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ASSOCIATION OF A NEW 16SrVI SUBGROUP PHYTOPLASMA WITH LITTLE LEAF OF BRINJAL (*Solanum melongena L.*) GROWN IN KARNATAKA STATE (INDIA)

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Abstract- Introduction: Little leaf of brinjal is one of the devastating disease effecting brinjal cultivars worldwide. So far no report is available on molecular detection and characterization of phytoplasma associated with brinjal from South India. **Aims and objectives-** This study was conducted to detect, characterize and group phytoplasma associated with little leaf of brinjal from Karnataka. **Materials and methods** –Field survey was conducted to collect symptomatic, asymptomatic and healthy samples. Genomic DNA was isolated and subjected to nested PCR with universal primer pairs P1/P7 and R16nF2/R16nR2 respectively for amplification of highly conserved 16SrRNA gene. The amplified products were analyzed on 1.5% agarose gel and the expected nested PCR product of 1250 bp were purified, sequenced in both the directions by Sanger sequencing method and were assembled using Codon Code Aligner software. Multiple sequence alignment was done. Virtual RFLP pattern, restriction map was obtained and phylogenetic tree was constructed. **Results and Conclusion -** The nBLAST search and phylogenetic analysis of the sequence showed that, all the three samples shared maximum similarity with Clover proliferation (16SrVI) group. The virtual RFLP pattern obtained using *i*Phyclassifier revealed the association of a new 16SrVI subgroup phytoplasma with one isolate and 16SrVI-D group phytoplasma with other two isolates. This study reports the association of 16SrVI-D and a new sub-group phytoplasma associated with little leaf of brinjal from Karnataka.

Keywords: Little leaf of brinjal, Phytoplasma, Molecular Detection, Molecular Diversity, Karnataka State, 16SrVI-D group, Clover proliferation group, India

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Introduction

Phytoplasma are large group of plant pathogenic, non-helical, pleomorphic wall less bacteria, belonging to the class Mollicutes that inhabit the phloem tissues [5] and are known to cause diseases on hundreds of economically important crop plants worldwide. They are closely related to *Acholeoplasma* and were assigned provisional genus level taxa *Candidatus* phytoplasma [15]. They have very small genome size ranging from 530 kb to 1350 kb with G + C content of less than 28% [12]. They are generally spread by phloem-feeding insect vectors, which belong to the order Hemiptera, as they cannot be cultured in cell free media, their identification is mainly based on symptoms and molecular methods [4,6,18]. Phytoplasmas are classified into groups and subgroups based on the RFLP pattern of 16SrRNA sequences and '*Candidatus* Phytoplasma' species is assigned based on the nucleotide sequence similarity of 16SrRNA gene [7,9,10,14].

Brinjal (*Solanum melongena* L.) is the most important and common vegetable grown worldwide. India is the second largest producer of Brinjal in the world and Karnataka state is among top 10 Brinjal growing states of India [12]. The crop is affected by several diseases caused by fungi, bacteria, viruses and other insect pests of which little leaf of brinjal (LLB) is one of the most important diseases causing considerable economic losses as the infected plant fails to produce yield. The association of two groups of phytoplasma 16SrVI-D and 16SrI with Brinjal has been studied and reported from Northern India [3,13]. The present investigation was aimed to detect, characterize and to know the phylogeny of Phytoplasma affecting little leaf of Brinjal in Karnataka.

Materials and Methods

Collection of LLB samples

Field survey was conducted to different places of Karnataka and incidence was found to vary from 5-18% in all studied regions. Little leaf of brinjal samples were collected from Mysore (KA03), Mandya (KA10) and Dharwad (KA12) districts of Karnataka state [Fig-1] during the year 2012-13 along with healthy Brinjal leaf samples. Phytoplasma affected periwinkle (*Catharanthus roseus* (L.) G. Don) sample was also collected and used as positive control.

Genomic DNA isolation and PCR amplification

The genomic DNA was isolated from fresh LLB leaf samples (100mg) of after homogenizing in liquid nitrogen using Gen Elute Plant Genomic DNA Mini prep Kit (Sigma-Aldrich, USA) as per manufacturer's instructions and used directly for PCR. Phytoplasma 16S rRNA gene was amplified by direct and nested PCR by using Phytoplasma universal primers (P1/P7) and (R16F2n/R16R2) primers respectively [1,8]. Briefly, 25 µl of PCR mixture contained 1µl of DNA sample, 1 µl each of forward and reverse primers (10 pmol), 12.5 µl 2X Ready mix Taq reagent and 9.5 µl of nuclease free water. Amplification was performed using Advanced Primus 25 Thermo Cycler (Peqlab, Germany). The PCR conditions included, initial denaturation at 94˚C for 3 min. and 35 cycles of denaturation at 94˚C for 1 min., annealing at 50˚C for 1 min. and extension at 72˚C for 2 min. followed by final extension at 72˚C for 10 min. The first PCR products were diluted 1:30 with nuclease free water and 1µl of DNA sample was used for nested PCR employing the same PCR conditions above. DNA samples from healthy brinjal and phytoplasma affected periwinkle were used as negative and positive control respectively. The nested PCR products were separated on 1.5% agarose gel and stained with 1% ethidium bromide and image was documented using gel documentation system (Biorad, USA).

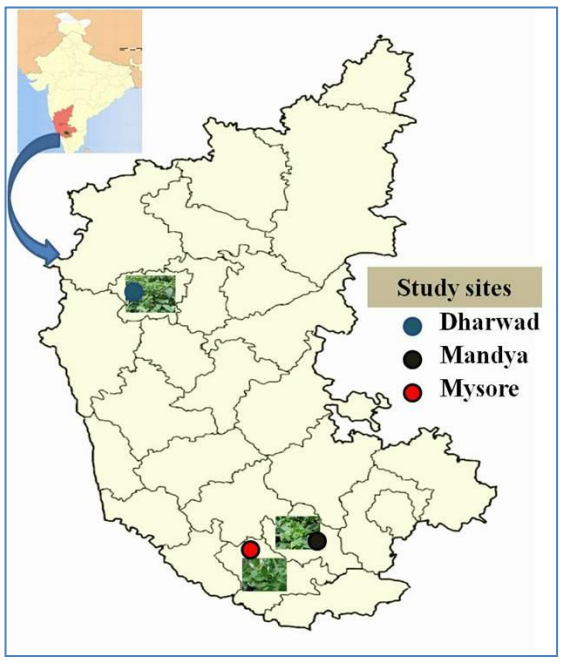

Fig-1 Karnataka state map showing places where little leaf of Brinjal samples were collected for Phytoplasma detection and characterization.

Sequence assembly, identification, sequence annotation and virtual RFLP analysis

The nested PCR products of ~1250bp were purified and subjected to direct sequencing from both the directions by Sanger sequencing method. Both forward and reverse direction sequences were aligned using Codon Code aligner. The 16S rRNA sequences from three LLB samples (KA-04, KA-10 and KA-12) were deposited in the GenBank with accession numbers KP027495, KP027497 and KP027498 respectively. The ~1250bp sequences of 16SrRNA (R16F2/R16R2 primer primed) were subjected to nBLAST search and analysed in *i*PhyClassifier online tool (http://www.plantpathology.ba.ars.usda.gov/cgibin/resource/iphyclassifier.cgi The sequences under this study and 16SrVI Phytoplasma subgroups reference strains obtained by NCBI GenBank, virtual gel plots for 17 restriction endo nucleases (AluI, BamHI, BfaI, BstUI , DraI, EcoRI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, Sau3AI , MseI, RsaI, SspI and TaqI) were obtained and analysed . Based on the RFLP pattern of these 17 restriction enzymes, 16SrRNA groups and subgroups are being assigned to phytoplasma. [23,24].

Multiple sequence alignment, restriction mapping, motif scan and phylogenetic analysis

All the three sequences were subjected to multiple sequence alignment using Discovery Science (DS) Gene v.1.5 (USA) software package and analyzed for the presence of single nucleotide polymorphisms (SNPs). Restriction maps were obtained for primer primed 16S rRNA gene sequence after putative restriction site analysis with restriction endo nucleases for LLB samples from this study and of the reference strain X38431. For phylogenetic analysis, 16SrRNA genes sequences from different groups of phytoplasma and sequences from the present study were aligned using CLUSTAL W version [22]. A phylogenetic tree was constructed by the NJ method using MEGAv6 [21]. *Acholeoplasma laidlawii* was used as an out-group to root the tree. Bootstrapping was performed 1000 times for estimation of stability and support for the clades. Phylogenetic interrelationships among the little leaf of brinjal Phytoplasma isolates representing all previously described Phytoplasma groups were analyzed based on 16S rRNA gene sequences. Reference sequences used in the present study are listed in [Table-1].To locate the position of conserved region, motif scan was run using Gene tool analysis software for all the three LLB samples along with reference strain (X38431)

Results and Discussion

The three samples of little leaf of Brinjal used in the present study, showed typical symptoms caused by phytoplasma showing stunted growth, greenish, sometimes yellowish malformed leaves with rosette appearance of upper portion of the plants with disease incidence of 10 to 15 in three Brinjal growing fields of Karnataka state. Petioles of such affected leaves were short, narrow, soft, glabrous, and yellowish and internodes were shortened with profuse axillary buds. Samples from Mysore region showed the formation of giant persistent calyx with sterile deformed flowers [Fig-2]. The results of nested PCR amplification of all three LLB samples yielded ~1250bp length amplicons [Fig-3] and no such amplicons were detected in healthy brinjal sample. The nBLAST analyses of the sequences revealed that, the 16S rRNA gene sequence of Mysore (KA-04 - KP027495) shared 100% homology with reference strains JX104336.1, KC178679.1, AF228053.1, AF228052.1 and X83431.1 respectively. Phytoplasma isolate from Mandya (KA10 -KP027497) and Dharwad (KA12 -KP027498) shared 99% homology with reference strains JX104336.1, KC178679.1 AF228053.1, AF228052.1 and X83431.1 respectively obtained from the Gene Bank.

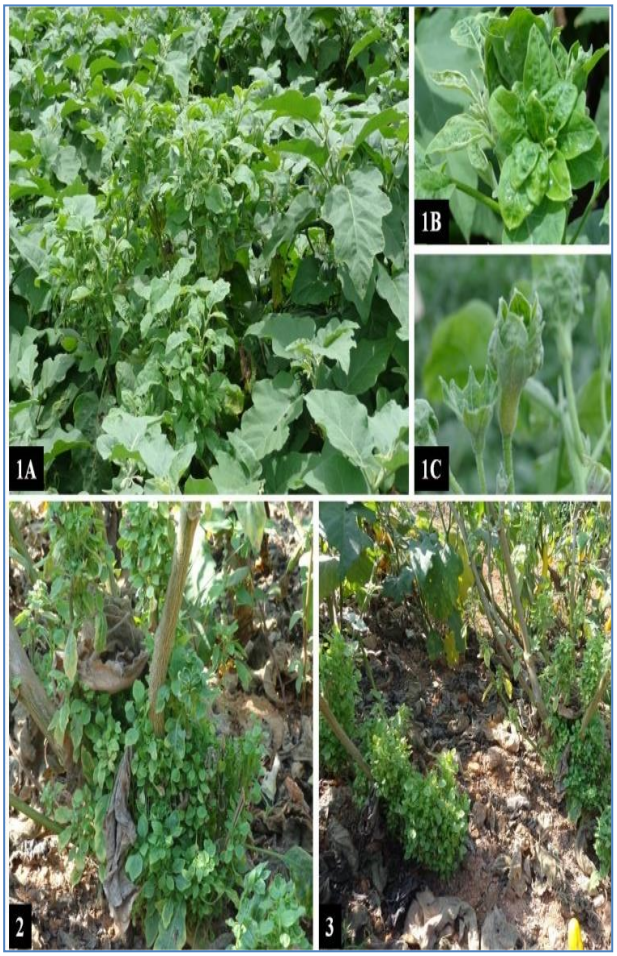

Fig-2 Symptoms of Little leaf of Brinjal: 1.A, B & C, - Malformed greenish appearance of mature affected shoot, malformed inflorescence and flower (Mysore District- KA04), 2-Little leaf symptoms in Mandya District –KA-10, 3-Little leaf sample from Dharwad District -KA12.

The similarity coefficient values obtained by iPhyclassifier indicated that, samples from Mysore (KA-04), Mandya (KA10) and Dharwad (KA12) shared 100, 99.2 and 98.8% similarity respectively with reference strain AY39026. Further, virtual RFLP analysis for 17 restriction enzymes of LLB samples in this and reference strain (X83431) through iPhyclassifier suggested that, LLB samples from Mysore and Dharwad were identical to that of reference strain (X83431) with similarity coefficient 1.0 and a sample from Mandya district showed different RFLP pattern with similarity coefficient 0.96 [Fig-4]. The putative restriction site analysis of 16S rRNA sequences from little leaf brinjal phytoplasma isolates from Karnataka with

that of reference strains (X83431) revealed that, restriction maps of Mysore and Dharwad samples were identical and in Mandya isolate lack of Eco RI site was identified [Fig-5].The motif scan further revealed the fact that, the region sigma 29 factor was conserved in all the three isolates of phytoplasmas [Fig-6]. A phylogenetic tree constructed based on 16S rRNA gene for three Phytoplasma isolates of this study along with representative strains of Phytoplasma from the Gen Bank revealed that, all the three little leaf of Brinjal samples from Karnataka state shared a common ancestor with *Acholeoplasma laidlawii* which formed as an out group and three isolates clustered with reference strains from group 16SrVI, showing maximum similarity with 16SrVI-D [Fig-7].

Table-1 *Phytoplasma 16SrRNA gene sequences used in this study*

Sl.No.	Strain Name	PhytoplasmaGroup	GenBank accession number	Reference
1.	'Ca. phytoplasmaasteris'	16SrI-B	M30790	Lee et al., 2004a
2.	Clover PhyllodyPhytoplasmaCPh	16SrI-C	AF222065	Dally et al.; Lee et al., 1998
3.	'Ca. phytoplasmaasteris' related strain PaWB	16SrI-D	AY265206	Lee et al., 1998
4.	Blue berry stunt phytoplasma strain BBS3	16SrI-E	AY265213	Lee et al., 1998
5.	'Ca. phytoplasmaasteris' related strain ACLR-AY	16SrI-F	AY265211	Lee et al., 1998
6.	Peanut witches'-broom phytoplasma	16SrII-A	L33765	Lee et al., 1998
7.	Ca.Phytoplasmalycopersici	-	EF199549	Arocha al., 2007
8.	'Ca. phytoplasmaaurantifolia'	16SrII-B	U15442	Zreik et al., 1995
9.	'Ca. Phytoplasmaaustralasiae'	16SrII-D	Y10097	White et al., 1998
10.	'Ca. Phytoplasmapruni'	16SrIII-A	L04682	Schneider et al.,1995
11.	Clover yellow edge phytoplasma	16SrIII-B	AF189288	Lee et al.,1998
12.	'Ca. Phytoplasmapalmae'	16SrIV-A	U18747	Harrison et al., 1994
13.	Phytoplasma sp. LfY5(PE65)-Oaxaca	16SrIV-B	AF500334	Harrison et al., 2002a; Wei et al., 2007
14.	Carludovicapalmata leaf yellowing phytoplasma	16SrIV-D	AF237615	Harrison et al.,2002b; Wei et al., 2007
15.	'Ca. Phytoplasmaulmi'	16SrV-A	AY197655	Lee et al., 2004b
16.	'Ca. Phytoplasmaziziphi' JWB-G1	16SrV-B	AB052876	Jung et al., 2003a
17.	'Ca. Phytoplasmavitis'	16SrV-C	AF176319	Davis and Dally, 1999
18.	Alder yellows phytoplasma strain ALY882	16SrV-C	AY197642	Lee et al. , 1998
19.	'Ca. Phytoplasmaziziphi'-related strain JWB-Kor1	16SrV-G	AB052879	Jung et al. , 2003a; Wei et al., 2007
20.	'Ca. Phytoplasmatrifolii'	16SrVI-A	AY390261	Hiruki& Wang , 2004
21.	Strawberry multiplier disease (MC)	16SrVI-B	AF190224	Jomantiene, et al.,1998
22.	Illinois elm yellows (EY-IL1)	16SrVI-C	AF409069	Jacob et al., 2003
23.	Periwinkle little leaf (PLL-Bd)	16SrVI-D	AF228053	Siddique et al., 2001
24.	*Centaureasolstitialis*virescence (CSVI)	16SrVI-E	AY270156	Faggioli et al., 2004
25.	Catharanthusphyllodyphytoplasma (CPS)	16SrVI-F	EF186819	Martini et al., 2007
26.	Portulaca little leaf phytoplasma (PLL-Ind)	16SrVI-H	EF651786	Samad et al., 2008
27.	Passionfruit (WB-Br4) '*Ca.* P. sudamericanum'	16SrVI-I	GU292081	Valiunas et al., 2009
28.	'Ca. Phytoplasmafraxini'	16SrVII-A	AF092209	Griffiths et al., 1999
29.	'Ca. Phytoplasmaluffae'	16SrVIII-A	AF086621	Chung et al., 2001
30.	'Ca. Phytoplasmaphoenicium'	16SrIX-D	AF515636	Verdin et al., 2003
31.	Pigeon pea witches'-broom	16SrIX-A	AF248957	Davis and Dally, 2000
32.	'Ca. Phytoplasmamali'	16SrX-A	AJ542541	Seemu¨ller and Schneider, 2004
33.	'Ca. Phytoplasmaspartii'	16SrX-D	X92869	Marcone et al. , 2004a
34.	'Ca. Phytoplasmaprunorum'	16SrX-F	AJ542544	Seemu¨ller and Schneider, 2004
35.	'Ca. Phytoplasmaoryzae'	16SrXI-A	AB052873	Jung et al., 2003b
36.	'Ca. Phytoplasmasolani'	16SrXII-A	AF248959	Davis and Dally, 2000
37.	Allocasuarinamuelleriana' Phytoplasma	-	AY135523	Gibb et al., 2003
38.	'Ca. Phytoplasmaaustraliense'	16SrXII-B	L76865	Davis et al., 1997
39.	'Ca. Phytoplasmajaponicum'	16SrXII-D	AB010425	Sawayanagi et al., 1999
40.	'Ca. Phytoplasmafragariae'	16SrXII-E	DQ086423	Valiunas et al.,2006
41.	Mexican periwinkle virescencephytoplasma	16SrXIII-A	AF248960	Dally et al., 2001
42.	Chinaberry yellows Phytoplasma	16SrXIII	AF495882	Harrison et al., 2002
43.	'Ca. Phytoplasmacynodontis'	16SrXIV-A	AJ550984	Marcone et al. , 2004b
44.	'Ca. Phytoplasmabrasiliense'	16SrXV-A	AF147708	Montano et al., 2001
45.	'Ca. Phytoplasmagraminis'	16SrXVI-A	AY725228	Arocha et al., 2005
46.	'Ca. Phytoplasmacaricae'	16SrXVII-A	AY725234	Arocha et al., 2005
47.	'Ca. Phytoplasmaamericanum'	16SrXVIII-A	DQ174122	Lee et al., 2006
48.	'Ca. Phytoplasmacastaneae'	16SrXIX-A	AB054986	Jung et al.,2002; Wei et al.,2007
49.	'Ca. Phytoplasmarhamni'	16SrXX-A	X76431	Marcone et al., 2004a; Wei et al., 2007
50.	'Ca. Phytoplasmapini'	16SrXXI-A	AJ632155	Schneider et al., 2005; Wei et al., 2007
51.	'Ca. Phytoplasmacocosnigeriae'	16SrXXII-A	Y14175	Wei et al., 2007
52.	Phytoplasma species	-	X80117	Tymon et al., 1998
53.	Buckland valley grapevine yellows phytoplasma	16SrXXIII-A	AY083605	Wei et al., 2007
54.	Sorghum bunchy shoot phytoplasma	16SrXXIV-A	AF509322	Wei et al., 2007
55.	Sorghum bunchy shoot phytoplasma	16SrXXV-A	AF521672	Wei et al., 2007
56.	Sugar cane phytoplasma D3T1	16SrXXVI-A	AJ539179	Wei et al., 2007
57.	Sugar cane phytoplasma D3T2	16SrXXVII-A	AJ539180	Wei et al., 2007
58.	Derbidphytoplasma	16SrXXVIII-A	AY744945	Wei et al., 2007
59.	'Ca. Phytoplasmaomanense'	16SrXXIX-A	EF666051	Al-Saady et al.,2008
60.	'Ca. Phytoplasmatamaricis'	16SrXXX	FJ432664	Zhao et al.,2009
61.	*Acholeplasmaladwii*	-	U14905.1	Artiushin et al., 1995Zhao et al.,2009

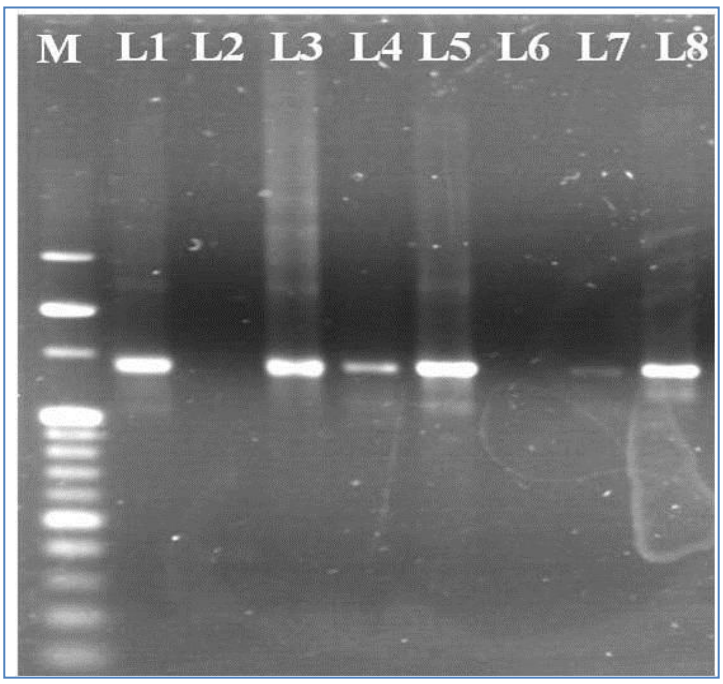

Fig-3Agarose gel (1.5%) showing the presence of ~1250 nested PCR products of LLB samples (Lane-1: Periwinkle; Lane 2- water as negative control; Lane-3: LLB (KA 04); Lane-4: LLB (KA 10); Lane-5: LLB (KA12); Lane-6 and Lane-7: Healthy brinjal ;Lane-8 : Periwinkle)

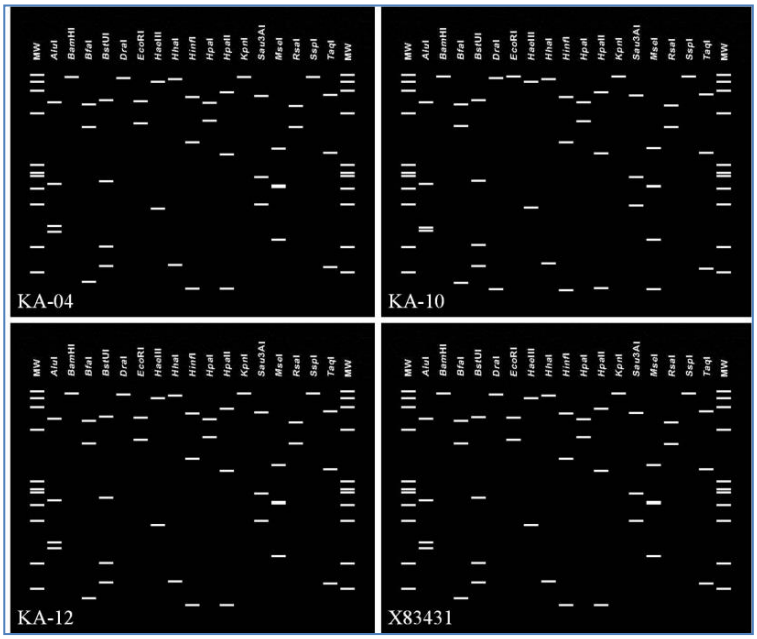

Fig-4 Virtual RFLP pattern of R16F2/R16R2 primer primed sequences of little leaf of brinjal Phytoplasma strains (KA-04, KA-10, KA-12) and reference strain X83431obtained from in-silico digestions using 17 restriction enzymes.

Little leaf and yellow's type diseases caused by phytoplasma are one of the limiting biotic factors for the productivity of many important crops which isresponsible for huge economic losses all around the world. Association of phytoplasma with brinjal has been reported from many countries. Brinjal is grown on nearly 7,22,000ha with annual production of 13,443.6 MT during the year 2013-14, making the country as the second largest producer with 26% world production share [2]. The little leaf of brinjal is a serious concern recently as it is spreading in many brinjal growing regions of South India. The association of phytoplasma in brinjal and its detection, characterization of the associated phytoplasma have been reported from all over the world. Eggplant dwarfing in Japan has been affiliated to group 16SrI phytoplasma [14,17], Group 16SrI (*Candidatus Phytoplasma asteris*) with Brinjal in Bangladesh [11,20], giant calyx symptom of eggplant in Brazil with 16SrIII- J and 16SrIII-U subgroups [16] phytoplasma associated with little leaf in Turkey belongs to Clover proliferation group (16SrVI-A) [19]. Occurrence of two groups of phytoplasma 16SrVI-D and 16SrI have been detected in little leaf of Brinjal from two North Indian states such *viz.*, New Delhi and Bihar [3,13].In the present study, association of phytoplasma with little leaf of Brinjal from south Indian state of Karnataka was confirmed with extensive field survey in Mysore, Mandya and Dharwad districts with 5-15% disease incidence. Though the exact incidence and the extent of loss due to LLB from all over India are yet to be established and similar is the case with all major brinjal growing regions in the world. The LLB was identified based on morphological symptoms and presence of phytoplasma was confirmed through nested PCR using phytoplasma specific R16F2n / R16R2 primer pair. The typical morphological symptoms were

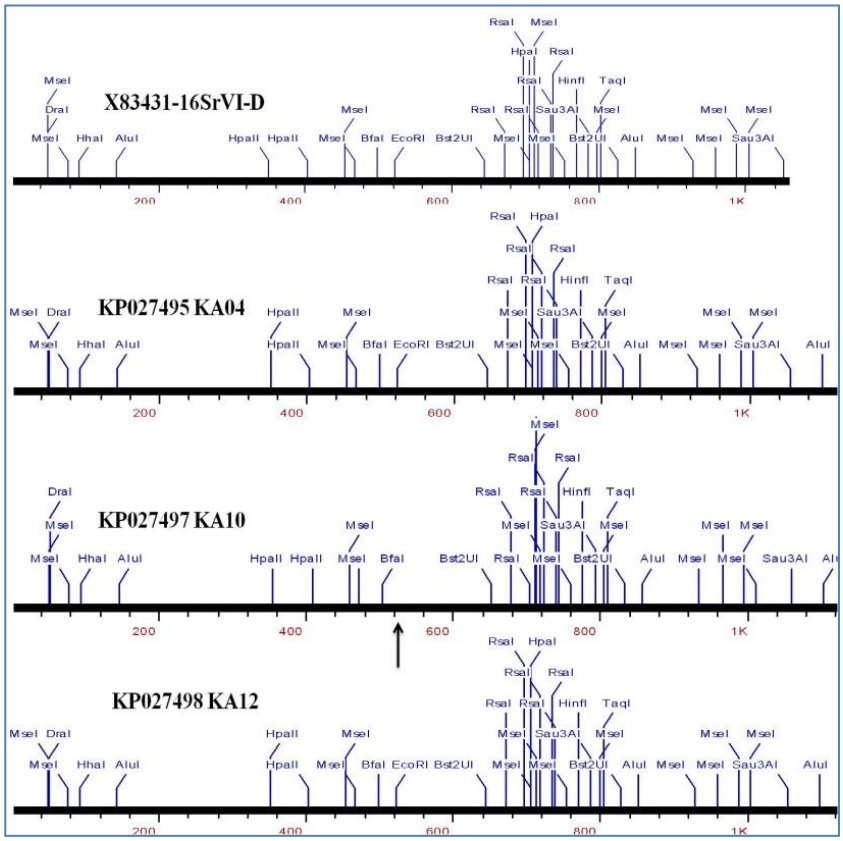

Fig-5 Comparative analysis of putative restriction sites in 16SrRNA gene sequences on LLB samples (KA-04, KA-10, KA-12) along with reference strain X83431. Arrow indicates absence of EcoRI restriction site only on KA10 sample.

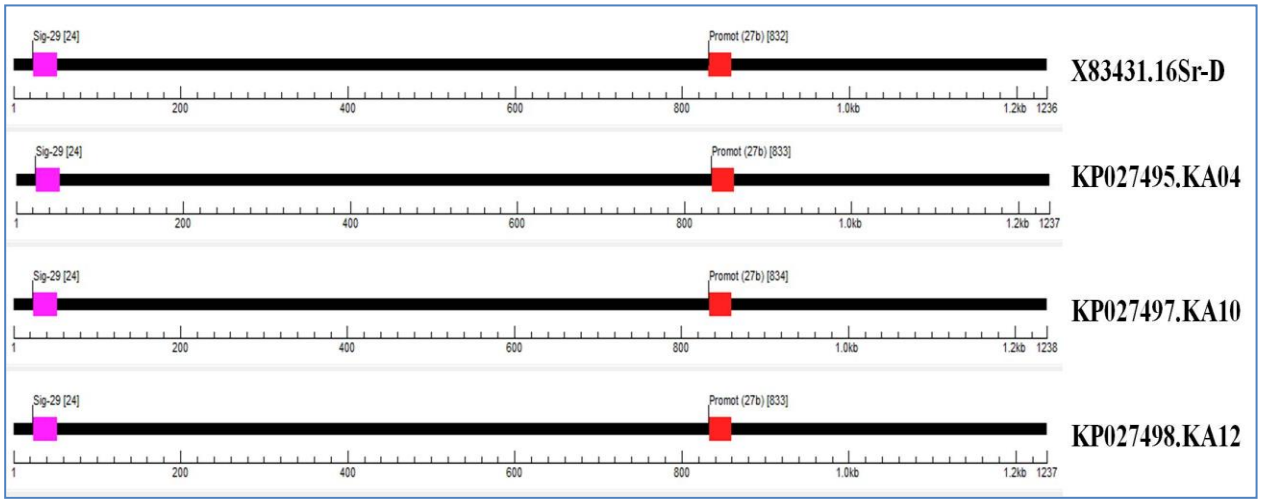

Fig-6 Comparative analyses of 16SrRNA gene sequences of LLB samples compared with reference strain X83431 for motif scan using the prokaryotic motif builder.

comparable to other LLB samples described earlier in other parts of North India. The study confirmed two isolates of Phytoplasma associated with LLB from Mysore and Dharwad districts belongs to 16SrVI-D group and isolate from Mandya appeared to represent new subgroup as it has shared 0.96 similarity coefficient with the reference strain. A key enzyme coding region distinguishing the strain was Eco RI, site of which was absent in the sequence of Mandya sample. The phylogenetic tree constructed by Neighbour-Joining (NJ) method using 16SrRNA gene sequences from three LLB samples with other 61 representative phytoplasma strains using *Acholeoplasma laidlawii* as an out-group indicated that LLB phytoplasma isolates had the highest similarity with phytoplasma strains from 16SrVI Clover proliferation group, and clustered with 16Sr VI-D group (reference strains X83431, AF228053). The phylogenetic tree constructed very well supported the results of nBLAST and iPhyclassifier. All bacteria contain a primary σ factor responsible for transcription of housekeeping genes necessary for growth and survival. The motif analysis of isolates and the reference strain (X83431) showed that position of sigma factor was conserved in all the three samples. Based on the genetic analysis and Phylogenetic tree, it was found that the LLB samples from Mysore and Dharwad belongs to 16SrVI-D and LLB sample from Mandya was found to be a new sub group based on Virtual RFLP analysis.(23)

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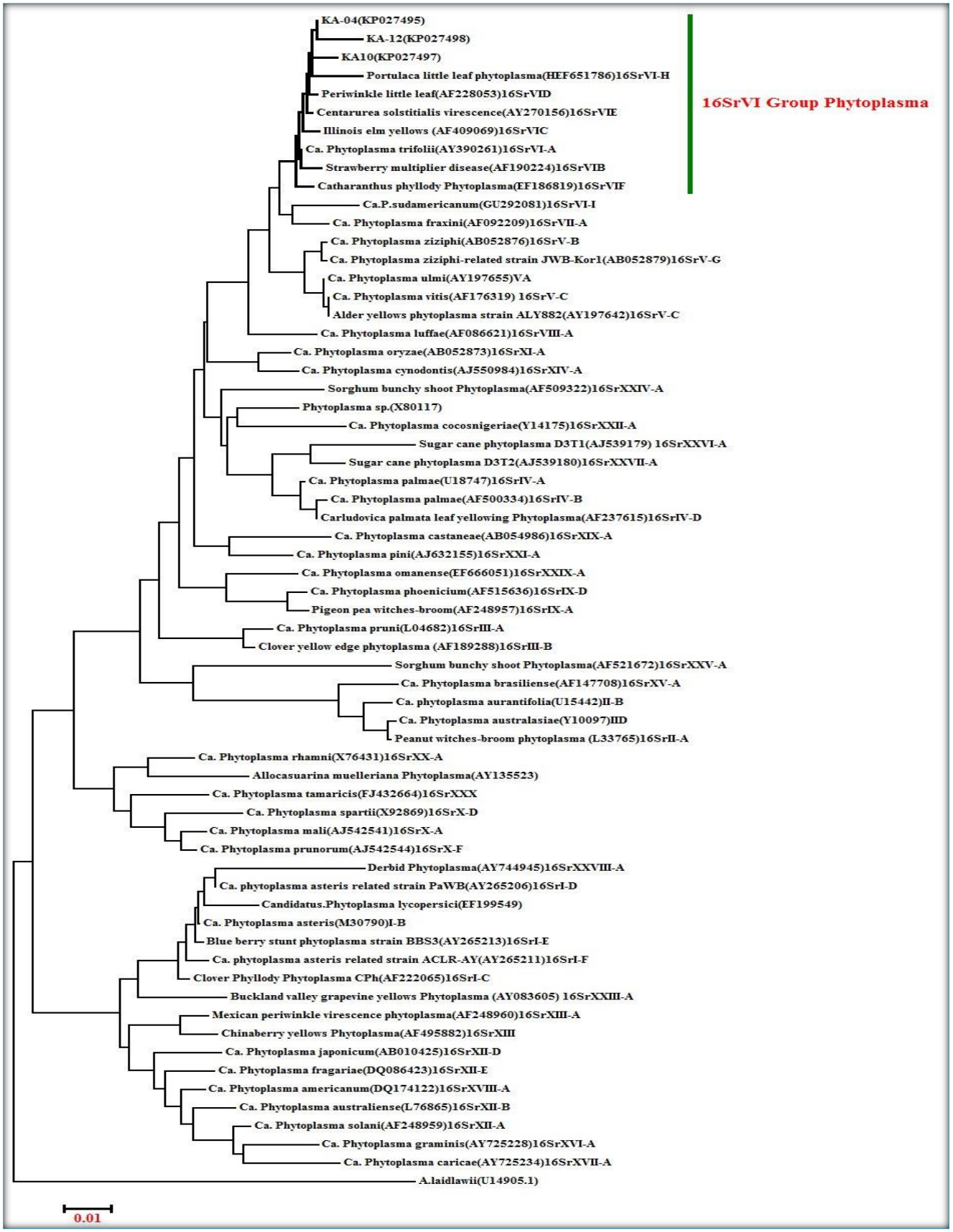

Fig-7 Phylogenetic tree constructed by Neighbour-Joining method based on multiple sequence alignments of 16SrDNA (1.25 kb) sequences of strains of LLB-KA-04, KA-10, KA-12 and other Phytoplasma strains (accession numbers mentioned in parentheses)obtained from GenBank which represent different groups. *Acholeoplasma laidlawii* is used as out-group. GenBank accession numbers for sequences obtained in this study are shown in bold letters.

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