoplasma*	16SrII	KU170534	Taiwan	
	Cowpea	Peanut Witches broom group	*Vigna unguiculata* subsp. *Sesquipedalis phytoplasma*	16SrII	KU170533	Taiwan	
	Cowpea	Peanut Witches broom	*Cowpea phyllody phytoplasma*	16SrII-A	KC953001	China	Unpublished
	Medagaskar	Peanut		16SrII-A	L33765	USA	Gundersen

periwinckle	Witches broom phytoplasma					et al. 1994
Key Lime	Peanut Witches broom	*Ca. P. aurantifolia*	16SrII-B	U15442	France	Zreik et al. 1995
Faba bean	Fababean phyllody		16SrII-C	X83432	Germany	Choueiri et al. 2005
Papaya	papaya yellow crinkle disease	*Ca. P. australasia*	16SrII-D	Y10097	Australia	White et al. 1998
Bristy oxtongue	Picris echiodes phyllody phytoplasma		16SrII-E	Y16393	Italy	Seemueller et al. 1998
Cactus	Peanut Witches broom group	*Cactus Witches broom-YN11*	16SrII-F	EU099556	USA	Cai et al. 2008
Cactus	Peanut Witches broom group	*Cactus Witches broom-YN23*	16SrII-G	EU099568	USA	
Cactus	Peanut Witches broom group	*Cactus Witches broom-YN24*	16SrII-H	EU099569	USA	
Cactus	Peanut Witches broom group	*Cactus Witches broom-YN06*	16SrII-I	EU099551	USA	

Cactus	Peanut Witches broom group	*Cactus Witches broom-YN07*	16SrII-J	EU099552	USA	
Cactus	Peanut Witches broom group	*Cactus Witches broom-YN28*	16SrII-K	EU099572	USA	
Cactus	Peanut Witches broom group	*Cactus Witches broom-YN01*	16SrII-L	EU099546	USA	
Tephrosia purpurea	Tephrosia purpurea Witches broom		16SrII-M	HG792252	India	Yadav et al. 2014
Papaya	Bunchy top symptom phytoplasma	*Papaya bunchy top phytoplasma*	16SrII-N	JF781309	Cuba	Lopez et al. 2016
Tabebuia pentaphylla	Tabebuia pentaphylla Witches broom phytoplasma	*Tabebuia pentaphylla phytoplasma*	16SrII-O	EF647744	UK	Mafia et al. 2007
Papaya	Cuban papaya phytoplasma		16SrII-P	DQ286948	Cuba	Arocha et al. 2006
Papaya	Papaya bunchy top phytoplasma		16SrII-Q	JF781310	Cuba	Lopez et al. 2015
Easter lily cactus	Echinopsis sp. yellow		16SrII-R	DQ535900	Mexico	-

	patch					
Amaranthus hypochondriacus		*Amaranthus hypochondriacus phytoplasma*	16SrII-S	FJ357164	Mexico	-
Tomato	Tomatillo Witches broom phytoplasma		16SrII-T	EU125185	Mexico	-
Mountain Papaya	Peanut Witches broom	*Vasconcellea cundinamarcensis little leaf phytoplasma*	16SrII-U	KP057205	China	-
Cowpea	Peanut Witches broom	*Ca. P. australasiae*	16SrII-V	MZ831316	China	-
Praxelis clematidea	Peanut Witches broom	*Praxelis clematidea phyllody phytoplasma*	16SrII-V	KY568717	China	-
Crotalaria aegyptiaca	Peanut Witches broom group	*Crotalaria witches-broom phytoplasma*	16SrII-W	KY872734	Oman	Al-Subhi et al. 2017
Potato	Peanut Witches broom group	*Ca. P. aurantifolia*	16SrII-X	MH423498	Soudi Arabia	
Cowpea	Peanut Witches broom	*Ca. P. aurantifolia*	16SrII-Y	MW827058	India	**This study**
Cowpea	Peanut Witches	*Ca. P. aurantifolia*	16SrII-Y	MW827059	India	

	broom					
Soybean	X disease group	*Soybean veinal necrosis phytoplasma*	16SrIII	AF177383	Lithuania	-
Veitchia merillii	Coconut lethal yellows group	*Coconut lethal yellowing phytoplasma*	16SrIV	U18747	USA	-
Elmyellow	Elm yellows group	*Ca. P. ulmi*	16SrV	AY197655	USA	Lee et al. 2004
Alsikeclover	Clover proliferation group	*Ca. P. trifolii*	16SrVI	AY390261	Canada	Hiruki and Wang 2004
Cowpea	Clover proliferation group	*Vigna unguiculata virescence phytoplasma*	16SrVI	MK088178	Iran	-
Cowpea	Clover proliferation group	*Vigna unguiculata virescence phytoplasma*	16SrVI	KC633094	Iran	
Cowpea	Clover proliferation group	*Vigna unguiculata virescence phytoplasma*	16SrVI	KC633093	Iran	
Cowpea	Clover proliferation group	*Vigna unguiculata virescence phytoplasma*	16SrVI	KC633092	Iran	
Chestnut	Clover proliferation group	*Ca. P. castaneae*	16SrVI	AB054986	South korea	Jung et al. 2002
Medagaskar periwinckle	Ash yellows group	*Ca. P. fraxini*	16SrVII	AF092209	USA	-
StraWitches		*Phytoplasma* sp.	16SrVIII-	U96614	USA	Jomantiene

broomerry		*Stra Witches broom1*	B			et al. 1998
StraWitches broomerry	Ash yellows group	*Argentinean straWitches broomerry phyllody phytoplasma*	16SrVIII-C	JN368423	Argentina	-
Cowpea	Pigeonpea witches broom group	*Ca. P. phoenicium*	16SrIX	MH547072	India	-
Peach	Eeuropean stone fruit yellows group	*Ca. P. prumorum*	16SrX-F	AJ542544	Germany	Seemuller et al. 2004
Rice	Rice yellow dwarf group	*Ca. P. oryzae*	16SrXI	AB052873	Thailand	Jung et al. 2003
Potato	Potato purple top ru group	*Russia potato purple top phytoplasma*	16SrXII-A	EU344890	Russian federation	Girsova et al. 2008
Brocolli	Brocolli stunt group	*Pytoplasma asteris* related strain	16SrXIII-A	JX626328	Brazil	-
Cowpea	Bermuda white leaf group	*Ca. P. cynodontis*	16SrXIV	MK367419	Iraq	-
Cowpea	Bermuda white leaf group	*Ca. P. cynodontis*	16SrXIV	MK367418	Iraq	
Cowpea	Bermuda white leaf group	*Ca. P. cynodontis*	16SrXIV	MK367417	Iraq	

	Cowpea	Bermuda white leaf group	*Ca. P. cynodontis*	16SrXIV	MK367416	Iraq	
	Cowpea	Peanut Witches broom group	*Vigna unguiculata' flat stem phytoplasma*	16SrXIV-A	KY439870	India	-
	Hemp	Hemp little leaf group	*Ca. P. cynodontis*	16SrXIV-A	KM220612	India	-
	Sunhemp	Sunhem yellowing group	*Phytoplasma brasiliense related strain*	16SrXV-A	KF878382	Brazil	Bianco et al. 2014
	Bermuda grass	Cynodon white fly group	*Ca. P. graminis*	16SrXVI-A	AY742327	Cuba	Arocha et al. 2005
	Potato	Texas potato top wilt	*Ca. P. americanum*	16SrXVIII-A	DQ174118	USA	Lee et al. 2006
	Stervia		*Stevia rebaudiana phytoplasma*	16SrXXIV-A	JF970603	India	Samad et al. 2011

Figures

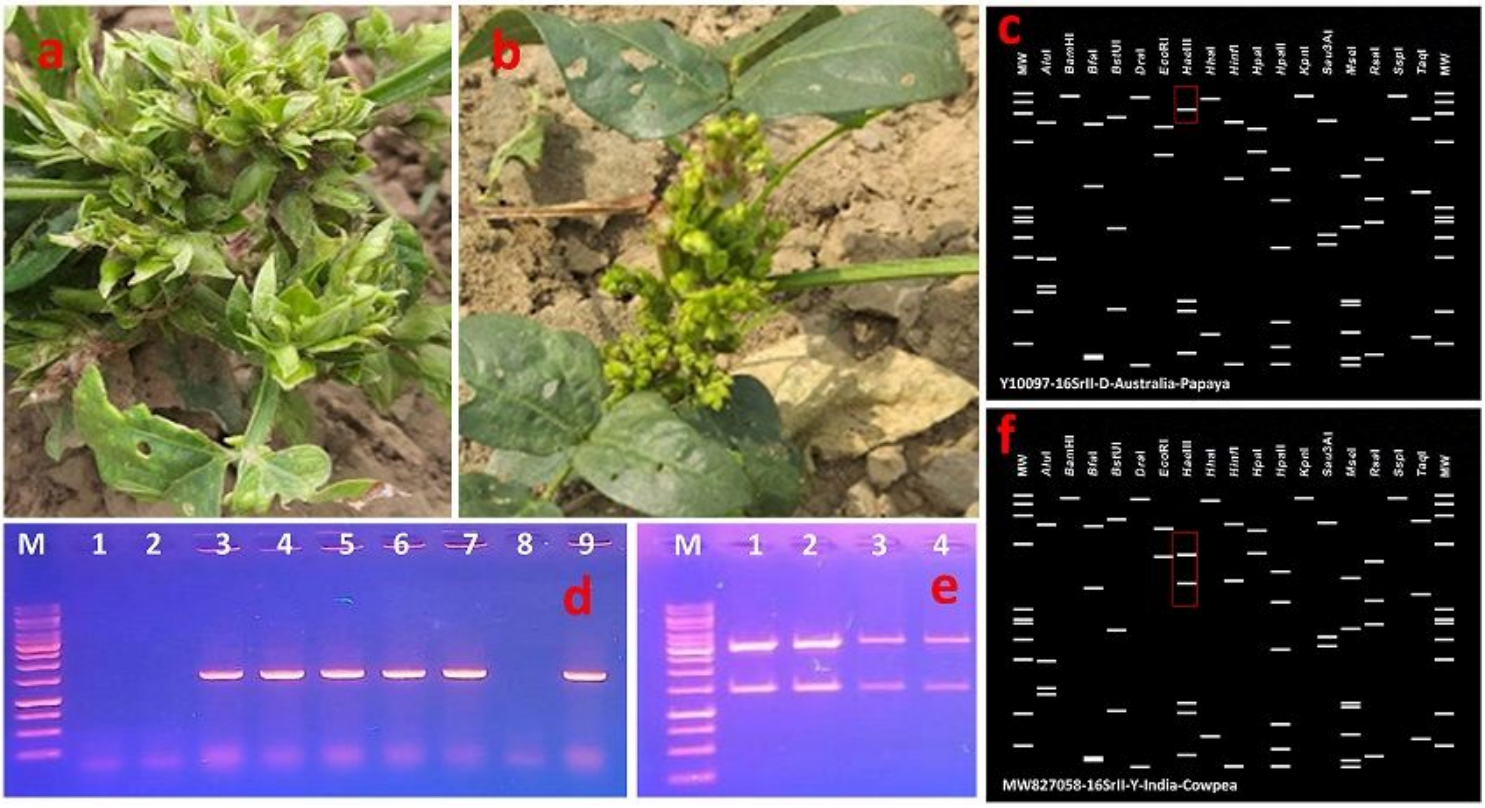

Figure 1

Symptoms of bud proliferation and stunting on cowpea plants (a, b); PCR amplification of 16SrII DNA fragments using P1/P7 and R16nF/R16nR primers (M=1kb DNA marker, Lane 1 and 2=Healthy cowpea samples, Lane 3-7=phytoplasma infected samples, Lane 8=negative control and Lane 9=Positive control (d); restriction digestion of positive clones plasmids (M=1kb DMA marker, Lane 1-4=positive clones releasing the desired fragments ~1250bp (e); virtual RFLP pattern of cowpea phytoplasma strain understudy (MW827058) and reference phytoplasma strain (c and f). Among 17 restriction enzymes, *Hae*I RE give different RFLP pattern from reference strain (Y10097-16SrII-D-Australia-Papaya).

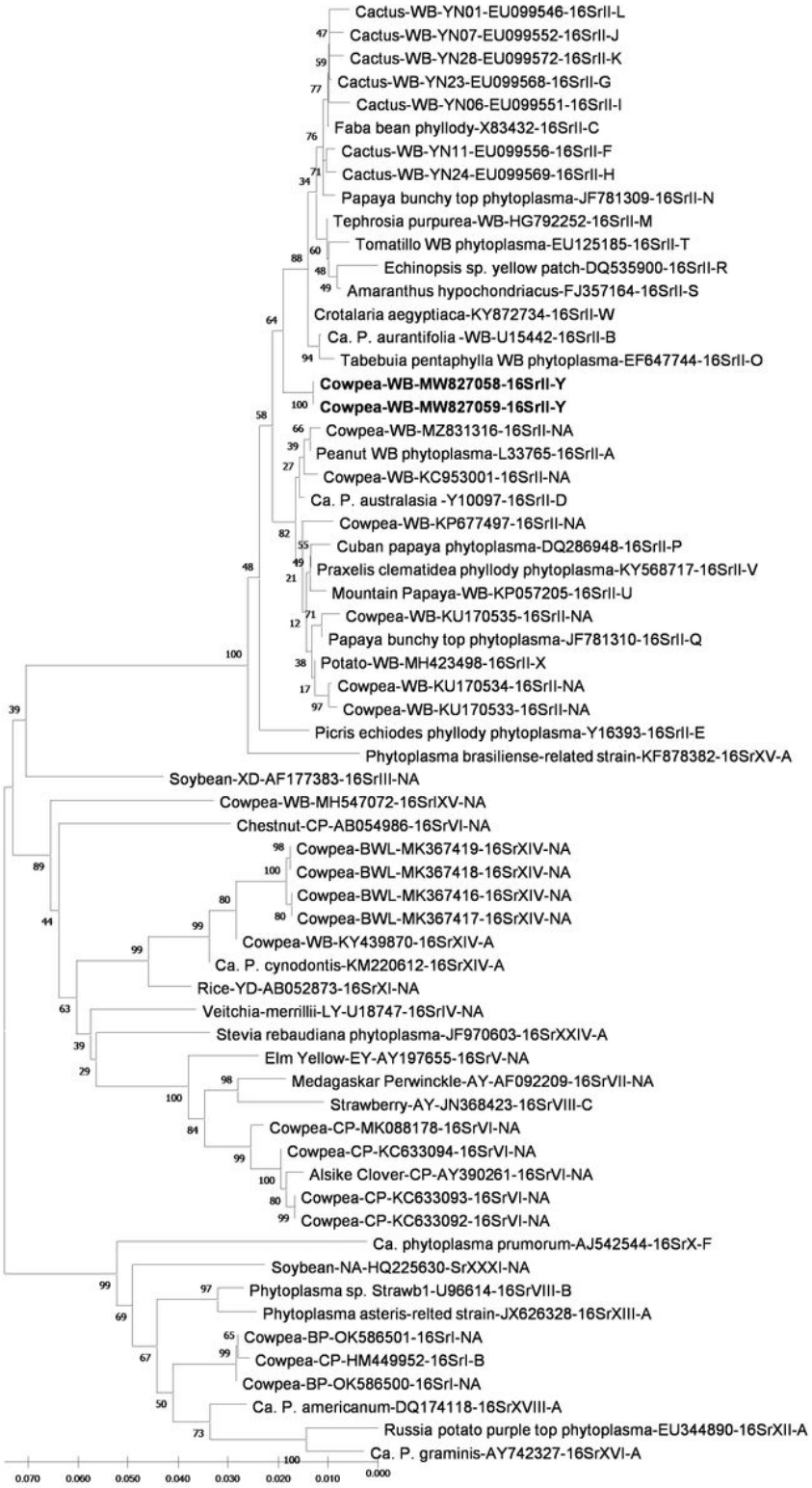

Figure 2

Phylogentic relationship between cowpea phytoplasma understudy (16SrII-Y) with other selected phytoplasma groups and subgroups constructed from 16S rDNA sequences. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the

evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 63 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1877 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. WB= Witches broom group; BWL=Bermuda white leaf group; CP=Clover proliferation group; BP= Bud proliferation group; AY=Ash yellows group; EY=Elm yellows group; YD=Yellow dwarf group; LY= Lethal yellows group; XD= X disease group; YCD= Yellow crinkle disease; NA= Not available