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Molecular identification of 16SrXI-B subgroup of phytoplasma related strain with yellow leaf disease of sugarcane (*Saccharum officinarum*) in India

KUMAR A.[1,2], HOLKAR S. K.[1,¶,*], SINGH R.[2], SINGH P. K.[3], MITRA S.[4], KUMAR S.[4], BASAVARAJ Y. B.[4] AND PATHAK A. D.[3]

[1]*ICAR-Indian Institute of Sugarcane Research*
Biological Control Centre, Pravaranagar, Maharashtra - 413 712, India
**(e-mail : somnathbhu@gmail.com)*

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ABSTRACT

In India, yellow leaf disease (YLD) is known to be caused by *Sugarcane yellow leaf virus* (SCYLV) and *Sugarcane yellows phytoplasma* (SCYP; 16SrI-B). Therefore, present study was conducted to confirm the association of specific phytoplasma with YLD in Co 0238 and CoS 510 sugarcane genotypes during 2019-2020 at Indian Institute of Sugarcane Research, Lucknow. The YLD affected genotypes were characterized by yellowing of leaf mid-ribs and leaf-lamina, leaf-tip necrosis, bunching of leaves at crown region and drying of plants with an incidence ranged from 28.26-35.81%. YLD symptomatic leaf samples were collected from the two genotypes and subjected to genomic DNA extraction by DNeasy Plant Mini Kit followed by nested PCR assay using universal phytoplasma primers (16S rDNA region). The nested PCR assays of samples from both the genotypes showed specific amplification. 16S rRNA gene sequence analyses of CoS 510 isolate (MN913611) revealed 100% identity with *Sugarcane white leaf phytoplasma* (SCWLP) belongs to 16SrXI-B subgroup. Similarly, virtual RFLP analyses revealed 1.00 similarity coefficient with 16SrXI-B subgroup. Thus, present study first time reports the association of SCWLP related strain with YLD of sugarcane in India. Phytoplasma infecting sugarcane are more diverse and needs systematic studies on their single or mixed infections in different varieties in India.

Key Words : 16SrI-B, 16SrXI-B, sugarcane white leaf phytoplasma, sugarcane yellows phytoplasma

INTRODUCTION

Sugarcane (*Saccharum officinarum*) belongs to the family *Poaceae* and one of the most important commercial cash crops in India. Globally, sugarcane is known to infect by different fungal, bacterial, nematode, viral, and phytoplasma diseases and thus play an important role in reducing its production and productivity (Singh *et al.,* 2009; Viswanathan and Rao, 2011; Ou *et al.*, 2017). Besides these diseases, phytoplasma diseases are one of the most serious constraints causing economic yield losses in sugarcane including Sugarcane grassy shoot phytoplasma (SCGSP) (Rao *et al.,* 2008), *Sugarcane white leaf phytoplasma* (SCWLP) (Rao *et al.,* 2008; Soufi *et al.,* 2013) and Sugarcane yellows phytoplasma (SCYP) (Gaur *et al.,* 2008; Kumar *et al.,* 2015). Among these, SCYP evidenced an economically important pathogen causing drastic reduction in sugarcane growth and yield contributing parameters (Cronje *et al.,* 1998).

The yellow leaf disease (YLD) was first recorded in Hawaii on sugarcane (*S. officinarum*) cv. H 65-7052 and complete fields were exhibiting severe yellowing (Schenck, 1990) and likewise, Comstock *et al.* (1994) was also observed similar symptoms in sugarcane in Florida. Since then, diagnosis of YLD and

[2]Amity Institute of Biotechnology, Amity University, Uttar Pradesh Campus, Lucknow-226 028, India.
[3]Division of Crop Improvement, ICAR-Indian Institute of Sugarcane Research, Lucknow-226 002, India.
[4]Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi-110 012, India.
[¶]Current address: ICAR-National Research Centre for Grapes, Pune -412 307, Maharashtra, India.

its symptoms have been recorded in more than 20 countries including Australia, Brazil, Cuba, Mauritius, Reunion, South Africa and USA (Bailey *et al.,* 1996; Cronje *et al.,* 1998). YLD was earlier known as yellow leaf syndrome (YLS) that was characterized by yellowing of the leaf midribs and leaf lamina of sugarcane.

The association of SCYP with YLD have been reported from various sugarcane growing countries including Australia, Cuba and Mauritius (Cronje *et al.,* 1998; Arocha *et al.,* 1999; Aljanabi *et al.,* 2001). Recently, SCYP strains of *Aster yellows phytoplasma* belonging to 16SrI group and *Rice yellow dwarf phytoplasma* belonging to 16SrXI group of phytoplasma have been detected in asymptomatic sugarcane cultivars in Hawaii (Soufi and Komor, 2014). Moreover, the evidence of association of SCYP belonging to 16SrI-B subgroup (*Ca.* phytoplasma asteris) was reported from Brazil, Egypt and India (Silva *et al.,* 2009; Elsayed and Boulila, 2014; Kumar *et al.,* 2015). The association of SCWLP belonging to 16SrXI group was detected in YLD affected sugarcane from Thailand (Soufi *et al.,* 2013). Findings from the present study confirms the first report of association of SCWLP with YLD in sugarcane in India.

Primary transmission of phytoplasma occurs due to vegetative propagation through seed cane while, secondary transmission occurs through phloem-feeding planthoppers (family: *Cicadellidae*) in a circulative manner from infected to healthy sugarcane (Gatineau *et al.,* 2001; Kavita *et al.*, 2018). Arocha *et al.* (2005) studied that the SCYP belongs to 16SrI-A subgroup (*Aster yellows phytoplasma*) is mainly transmitted by delphacid plant hopper (*Saccharosydne sachharivora*).

YLD is known to be caused either by SCYP and/or *Sugarcane yellow leaf virus* (SCYLV; genus: *Polerovirus*, family: *Luteoviridae*). Therefore, in India YLD was first described during 1999 and identified the association of SCYLV as a causal agent of the disease (Viswanathan *et al.,* 1999; Rao *et al.,* 2001; Viswanathan, 2002; Kumar *et al.,* 2020). Later, Gaur *et al.,* (2008) reported the association of SCYP belonging to 16SrXII group of phytoplasma with YLD in India. In the recent past, the presence of '*Ca.* phytoplasma asteris' belonging to 16SrI-B subgroup was detected and confirmed in two YLD affected sugarcane varieties *viz.,* CoLk 94184 and CoSe 92423 (Kumar *et al.,* 2015) and other sugarcane varieties from different states of India (Kumar *et al.,* 2018). The association of SCWLP with sugarcane white leaf disease is well known in India and elsewhere (Rao *et al.,* 2008; Soufi *et al.,* 2013) but there was no information available on the association of SCWLP with the YLD affected sugarcane genotypes in India.

Therefore, by keeping in mind that the association of diverse group of phytoplasma with YLD, the present study was conducted for the identification of associated phytoplasma with YLD based on 16Sr RNA gene sequence information in two major sugarcane varieties *viz.,* Co 0238 and CoS 510 cultivated in sub-tropical conditions.

MATERIALS AND METHODS

The present investigation was conducted during 2019-20 at Indian Institute of Sugarcane Research, Lucknow to identify the specific sub-group of phytoplasma associated with YLD in Co 0238 and CoS 510 sugarcane varieties largely cultivated in subtropical conditions in India.

Sample Collection and Genomic DNA Extraction

During the surveys conducted in November 2019, the YLD affected samples from two popular sugarcane varieties *viz.,* Co 0238 and CoS 510 were collected from the experimental fields at ICAR- Indian Institute of Sugarcane Research (IISR), Lucknow, India. The sugarcane leaves showing typical YLD symptoms were collected and subjected to the genomic DNA extraction using DNeasy Plant Mini Kit (Qiagen, Germany). Before proceeding to DNA extraction, the YLD affected sugarcane leaves were surface cleaned with 70% ethanol to avoid contamination.

Nested Polymerase Chain Reaction

The isolated genomic DNA from leaf samples collected from two YLD affected sugarcane varieties *viz.*, Co 0238 and CoS 510 were subjected to nested PCR assay using universal primer pairs. For Co 0238 samples P1: 5′-AAGAGTTTGATCCTGGCTCAGGATT-3′ and P7: 5′-CGTCCTTCATCGGCTCTT-3′ (Deng and Hiruki, 1991; Schneider *et al.,* 1995), R16F2n: 5′-GAAACGACTGCTAAGACTGG-3′ and R16R2: 5′-TGACGGGCGGTGTGTACA AACCCCG-3′ (Gunderson and Lee, 1996), for CoS 510 samples P1/P7 and 3F: 5′-ACCTGCCTTTAAGACGAGGA-3′ and 3R: 5′-AAAGGAGGTGATCCATCCCCACCT-3′ (Manimekalai *et al.,* 2010) were used.

The first round PCR reactions of the YLD affected leaf samples collected from Co 0238 and CoS 510 varieties were carried out in a thermal cycler (Vapo-Master Cycler, Eppendorf, Germany). Temperature profile using P1/P7 primer pair for Co 0238 samples followed as under: initial denaturation at 94°C for 4 min followed by 30 cycles of 94°C for 30 sec; primer annealing at 55°C for 30 sec; extension at 72°C for 1 min and final extension at 72°C for 10 min. Using the first round PCR product as a template, the second round nested PCR was performed using R16F2n/R16R2 with the similar temperature profile as mentioned above except the primer annealing temperature at 56°C for 30 sec. Similarly, for first round PCR of CoS 510 samples P1/P7 primer pair and for second round PCR 3F/3R primers were used. PCR profile of profile 3F/3R includes 94°C for 5 min followed by 35 cycles of 94°C for 45 sec, 63°C for 1 min and 72°C for 10 min.

The 25 µl PCR reaction recipe for the first round of PCR in two isolates contained 2 µl of the template DNA (100 ng/µl), 1.5 µl of MgCl2 (25 mM), 0.5 µl of dNTP (10 mM) mixture, 0.5 µl of each primer, 0.5 µl (5U/µl) of Taq DNA polymerase (G Biosciences; USA), 2.5 µl of 10X buffer and 17 µl SDW to makeup final volume. For second round PCR in two isolates the reaction recipe contained, 2 µl of first round PCR product was used as a template at a dilution of 1:10 with similar reaction ingredients as described for the first round nested PCR assay. The genomic DNA extracted from YLD symptomatic leaf samples were stored in deep freezer (-80°C) and used as positive control. In negative control sterile water was used instead of template DNA. The amplified PCR products of both the first and second rounds were subjected to 1% agarose gel electrophoresis stained with ethidium bromide (0.01%) and observed under a UV trans-illuminator.

Sequence Analyses

The PCR products of ~1200 bp and ~1300 bp corresponding to phytoplasma association against the leaf samples collected from Co 0238 and CoS 510, respectively were purified using the Nucleospin Gel and PCR Clean-up kit (Macherey Nagel, Germany) and subjected for sequencing. The 16S rDNA sequence of these two phytoplasma samples obtained were aligned using CLUSTAL W multiple alignment method (Hall, 1999). And the sequences were aligned using BioEdit software program version (BioEdit 7.0.5.3). The final consensus sequence of the two phytoplasma strains were submitted to the NCBI GenBank (Acc. Nos: MN913611, MN913612). The sequence identity matrix and phylogenetic trees were constructed using the Neighbour-Joining method in MEGA 6.0 software version at 1000 bootstrap replications (Tamura *et al.,* 2004, Kumar *et al.,* 2016). The list of sequences was used for comparison and phylogenetic analyses were retrieved from NCBI GenBank (url: https://www.ncbi.nlm.nih.gov/; Table 1). In order to determine the group and subgroup of the associated phytoplasma, the virtual restriction fragment length polymorphism (RFLP) based on 16S rRNA gene segment and similarity coefficient was performed using *i*PhyClassifier online program (url: https://plantpathology.ba.ars.usda.gov/; Lee *et al.,* 1998; Wei *et al.,* 2007; Zhao *et al.,* 2009).

RESULTS AND DISCUSSION

Symptomatology

The YLD affected sugarcane varieties *viz.,* Co 0238 and CoS 510 exhibited various symptoms including mild to prominent yellowing of the leaf mid-ribs which later extended towards the leaf lamina. Subsequently, necrosis of the leaves started from leaf-tip towards leaf base (Fig. 1A-B). In addition to this, sugarcane variety CoS 510 exhibited smaller leaves, bunching in the crown region and stunted growth of the plants (Fig. 1B). The YLD incidence in these two sugarcane genotypes was ranged from 28.26% to 35.81%.

In the present investigation, symptoms of YLD were recorded in two sugarcane varieties *i.e.,* Co 0238 (known as wonder cane) and CoS 510 were found similar as described earlier in India (Gaur *et al.,* 2008; Viswanathan and Rao, 2011; Rao *et al.,*

Table 1. List of phytoplasma sequences retrieved from NCBI, GenBank for comparison and construction of phylogenetic tree with the phytoplasma isolates identified as sugarcane yellows and white leaf phytoplasma from the present study

S. No.	Phytoplasma name	Acronym	Crop/Vector	Country	Accession number	Group
1.	*Sugarcane yellows phytoplasma**	SCYP	Sugarcane	India	MN913612	16SrI-B
2.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	Mexico	MH891144	16SrI-B
3.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	Brazil	EU423900	16SrI-B
4.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	India	KJ599656	16SrI-B
5.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	India	KJ599657	16SrI-B
6.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	Mexico	MH891145	16SrI-B
7.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	Mexico	MH891146	16SrI-B
8.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	Mexico	KJ491099	16SrI-B
9.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	Mexico	KJ491100	16SrI-B
10.	*Onion yellows phytoplasma*	OYP	Onion	Japan	AP006628	16SrI-B
11.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	Mauritius	EF413056	16SrIII-A
12.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	Mauritius	AJ539178	16SrIV-A
13.	*Sugarcane white leaf phytoplasma*	SCWLP	Sugarcane	Thailand	AB052874	16SrXI
14.	*Sugarcane grassy shoot phytoplasma*	SCGSP	Sugarcane	India	JX862179	16SrXI
15.	*Sugarcane white leaf phytoplasma**	SCWLP	Sugarcane	India	MN913611	16SrXI-B
16.	*Sugarcane white leaf phytoplasma*	SCWLP	Sugarcane	Sri Lanka	MN174860	16SrXI-B
17.	*Sugarcane white leaf phytoplasma*	SCWLP	Sugarcane	China	KR020691	16SrXI-B
18.	*Sugarcane white leaf phytoplasma*	SCWLP	Sugarcane	China	KR020690	16SrXI-B
19.	*Sugarcane white leaf phytoplasma*	SCWLP	Sugarcane	China	KR020686	16SrXI-B
20.	*Sugarcane white leaf phytoplasma*	SCWLP	Sugarcane	Thailand	FM208260	16SrXI-B
21.	*Sugarcane white leaf phytoplasma*	SCWLP	Sugarcane	China	KR020694	16SrXI-B
22.	*Cymbopogon citratus' little leaf phytoplasma*	CCLLP	Lemon grass	India	MT127618	16SrXI-B
23.	*Candidatus Phytoplasma oryzae*	CaPO	Gallow grass (Hemp)	India	MN719898	16SrXI-B
24.	*Candidatus Phytoplasma oryzae*	CaPO	Sugarcane	India	MG745912	16SrXI-B
25.	*Sugarcane white leaf phytoplasma*	SCWLP	Sugarcane	China	KP638415	16SrXI-B
26.	*Saccharum officinarum Mollicutes*	MOs	Sugarcane	Germany	X76432	16SrXI-B
27.	*Sugarcane white leaf phytoplasma*	SCWLP	Sugarcane	China	KR020685	16SrXI-D
28.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	India	EU170474	16SrXVI-A
29.	*Acholeplasma laidlawii*	APLi	NA	NA	M23932	16SrXXVI
30.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	Mauritius	AJ539179	16SrXXVI-A
31.	*Sugarcane yellows phytoplasma*	SCYP	Sugarcane	Mauritius	AJ539180	16SrXXVII-A

*: Phytoplasma isolate from the present study are indicated in bold letters; NA : Not available in GenBank.

2012; Kumar *et al.*, 2015). In the recent past, Holkar *et al.* (2016) has recorded the incidence of YLD in more than 50 sugarcane genotypes and were characterized by similar disease symptoms as described in the present study. Likewise, the YLD symptoms were described by Kumar *et al.* (2015) on two sugarcane genotypes including CoSe 92423 and CoLk 94184. Moreover, the symptoms of YLD were recorded on 40 sugarcane genotypes collected from 11 major sugarcane growing states of India including Andhra Pradesh, Assam, Bihar, Chhattisgarh, Haryana, Karnataka, Maharashtra, Punjab, Tamil Nadu, Uttarakhand and Uttar Pradesh (Kumar *et al.*, 2018). In addition to this, during 2016-17 and 2017-18 crop seasons recorded the similar symptoms of yellowing of midribs, drying of the leaf tips towards base

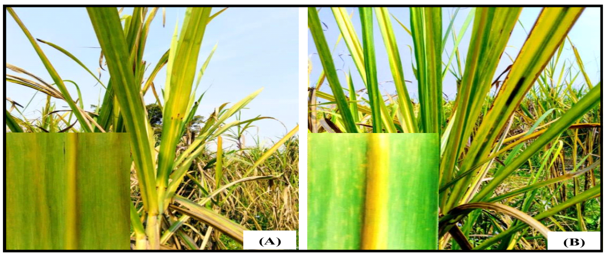

Fig. 1. Symptoms of yellow leaf disease on two sugarcane genotypes *viz.*, Co 0238 (A) and CoS 510 (B) at Indian Institute of Sugarcane Research, Experimental Farm. YLD affected genotypes exhibiting yellowing of the leaf midribs extending towards leaf lamina which was, followed by leaf- tip necrosis and bunching of the leaves at the crown region.

of the leaves followed by complete drying of the leaves were recorded in 103 sugarcane genotypes from tropical and sub-tropical conditions in India (Kumar *et al.,* 2020). The symptoms of YLD described in the present study are in agreement with the YLD symptoms described from Africa (Arocha *et al.,* 1999; Cronje and Bailey 1999), Brazil (Silva *et al.,* 2009), Cuba (Aljanabi, 2001), Egypt (Elsayed and Boulila, 2014), USA-Hawaii (Schenck, 1990) and USA-Florida (Comstock *et al.,* 1994), Mauritius (Aljanabi, 2001) and Thailand (Soufi *et al.,* 2013).

Nested PCR Assay and Sequence Information

The quality of the isolated genomic DNA from the YLD symptomatic samples was determined by 260/280 absorbance ratio which ranged from 1.7 to 1.8, suggesting the DNA samples were without any polyphenolic compound and protein contamination. Moreover, visualization of the genomic DNA samples were assessed by 1% agarose gel electrophoresis and found intact amplicons. The first round nested PCR assay results revealed the amplification of ~1800 bp size amplicons in both of the leaf samples collected from YLD affected sugarcane varieties *viz.,* Co 0238 and CoS 510, respectively (data not shown). Likewise, the second round PCR results revealed amplification of ~1200 bp and ~1300 size amplicons, corresponding to presence of

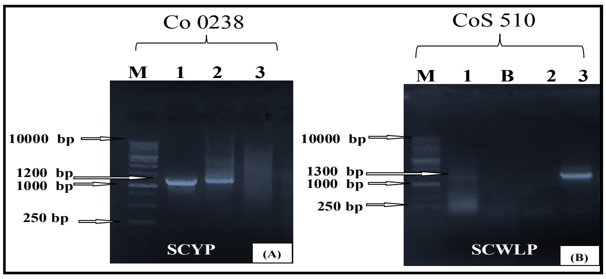

Fig. 2. 1% agarose gel electrophoresis of the second round nested PCR amplification of *Sugarcane yellows phytoplasma* (SCYP) and *Sugarcane white leaf phytoplasma* (SCWLP) using R16F2n/R16R2 and 3F/3R primers showed ~1200 bp (A) and ~1300 bp (B) size amplicons, respectively in two sugarcane samples. (A) Lanes: 1: positive control of Co 0238, 2: sample (YLD affected), 3: negative control of Co 0238 and M: 1 Kb DNA ladder. (B) Lanes: 1: positive control of CoS 510, B: blank (sample not loaded) 2: negative control of CoS 510, 3: sample (YLD affected) and M: 1 Kb DNA ladder.

phytoplasma in YLD affected Co 0238 (Fig. 2A) and CoS 510 (Fig. 2B) sugarcane varieties, respectively. The sequence information of first isolate evidenced the association of SCYP belonging to 16SrI-B subgroup of phytoplasma in the YLD affected Co 0238 (GenBank Acc. No. MN913612) sugarcane variety. Whereas the second isolate evidenced association of SCWLP (GenBank Acc. No. MN913611) belonging to rice yellow dwarf (RYD) group and 16SrXI-B subgroup with the YLD affected CoS 510 sugarcane variety.

YLD is known to be caused by single or mixed infections of SCYLV (Schenck 1990; Comstock *et al.,* 1994; Viswanathan *et al.,* 1999; Rao *et al.,* 2001; Viswanathan, 2002) and/or SCYP (Cronje *et al.,* 1998; Gaur *et al.,* 2008; Kumar *et al.,* 2015). Therefore, by symptomatology alone it becomes difficult to identify the causal agent responsible for YLD. However, the samples collected from two sugarcane genotypes *viz.,* Co 0238 and CoS 510 showed similar symptoms as described earlier (Kumar *et al.,* 2015; Kumar *et al.,* 2020). In addition to this, the association of phytoplasma with YLD was described by different researchers, including Arocha *et al.* (1999) have detected the presence of SCYP with YLD belonging to 16SrI group (16SrI-A subgroup) and Cronje and Bailey (1999) have detected the association of SCYP with YLD and which had two different strains belonging to 16SrIII and 16SrXI groups of phytoplasma from South Africa. The association of 16SrI (16SrI-A subgroup) and 16SrIII groups of phytoplasma were reported with YLD from South Africa (Arocha *et al.,* 1999). Similarly, from Brazil confirmed that the 16SrI-B subgroup of phytoplasma associated with YLD in sugarcane (Silva *et al.,* 2009).

In India, Gaur *et al.* (2008) has confirmed the association of SCYP belonging to 16SrXII group with YLD in sugarcane in India. Subsequently, the association of SCYP belonging to 16SrI group in different YLD affected sugarcane genotypes was confirmed (Kumar *et al.,* 2015; Kumar *et al.,* 2018; Rao *et al.,* 2012). Similarly, in the present investigation re-confirmed the presence of SCYP (16SrI group) in Co 0238 in India based on 16S rRNA genes and confirmed the association of SCWLP belonging to 16SrXI group of phytoplasma with YLD in CoS 510 sugarcane variety in India.

Sequence Identity, Virtual RFLP, Similarity Coefficient and Phylogeny

The NCBI BLAST results of nucleotide sequence revealed that the Co 0238 phytoplasma isolate shared 98.90% sequence similarity with that of SCYP isolate available in the NCBI GenBank. Whereas the phytoplasma isolate originating from CoS 510 isolate shared 96.76% sequence similarity with the other SCWLP isolates available in the NCBI GenBank. Although, CoS 510 variety was not exhibiting the characteristic symptoms of white leaf disease. The nucleotide identity matrix of Co 0238 phytoplasma isolate shared 100% identity with Indian SCYP isolate (Acc. No. KJ599657) belonging to 16SrI-B subgroup (Table 2). Whereas isolate originating from CoS 510 shared 100% nucleotide identity with SCWLP isolate from Thailand (Acc. No. AB052874) belonging to 16SrXI-B subgroup (Table 2), which is a new subgroup of phytoplasma recorded in India with YLD of sugarcane.

Moreover, virtual restriction fragment length polymorphism (RFLP) profiles of 16Sr RNA genes of first isolate (Co 0238), through computer- simulated *i*PhyClassifier program revealed 1.00 similarity coefficient (Table 3), with *Onion yellows phytoplasma* belonging to 16SrI-B subgroup (Acc. No. AP006628; Fig. 3A). Similarly, sequence comparison of second isolate (CoS 510) revealed the similarity coefficient of 1.00 with *Saccharum officinarum*

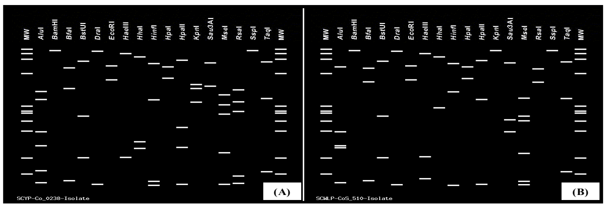

Fig. 3. Virtual restriction fragment length polymorphism (RFLP) patterns of 16S rRNA genes of sugarcane yellows phytoplasma (SCYP) and sugarcane white leaf phytoplasma (SCWLP) infecting sugarcane, using the computer- simulated *i*Phyclassifier program with defined set of 17 restriction endonuclease enzymes. (A) RFLP patterns of Co 0238 SCYP isolate corresponding to 16SrI-B subgroup (Ref. strain: Onion yellows phytoplasma*;* Acc. No. AP006628). (B) RFLP patterns of CoS 510 SCWLP isolate corresponding to 16SrXI-B subgroup (Ref. strain: *Saccharum officinarum mollecutes;* Acc. No. X76432).

Mollicutes belonging to 16SrXI-B subgroup (Acc. No. X76432; Fig. 3B). The CoS 510 isolates shared 0.93, 0.91 and 0.89 similarity coefficient with the 16SrXI-A, 16SrXI-D and 16SrXI-C subgroups of phytoplasma (Table 4).

In the present study, evolutionary history of the Co 0238 and CoS 510 phytoplasma isolates was inferred with the other phytoplasma sequences retrieved from NCBI GenBank (Table 1). Results revealed that the clustering of Co 0238 and CoS 510 isolates designated distinct clades corresponding to 16SrI and 16SrXI group of phytoplasma, respectively. The Co 0238 phytoplasma isolate clustered with the 16SrI group of phytoplasma which was known by SCYP reported earlier from Brazil, Egypt, India, and Netherland (Fig. 4). Interestingly, CoS 510 phytoplasma isolate from the present study clustered closely with *Sugarcane white leaf* and *Sugarcane grassy shoot phytoplasma* belonging to the 16SrXI group of phytoplasma associated with white leaf and grassy shoot disease, respectively in China and Thailand (Fig. 4). The CoS 510 phytoplasma isolate from the present study closely clustered with the SCWLP phytoplasma isolates belonging to 16SrXI-B subgroup of phytoplasma reported from Thailand, which is known to cause white leaf disease in sugarcane. Thus, the findings from the present study confirms the association of a novel 16SrXI-B subgroup of phytoplasma with YLD in CoS 510 in India.

The phytoplasma isolate originated from Co 0238 shared 99% to 100% sequence identity with the other SCYP isolate available in the NCBI GenBank which belonged to 16SrI-B subgroup of phytoplasma. Therefore, findings from the present investigation were in agreement with earlier studies (Kumar *et al.,* 2015). Moreover, the phytoplasma isolate originated from YLD affected CoS 510 shared 98% to 100% sequence identity at nucleotide level with the other strains of SCWLP are known to cause white leaf disease in sugarcane in India (Rao *et al.,* 2008). Prior to present investigation, similar findings were reported from Thailand, where the SCWLP belonging to 16SrXI-B subgroup was associated with the YLD but the plant exhibited the white leaf symptoms at the initial stage of occurrence of the disease (Soufi *et al.,* 2013). Whereas in the present study, no white leaf symptoms were observed on CoS 510, but we could detect the SCWLP. However, to our knowledge this is

Table 2. Sequence identity matrix of *Sugarcane yellows phytoplasma* originating from Co 0238 isolate and *Sugarcane white leaf phytoplasma* from CoS 510 isolate from Lucknow associated with yellow leaf disease of sugarcane with the other reported phytoplasma isolates obtained from NCBI

S. No.	Phytoplasma species	Acc. No.	1	2	3	4	5	6	7	8	9	10
1.	*Sugarcane yellows phytoplasma* (SCYP)*	MN913612	100%	-	-	-	-	-	-	-	-	-
2.	*Sugarcane yellows phytoplasma* (SCYP)	KJ599657	100%	100%	-	-	-	-	-	-	-	-
3.	*Onion yellows phytoplasma* (OYP)	AP006628	100%	100%	100%	-	-	-	-	-	-	-
4.	*Sugarcane yellows phytoplasma* (SCYP)	JX157631	99%	99%	99%	100%	-	-	-	-	-	-
5.	*Sugarcane yellows phytoplasma* (SCYP)	MH891145	100%	99%	100%	99%	100%	-	-	-	-	-
6.	*Sugarcane yellows phytoplasma* (SCYP)	KJ491099	99%	99%	99%	98%	99%	100%	-	-	-	-
7.	*Sugarcane yellows phytoplasma* (SCYP)	EU423900	100%	100%	100%	99%	100%	99%	100%	-	-	-
8.	*Sugarcane yellows phytoplasma* (SCYP)	MH891144	100%	100%	100%	99%	100%	99%	100%	100%	-	-
9.	*Perkinsiella saccharicida phytoplasma* (PSP)	EF413058	100%	100%	100%	99%	99%	99%	100%	100%	100%	-
10.	*Sesame phyllody phytoplasma* (SPP)	AB558132	100%	100%	100%	99%	100%	99%	100%	100%	100%	100%
11.	*Sugarcane white leaf phytoplasma* (SCWLP)*	MN913611	100%	-	-	-	-	-	-	-	-	-
12.	*Sugarcane white leaf phytoplasma* (SCWLP)	AB052874	100%	100%	-	-	-	-	-	-	-	-
13.	*Saccharum officinarum Mollicutes* (MOs)	X76432	100%	100%	100%	-	-	-	-	-	-	-
14.	*Sugarcane white leaf phytoplasma* (SCWLP)	MN174860	100%	100%	100%	100%	-	-	-	-	-	-
15.	*Sugarcane white leaf phytoplasma* (SCWLP)	KR020691	100%	100%	100%	100%	100%	-	-	-	-	-
16.	*Sugarcane grassy shoot phytoplasma* (SCGSP)	HF586637	100%	100%	100%	100%	100%	100%	-	-	-	-
17.	*Sugarcane grassy shoot phytoplasma* (SCGSP)	HF586644	100%	100%	100%	100%	100%	100%	100%	-	-	-
18.	*Sugarcane grassy shoot phytoplasma* (SCGSP)	KY420066	100%	100%	100%	100%	100%	100%	100%	100%	-	-
19.	*Sugarcane grassy shoot phytoplasma* (SCGSP)	HF586640	100%	100%	100%	100%	100%	100%	100%	100%	100%	-
20.	*Candidatus Phytoplasma oryzae phytoplasma*	AB052873	98%	98%	98%	98%	98%	98%	98%	98%	98%	100%

*: Phytoplasma isolate from the present study are indicated in bold letters.

Table 3. Similarity coefficient *(F)* derived from *Sugarcane yellows phytoplasma* (SCYP) subgroup 16SrI-B identified in this study with other reference phytoplasma strains belonging to 16SrI and other groups of phytoplasma obtained from NCBI GenBank

S. No.	Phytopl-asma	Group	Acc. Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1.	SCYP*	16SrI-B	MN913611	1.00																								
2.	SP	16SrXII-A	AF248959	0.88	1.00																							
3.	FP	16SrI-AC	JQ730859	0.95	0.87	1.00																						
4.	OBP	16SrI-AD	DQ286577	0.92	0.78	0.89	1.00																					
5.	BP	16SrI-AE	DQ286953	0.97	0.85	0.92	0.91	1.00																				
6.	MPP	16SrI-AF	AY249247	0.94	0.82	0.89	0.88	0.91	1.00																			
7.	OYP	16SrI-B	AP006628	1.00	0.88	0.95	0.94	0.97	0.94	1.00																		
8.	CPP	16SrI-R	AF222065	0.93	0.88	0.90	0.87	0.90	0.87	0.93	1.00																	
9.	CPP	16SrI-S	AF222066	0.92	0.87	0.89	0.86	0.89	0.86	0.92	0.93	1.00																
10.	AYP	16SrI-D	AY265206	0.97	0.85	0.92	0.93	0.94	0.91	0.97	0.90	0.89	1.00															
11.	BSP	16SrI-E	AY265213	0.93	0.87	0.88	0.87	0.92	0.87	0.93	0.92	0.91	0.90	1.00														
12.	CaPF	16SrXII-E	DQ086423	0.90	0.89	0.87	0.83	0.87	0.83	0.90	0.88	0.83	0.87	0.83	1.00													
13.	AYP	16SrI-F	AY265211	0.88	0.81	0.83	0.76	0.85	0.90	0.88	0.87	0.88	0.85	0.87	0.77	1.00												
14.	CaPC	16SrXII-H	JN833705	0.88	0.92	0.87	0.78	0.85	0.82	0.88	0.92	0.87	0.85	0.87	0.85	0.81	1.00											
15.	CaPS	16SrI-K	U96616	0.88	0.83	0.81	0.64	0.83	0.80	0.86	0.85	0.86	0.85	0.90	0.76	0.90	0.83	1.00										
16.	OPP	16SrI-L	GU223209	0.97	0.85	0.92	0.91	0.94	0.91	0.97	0.90	0.89	0.94	0.90	0.87	0.85	0.85	0.83	1.00									
17.	AYP	16SrI-O	AF268405	0.87	0.74	0.82	0.80	0.83	0.80	0.87	0.80	0.80	0.85	0.80	0.78	0.74	0.74	0.80	0.83	1.00								
18.	AYP	16SrI-P	AF503568	0.94	0.87	0.89	0.88	0.91	0.88	0.94	0.93	0.94	0.91	0.93	0.83	0.94	0.87	0.92	0.91	0.80	1.00							
19.	CLLP	16SrI-Q	AY034089	0.92	0.82	0.87	0.86	0.89	0.88	0.92	0.89	0.84	0.89	0.85	0.85	0.86	0.85	0.78	0.89	0.80	0.86	1.00						
20.	CBLP	16SrI-R	HM067754	0.93	0.88	0.90	0.87	0.90	0.87	0.93	1.00	0.93	0.90	0.92	0.88	0.87	0.92	0.85	0.90	0.80	0.93	0.89	1.00					
21.	LLLP	16SrI-S	HM067755	0.92	0.87	0.89	0.86	0.89	0.86	0.92	0.93	1.00	0.89	0.91	0.83	0.88	0.87	0.86	0.89	0.80	0.94	0.84	0.93	1.00				
22.	ALLP	16SrI-T	HQ285917	0.92	0.82	0.87	0.86	0.89	0.88	0.92	0.85	0.84	0.89	0.85	0.82	0.80	0.82	0.78	0.89	0.81	0.86	0.90	0.85	0.84	1.00			
23.	MPPP	16SrI-U	FJ914650	0.93	0.83	0.90	0.87	0.90	0.87	0.93	0.88	0.87	0.90	0.87	0.87	0.81	0.83	0.80	0.90	0.84	0.87	0.87	0.88	0.87	0.88	1.00		
24.	MPPP	16SrI-V	FJ914642	0.93	0.81	0.88	0.87	0.90	0.87	0.93	0.87	0.85	0.90	0.87	0.83	0.81	0.81	0.80	0.92	0.82	0.87	0.87	0.87	0.85	0.88	0.94	1.00	
25.	AYP	16SrI-W	KJ413093	0.97	0.85	0.92	0.91	0.94	0.91	0.97	0.90	0.89	0.94	0.90	0.87	0.85	0.85	0.83	0.94	0.83	0.91	0.89	0.90	0.89	0.91	0.90	0.90	1.00

*: Phytoplasma isolate from the present study are indicated in bold letters, SP= *Stolbur phytoplasma*, FP= *Fraxinus sp. phytoplasma*, OBP= *Ocimum basilicum phytoplasma*, BP= *Broad bean phytoplasma*, MPP= *Marigold phyllody phytoplasma*, OYP= *Onion yellows phytoplasma*, CPP= *Clover phyllody phytoplasma*, AYP= *Aster yellows phytoplasma*, BSP= *Blueberry stunt phytoplasma*, CaPF= *Candidatus Phytoplasma Fragariae*, CaPC= *Candidatus Phytoplasma convolvuli*, CaPS= *Candidatus phytoplasma species*, OPP= *Onion proliferation phytoplasma*, CLLP= *Cherry little leaf phytoplasma*, CBLP= *Cherry bunchy leaf phytoplasma*, LLLP= *Lilac little leaf phytoplasma*, ALLP= *Azalea little leaf phytoplasma* and MPPP= *Mexican potato purple top phytoplasma*.

Table 4. Similarity coefficient *(F)* derived from *Sugarcane white leaf phytoplasma* (SCWLP) of 16SrXI-B subgroup identified in this study with other phytoplasma strains belonging to 16SrXI and other groups of phytoplasma obtained from NCBI GenBank

S. No.	Phytoplasma	Group	Acc. Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1.	SCWLP*	16SrXI-B	MN913611	1.00																								
2.	CaPT	16SrVI-A	AY390261	0.79	1.00																							
3.	AsYP	16SrVII-A	AF092209	0.76	0.85	1.00																						
4.	Ca.PL	16SrVIII-A	AF248956	0.83	0.89	0.74	1.00																					
5.	Ca. PO	16SrXI-A	AB052873	0.93	0.76	0.78	0.76	1.00																				
6.	BWLP	16SrXIV-A	AJ550984	0.80	0.81	0.74	0.74	0.87	1.00																			
7.	MPVP	16SrXXXII-A	EU371934	0.76	0.84	0.72	0.84	0.74	0.74	1.00																		
8.	Ca.PP	16SrIII-A	JQ044393	0.81	0.75	0.71	0.75	0.77	0.7	0.71	1.00																	
9.	Ca.PP	16SrIII-A rrn	JQ044392	0.77	0.72	0.67	0.72	0.73	0.67	0.67	0.97	1.00																
10.	CYEP	16SrIII-B	AF189288	0.80	0.76	0.69	0.76	0.75	0.71	0.72	0.93	0.90	1.00															
11.	SOM	16SrXI-B	X76432	1.00	0.79	0.76	0.83	0.93	0.8	0.76	0.81	0.77	0.80	1.00														
12.	YDCP	XXXII-B	EU498727	0.77	0.80	0.69	0.83	0.77	0.73	0.87	0.67	0.64	0.66	0.75	1.00													
13.	PBP	16SrIII-C	FJ376626	0.77	0.73	0.67	0.75	0.72	0.66	0.69	0.86	0.82	0.93	0.77	0.63	1.00												
14.	CPP	16SrVI-C	AF409070	0.82	0.97	0.82	0.85	0.8	0.84	0.82	0.78	0.75	0.79	0.82	0.77	0.74	1.00											
15.	Ca.RM	16SrXI-C	X76429	0.89	0.56	0.55	0.76	0.87	0.87	0.70	0.70	0.67	0.71	0.89	0.73	0.68	0.57	1.00										
16.	OPP	16SrXXXII-C	EU498728	0.77	0.84	0.73	0.87	0.75	0.73	0.90	0.69	0.66	0.70	0.77	0.90	0.67	0.81	0.70	1.00									
17.	Mollicutes	16SrVI-D	X83431	0.77	0.97	0.84	0.85	0.75	0.80	0.82	0.74	0.70	0.75	0.77	0.77	0.72	0.95	0.70	0.81	1.00								
18.	SCWLP	16SrXI-D	KP638414	0.91	0.79	0.69	0.83	0.84	0.78	0.78	0.72	0.69	0.73	0.91	0.75	0.70	0.82	0.80	0.77	0.77	1.00							
19.	CSVP	16SrVI-E	AY270156	0.78	0.95	0.83	0.84	0.77	0.81	0.81	0.75	0.71	0.76	0.78	0.76	0.73	0.96	0.65	0.80	0.96	0.78	1.00						
20.	Ca.PC	16SrXI-E	KR869146	0.85	0.73	0.72	0.73	0.85	0.78	0.70	0.73	0.69	0.74	0.85	0.71	0.71	0.76	0.87	0.71	0.71	0.76	0.72	1.00					
21.	MYP	16SrIII-F	AF510724	0.77	0.73	0.67	0.75	0.72	0.66	0.71	0.88	0.85	0.95	0.77	0.65	0.98	0.74	0.68	0.69	0.72	0.70	0.73	0.71	1.00				
22.	SCGSP	16SrXI-F	HF586648	0.87	0.54	0.51	0.58	0.80	0.76	0.54	0.60	0.58	0.62	0.87	0.57	0.60	0.57	0.84	0.73	0.52	0.80	0.53	0.76	0.6	1.00			
23.	WWBP	16SrIII-G	AF190226	0.80	0.76	0.69	0.76	0.75	0.71	0.72	0.97	0.94	0.92	0.80	0.68	0.85	0.79	0.71	0.72	0.75	0.71	0.76	0.74	0.87	0.52	1.00		
24.	Ca.PS	16SrVI-I	GU292081	0.78	0.90	0.86	0.79	0.78	0.76	0.76	0.74	0.71	0.75	0.78	0.70	0.72	0.89	0.69	0.75	0.93	0.71	0.90	0.74	0.72	0.53	0.75	1.00	
25.	CWBP	16SrIII-J	AF147706	0.77	0.75	0.69	0.75	0.72	0.68	0.71	0.90	0.87	0.97	0.77	0.65	0.96	0.76	0.68	0.69	0.74	0.70	0.75	0.71	0.98	0.60	0.89	0.74	1.00

*: Phytoplasma isolate from the present study are indicated in bold letters, SCWLP= *Sugarcane white leaf phytoplasma*, CaPT= *Candidatus phytoplasma trifoli*, AsYP= *Ash yellows phytoplasma*, CaPL= *Candidatus phytoplasma luffae*, CaPO= *Candidatus phytoplasma oryzae*, BWLP= *Bermuda grass white leaf phytoplasma*, MPVP= *Malaysian periwinkle virescence phytoplasma*, CaPP= *Candidatus phytoplasma pruni*, CYEP= *Clover yellow edge phytoplasma*, SOM= *Saccharum officinarum mollicutes*, YDCP= *Yellow dwarf coconut phytoplasma*, PBP= *Pecan bunch phytoplasma*, CPP= *Clover proliferation phytoplasma*, CaRM= *Candidatus roseus mollicutes*, OPP= *Oil palm phytoplasma*, CSVP= *Centaurea solstitialis virescence phytoplasma*, CaPC= *Candidatus phytoplasma cirsii*, MY= *Milkweed yellows phytoplasma*, SCGSP= *Sugarcane grassy shoot phytoplasma*, WWBP= *Walnut witches' broom phytoplasma*, CaPS= *Candidatus phytoplasma Sudamericanum* and CWBP= *Chayote witches broom phytoplasma*.

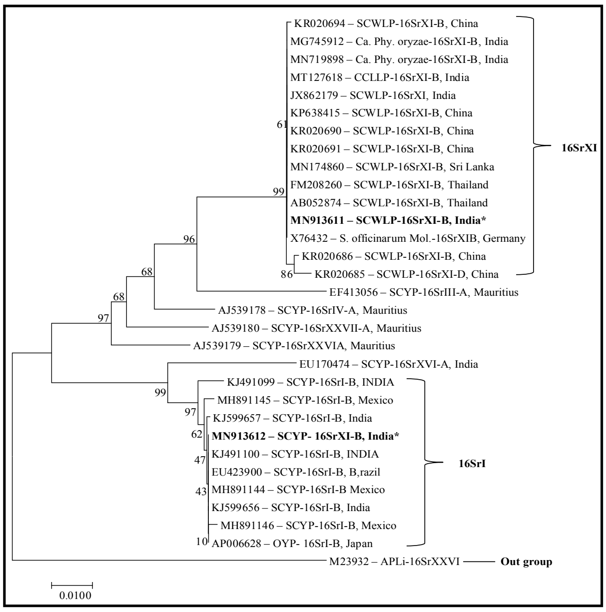

Fig. 4. Phylogenetic tree based on evolutionary history of 16SrRNA gene sequences of CoS 510 and Co 0238 isolate was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.31767934 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004). There was a total of 977 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7.4 (Kumar *et al.,* 2016). For the detailed information of sequences used for comparison is presented in table 1. *: indicated sequences are from the present study.

the first evidence of the association of SCWLP (16SrXI-B) with the YLD of sugarcane in India.

The association of the SCYP strain belonging to *Aster yellows phytoplasma* of 16SrI-A subgroup was described earlier by Arocha *et al.* (1999) from Africa and 16SrI-B subgroup from Cuba (Aljanabi, 2001), Brazil (Silva *et al.,* 2009) and Egypt (Elsayed and Boulila, 2014). Cronje *et al.* (1999) detected two different SCYP strains *viz.*, SCYP first belonging to *Western-X phytoplasma* (16SrIII group) and second was found to be SCWLP of 16SrXI group. Later, the SCYP strains from South Africa (Comstock *et al.,* 1994) and Mauritius (Cronje *et al.,* 1998), were identified and suggested the association of 16SrIII group.

CONCLUSION

From the present investigation, it has evidenced that the association of 16SrXI-B subgroup of phytoplasma with YLD in sugarcane (CoS 510) in sub-tropical conditions in India. Moreover, the association of SCYP belonging to 16SrI-B subgroup has confirmed with YLD in Co 0238, a prominent sugarcane variety cultivated in sub-tropical conditions in India.

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