10th Pathology Workshop

'Understanding Sugar Cane Diseases for their Efficient Management'

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- **Grassy shoot phytoplasma becoming a serious threat to commercial sugarcane varieties in Central-Eastern Uttar Pradesh, India**

  A. K. Tiwari, S.K. Vishwakarma and G.P. Rao

- **Progress in molecular characterization of sugarcane grassy shoot and sugarcane leaf yellows phytoplasmas in India**

  G.P. Rao, Madhupriya and A. Kumar

Grassy Shoot Phytoplasma becoming a serious threat to commercial sugarcane varieties in Central-Eastern Uttar Pradesh, India

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Phytoplasma Diseases in Sugarcane

- Phytoplasma have been reported to be associated with five sugarcane diseases viz. GSD, WLD, Sugarcane green grassy shoot, leaf yellows and Ramu stunt.
- GSD has been reported from Bangladesh , India , Malaysia, Nepal, Pakistan & Sudan (Rao et al., 2005; Viswanathan , 2000).
- Association of phytoplasma in YLS has been recorded from Africa, Australia, Mauritius and India (Aljanabi et al., 2001: Gaur and Rao, 2010).

Economic Importance of the SCGS Phytoplasma

- Wide occurrence in many promising commercial varieties
- 30-60% loss in germination (Madan et al, 1981; Dhumal, 1983)
- Very high yield loss (40-90%) (Bachchav et al.,1989; Viswantahan, 2000)
- 35% reduction in stalk height & 15% reduction in stalk girth
- 50-60% reduction in length of internodes
- Significant reduction in millable cane in ratoons
- 40% reduction in sugar yield (Viswantahan, 2000)
- No distinct symptoms at early stage on infection

Sugarcane Grassy Shoot Disease (GSD)

- GSD is known by various names like Albino, Yellowing, Bunchy disease, Leaf stuff, Grassy growth and **Grassy shoot**.
- The GSD was first observed in India by Barber (1919) from Maharastha
- It's incidence is always high in ratoon crops
- It's spread very rapidly in high moisture humidity and low temperature.
- GSD is a set borne disease and secondary infection has been carried out by leafhopper and possibly by weeds in sugarcane field.

Occurrence of Sugarcane Phytoplasma In India



Incidence Severity of GSD at different locations of Uttar Pradesh Province in Previous years (2007-2011)



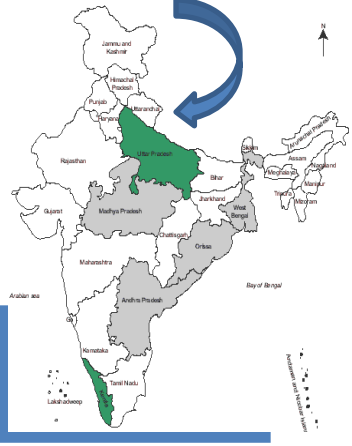
Year	Varieties affected	Incidence %	Districts
2007-08	CoS 767,8432,8436,88230,91269,95255, 96275,96268,97261,97264,CoSe 96436	>2	J.P. Nagar, Meerut, Saharanpur, Bareilly, Sitapur, Mathura, Raibareli, Balrampur, Gonda, Pilibhit, Barabanki.
2008-09	CoJ 64,CoS 96269,95253,96275,97261, 97264,767,8432,96268,88230,CoSe 92423	6-9	Shahjahanpur, Kheri, Sitapur, Raibareli, Sultanpur, Balrampur, Muzffarnagar, Saharanpur

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Survey Table continue.....



Year	Varieties affected	Incidence %	Districts
2009-10	CoJ 64, 85, CoS 767, 8432, 88230, 96269, 95253, 97261, 97264, 96268, CoSe 92423	12-15	Shahjahanpur, Lakhimpur, Sitapur, Bareilly, Pilibhit, Etawah, Muzffarnagar, Bijnor, Meerut
2010-11	CoS 8436, 95255, 767, 8830, 97261, 96269, 95422, 96436, 07250, CoSe 01424, 95422, 98231	Stay to 3	Pilibhit, Hargaon, Sultanpur, Gola, Pallia, Seohara, Rudrabilaspur, Dhampur.
	CoS 767	**18-56**	Ramala / Bagpat
	CoS 8436, 88230, 767, 92423	5-25	Amroha / J.P. Nagar
	CoS 1148,CoS767	**6-51**	Khatauli / M.Nagar
	CoS 767	4-11	Tikola / M.Nagar
	CoS 07250, CoS 95255, CoSe 01434, CoS 99259, CoS 98259, CoS 96275, CoS 97264	**10-48**	Shahjahnapur, Sitapur, Hardoi





Per cent Incidence of GSD/Leaf yellows (2011-12 harvesting season)

Varieties	GKP	SDR NGR	SPN
CoLk 8102	**16.8/ 23.6***	2.3 / 11.2*	**14.8/ 14.6***
CoS 767	- / 12. 6	- / 8.0	15.3/ 12. 0
CoSe 92423	8.6 / 12.0	6.2 /12.7	5.0/ 3. 6
CoS 8436	**15.4/ 13.8**	**23.7/ 36.7**	**/ 24.0**
CoS 95422	- / **24.0**		- / **26.5**
UP 1235	8.4 / 22.6	3.6 /16.8	4.0/ 16.6
BO 91	2.7 /10.6	18.4 /22.5	- / -
CoSe 98231	**11.0/32.2**	**15.3/ 24.6**	**32.2/28.0**

28.5

GSD

43.5

LY





GKP= Gorakhpur; SDR NGR= Sardarnagar; SPN= Shahjahanpur

* Per cent incidence on infected clump basis

Epidemiology

- Maximum incidence of the disease was noticed during the August month in Central and Western Uttar Pradesh (3 yrs data).
- In August month average humidity was recorded as 80.6% and average temp was 25.7/22.9^{o}C (max/min) (3 yrs data).
- Vector population ??????

Diagnosis

- Symptoms
- EM
- Serology
- PCR assays

Symptoms of Sugarcane Grassy Shoot Disease

- Chlorosis of spindle leaves and tillers.
- Excess tillering & stunting growth of clumps.
- The chlorotic tillers proliferate from the base of infected canes.
- The proliferation of diseased clumps make the plant bushy, grass like growth.
- No formation of millable canes and the clump dries out at later stage.

Symptoms



Leaf Chlorosis



Chlorosis of leaves



Grassy shoot proliferation

Symptoms ...









Grassy shoot appearance symptoms (without chlorosis) at Shahjahanpur District (Age 10 months)



Symptom showing chlorotic streaks on leaves in affected clump at Gola Res Station

Mixed Infection YLS & GSD



Effect of GSD on Yield Parameters in Sugarcane Varieties

S.N	Variety	Phenotypic					
		No of Internodes	Length of Internodes **cm**	Stalk height **cm**	Girth CM	Wt of Cane **(Kg)**	Wt of Juice **(Kg)**
1.	CoS 95255 **Healthy**	19	14.3	210	1.6	2.1	2.3
2.	Infected	14	13.4	148	1.5	0.57	1.2
3.	CoS 07250 **Healthy**	18	13.2	234	2.4	1.73	1.7
4.	Infected	12	10.5	138	18	0.42	0.95

(Average of 10 canes)

Juice Analysis

S.N	Variety	Juice Quality			
		Brix	Pol%	Purity	Sucrose
1.	CoS 95255 **Healthy**	19.27	72.64	90.25	17.50
2.	Infected	**14.98**	**52.36**	**85.30**	**12.88**
3.	CoS 07250 **Healthy**	17.80	65.38	88.62	15.88
4.	Infected	**15.24**	**47.34**	**75.59**	**11.61**

❖Extraction % of juice was reduced by up to 40% in infected canes as compared to healthy ones.

(Average of 10 canes)

Progress in molecular characterization of sugarcane grassy shoot and sugarcane leaf yellows phytoplasmas in India

G.P. Rao, Madhupriya and A. Kumar

Div Pl Pathol, IARI, New Delhi, India

Primers used

P1 5’-AAGAGTTTGATCCTGGCTCAGGATT -3’ (Deng and Hiruki ,1991)
P4 5’-GAGGGTGGCGGTTCT-3’ (Schneider *et al*.1995)
P7 5’-CGTCCTTCATCGGCTCTT-3’ (Schneider *et al*.1995)
P6 5’-TGGTAGGGATACCTTGTTACGACTTA-3’ (Deng and Hiruk,1991)
R16F2n 5'-GAAACGACTGCTAAGACTGG -3‘ (Gundersen and Lee,1996)
R16R2 5'-TGACGGGCGGTGTGTACAAACCCCG-3‘(Gundersen and Lee,1996)

- The highly conserved 16S rRNA gene sequence has been widely used as the very useful primary molecular tool for preliminary classification of phytoplasmas

SCGS Characterization

- Since the diseased plants exhibit various phenotypic symptoms under field conditions, diagnosis of the disease becomes difficult.

- primer pairs P1 and P7 were used for successfully detecting the SCGS phytoplasma in sugarcane leafhopper vector *Deltocephalus vulgaris* (Viswanathan *et al*., 2005; Srivastava et al., 2006; Viswanthan & Rao, 2011).

- Differential filtration approach was used to isolate and enrich the SCGS phytoplasma and its genomic DNA that was detected by PCR analysis for phytoplasmal 16S rDNA. Ratio of pathogen to host plant DNA was found in the order of 10^3 and 10^5 in infected tissue and enriched fraction respectively, offering 148-fold increase in sensitivity for their detection (Prabhu et al., 2008).

16SrDNA sequence similarity

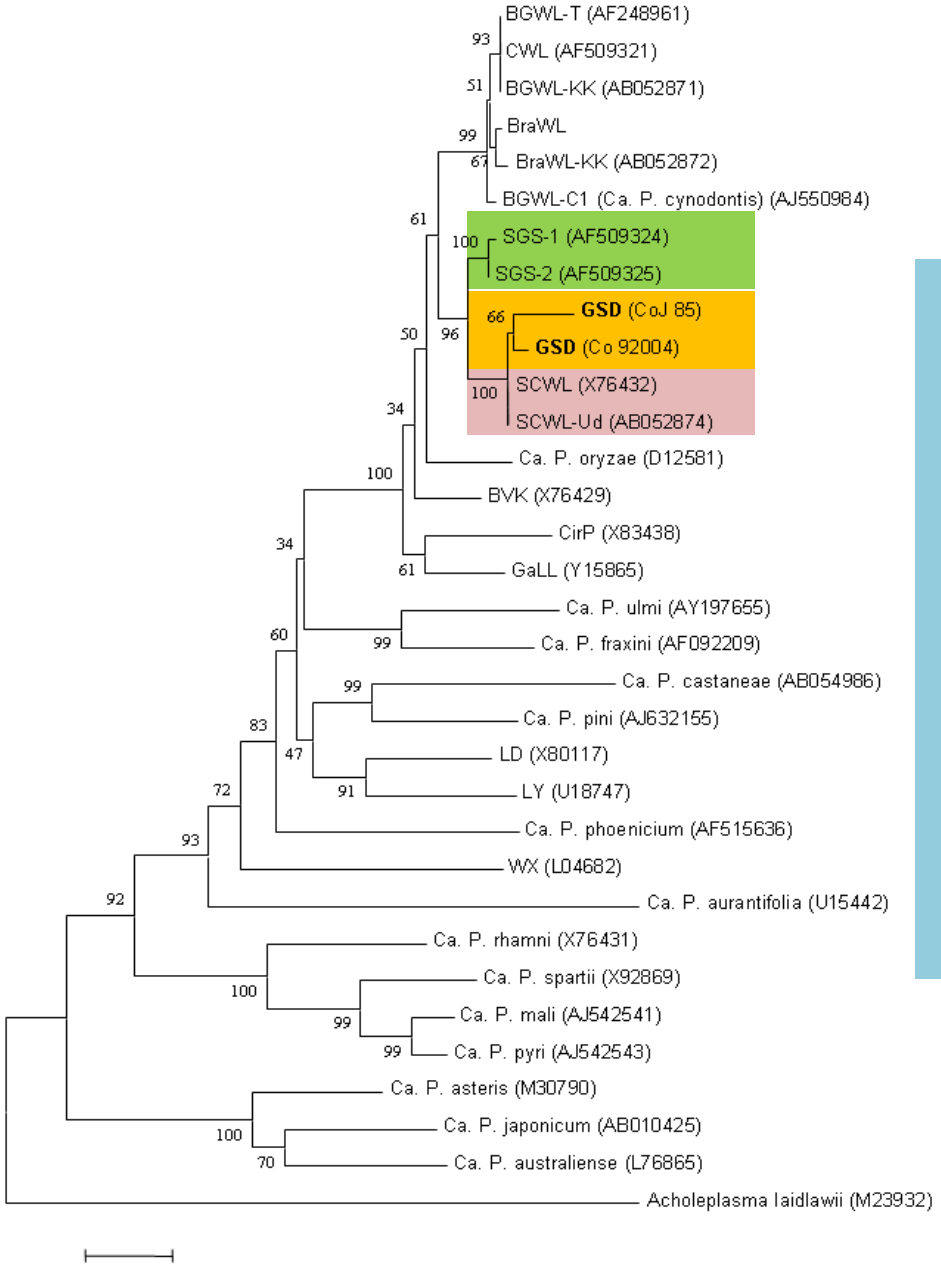
- SCGS phytoplasma affecting sugarcane crops in India is very closely related to the SCWL agent and is, thus, a member of the RYD phytoplasma group.
- **SCGS** and **SCWL** phytoplasmas shared a 16S rDNA sequence similarity which varied from of 97.5 to 98.8%.
- The other most closely related to SCGS phytoplasma are the **BGWL** (='*Candidatus* Phytoplasma cynodontis') and **brachiaria grass white leaf** (BraWL) agents, which shared 97.3 and 97.1% 16S rDNA sequence similarity, respectively (Rao et al., 2008).
- More distantly related to SCGS agent, is sorghum grassy shoot (SGS), RYD (= '*Candidatus* Phytoplasma oryzae')

SCGS genetic variability in India

(based on 16SrDNA & 16S-23S rRNA sequences)

- Sequence homology among SCGS-causing phytoplasma isolates in India is more than 99%, and their homology with SCWL and BGWL is from 98 to 99% (Nasare *et al.* ,2007)
- In another study, from South India concluded that phylogenetic relationships of SCGS isolates among themselves and related phytoplasmas based on 16S rRNA gene sequences showed 100% identity.
- No variation among 16S rRNA gene sequences of all the tested SGGS isolates of India were observed.
- Although there were significant variations in phenotypic expression of SCGS phytoplasmas on sugarcane, no genotypic variations could be established (Viswanathan et al. 2011)

Sugarcane & Related Phytoplasmas				
Sugarcane grassy shoot				
Sugarcane grassy shoot	16SrXI	AB239611 AB243298 AM269751 AM269750 AM266588 AM920754	Gorakhpur(UP) Uttar Pradesh, Maharasthra, Haryana and Tamil Nadu	Rao and Marcone (2007); Rao *et al*. (2005); Rao and Dhumal, (2002); Viswanathan, (2001)
Sugarcane yellow leaf 16SrXII				
Sugarcane yellow leaf	16SrXII	EU170474	Lucknow (UP)	Gaur *et al* . (2008); Rao et al. (2009)
Bermuda white leaf 16SrXIV				
Bermuda grass white leaf	16SrXIV	EU032485	Gorakhpur and Lucknow(UP)	Rao *et al* .(2007)
Dichanthium white leaf	16SrXIV	FJ348654	Gorakhpur(UP)	Rao *et al.* (2009)
Digitaria white leaf	16SrXIV	GQ403689	Delhi	Rao *et al.* (2009)
Oplismenus white leaf	16SrXIV	GQ403690	Gorakhpur (UP)	Rao *et al.* (2009)



The phylogenetic tree computed by the neighbor-joining method also revealed that all the phytoplasmas causing SCGS, SCWL, SGS, and BGWL form a stable phylogenetic subcluster, as judged by branch length and bootstrap values of 100% in the RYD 16S group clusters.

List of other phytoplasmas related to SCGS Phytoplasma

Phytoplasma (strain)	Geographical origin	Group	GenBank accession no	Similarity % observed
Other SCGS phytoplasmas				
Sugarcane grassy shoot (SCGS-Pun)	India	16SrXI	DQ459439	100
Sugarcane grassy shoot (SCGS)	India	16SrXI	DQ380341	100
Sugarcane grassy shoot (SCGS-CM1)	India	16SrXI	AM269740	100
Sugarcane grassy shoot (SCGS-SV1)	India	16SrXI	AM269742	100
Sugarcane grassy shoot (SCGS)	India	16SrXI	DQ380336	99.7
Sugarcane grassy shoot (SCGS)	India	16SrXI	AB243298	98.0
Sugarcane grassy shoot (SCGS)	India	16SrXI	AM085763	97.6
Sugarcane grassy shoot (SCGS)	India	16SrXI	AM085764	94.3
Other sugarcane phytoplasmas				
Sugarcane white leaf (SCWL)	Thailand	16SrXI	X76432	99.6
Sugarcane yellows phytoplasma (SCYP)	Mauritius	16SrIII	AJ539178	97.8
Related groups of phytoplasma				
Sorghum grassy shoot (SGS-1)	Australia	-	AF509324	99.6
Brachiaria white leaf (BraWL-KK)	Thailand	-	AB052872	99.6
Cynodon white leaf (CWL)	Australia	-	AF509321	99.1
Rice yellow dwarf (RYD)	Japan	16SrXI	D12581	97.5
Mollicutes	Germany	-	X76429	96.6
Sweet Potato witches broom (SPWB)	USA	16SrII	L33770	94.4
Ethiopian gliricidia little leaf (EGLL)	USA	-	AF361018	93.9
Bermuda grass white leaf (BGWL-C1)	Italy	16SrXIV	AJ550984	93.6
Ca.P.Pini	Spain	16SrXXI	AJ632155	85.7
Potato witches broom (PWB)	USA	16SrV1	AY500818	84.7
Stylosanthus little leaf (StLL)	Australia	16SrII	AJ289192	84.3
berry yellows (CBY)	Bolivia	16SrXIII	AF495882	84.3
Faba bean phylody (FBP)	Bolivia	16SrII	X83432	82.4

Weeds reported to be host of Different Phytoplasma groups in India

S N	Weeds as Host	Locations	16Sr groups
1	*Cynodon dactylon*	UP	16SrXIV
2	*Parthenium hysterophorus*	UP	16SrI
3	*Dactylotenium aegyptium*	UP	-----
4	*Dichanthium annulatum*	UP	16SrXIV
5	*Imperata arundinacea*	UP	----
6	*Ageratum conyzoides*	UP	16SrI
7	*Cannabis sativa*	UP	16SrI
8	*Oplismenus burmanni*	UP	16SrXIV
9	*Digitaria sanguinalis*	Delhi	16SrXIV
10	*Crotalaria tetragona*	Assam	16SrI
11	*Crotalaria spectabilis*	Assam	16SrI
12	*Achyranthes aspera*	UP	16SrI
13	*Amaranthus sp.*	Uttara Khand	16SrI
14	*Datura inoxia*	UP	16SrVI
15	*Calotropis procera*	UP	16SrI





15 weed species have been reported as host of Phytoplasma from India







Mall et al .,2011, Rao et al., 2012

Unexplored gene sequences and Methods Used for Characterizations of Phytoplasmas

- For subgrouping of phytoplasma up to strain level and subgroups different novel gens for group and subgroup specific characterization of phytoplasma
  -*Sec* A , *Sec* Y ,*gro*EL, *tuf*, *Rec A,D,R,G,U, ruv-A,B, Uvr A,B,C,D, per A, imp*, *dna*K *rpsJ-rplC-rplD-rplW-rplB* gene
  -*StAMP* gene encoding the antigenic membrane protein
  -Oligonucleotide microarray targeting 16S-23S spacer region
  -Real time PCR
  -Genes encoding 2 different immuno-dominant membrane proteins (*imp* genes)

- We have sequenced housekeeping genes in phytoplasma genome for further characterization of SCGS-phytoplasma into sub-group and/or strain level characterization of isolates

- ***Genes explored: gyrA, gyrB and secA genes***

Gene sequences were obtained from fully sequenced genome of Onion yellows phytoplasma OY-M DNA

Primers were designed using Primer3

Primers were in silico validated and used for sequencing SCGS

Feature	Onion Yellow-strain M
Length (bp)	860,631
G+C content (%)	28
Protein-coding region (%)	73
No. of protein-coding genes with assigned function	446
No. of conserved hypothetical genes	51
No. of hypothetical genes	257
Total no. of genes	754
Avg length of protein-coding genes (bp)	785
No. of tRNA genes	32
No. of rRNA operons	2

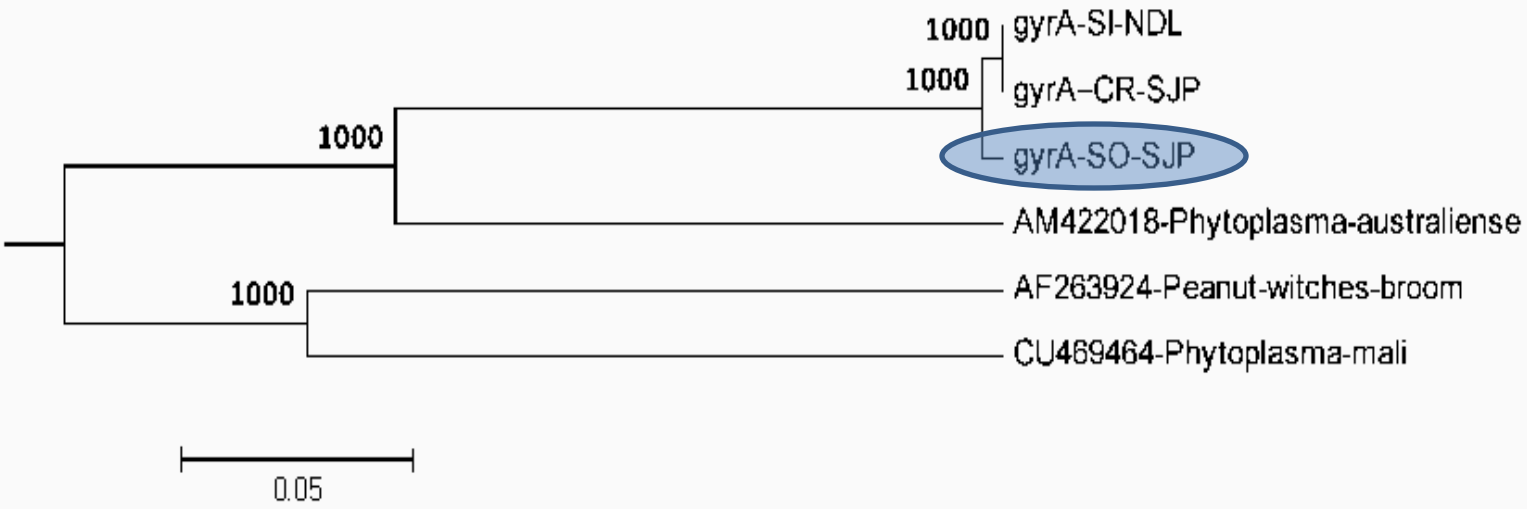
Phytoplasmas in general have distinctive genomic features such as a reduced chromosome, low GC content, fewer than 1,000 genes, and a limited metabolic capacity.

- Amplification of fragments containing phytoplasma *gyr*A, *gyr* B and sec A gene sequences with newly designed nested PCR primers were designed to amplify the respective genes in sugarcane grassy shoot

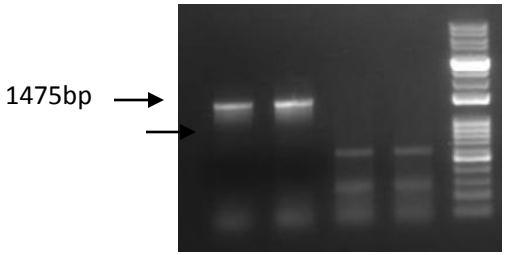
- Following amplification with these primers expected length amplicon of 1.4kb, 1.5kb and 0.496 kb of partial *gyr*A, *gyr* B and *dna*B genes, were obtained, respectively, confirming specificity of the nested PCR systems employed to the test SCGS phytoplasmas

- Various parameters such as polymorphic sites, mutations, nucleotide diversity and haplotype diversity were calculated

*gyr*A- DNA Gyrase alpha unit- as a candidate gene for phylogenetic analysis



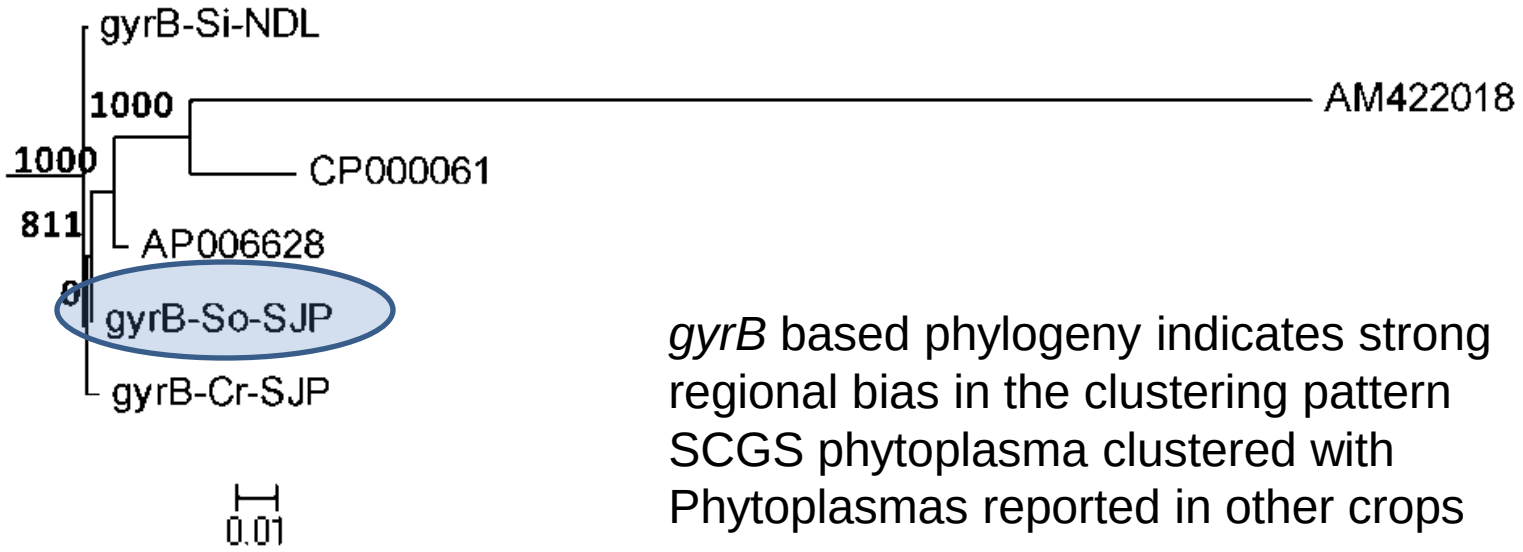
*gyr*A based phylogeny indicates strong regional bias in the clustering pattern SCGS phytoplasma clustered with Phytoplasmas reported in other crops



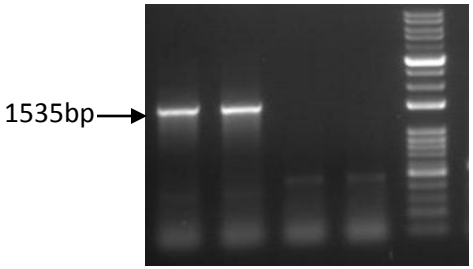
Haplotype Diversity (Hd): 0.933

Gene function: housekeeping

*gyr*B- DNA Gyrase beta unit- as a candidate gene for phylogenetic analysis



gyrB based phylogeny indicates strong regional bias in the clustering pattern SCGS phytoplasma clustered with Phytoplasmas reported in other crops



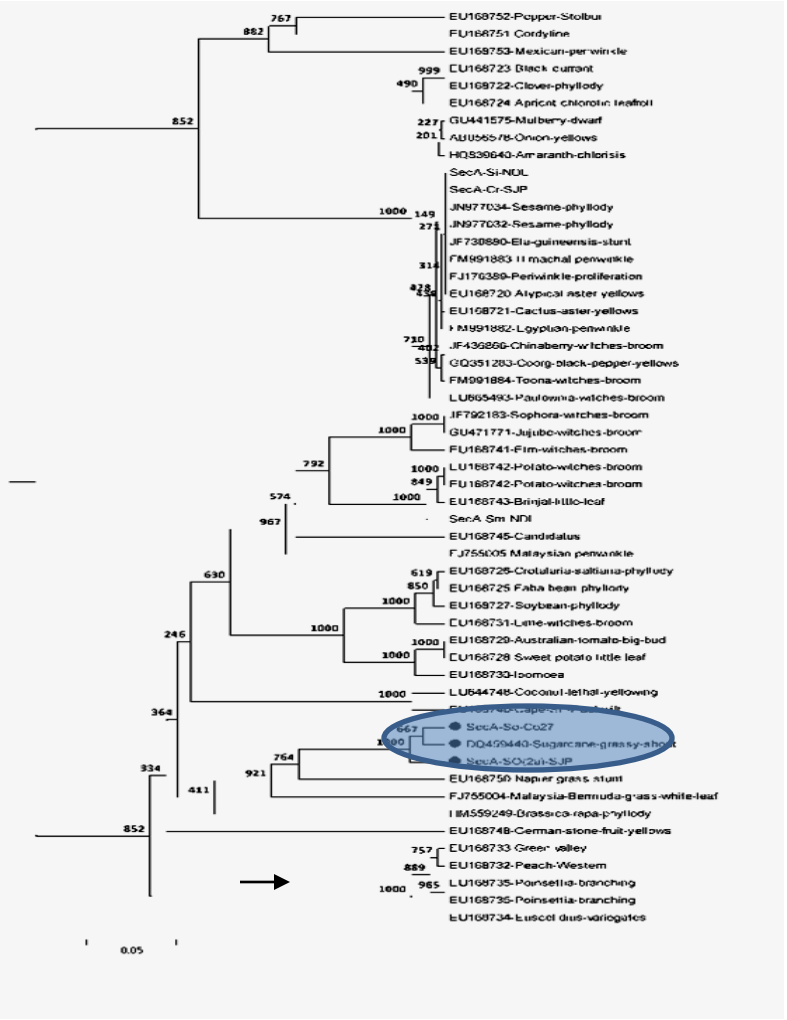
gyrB

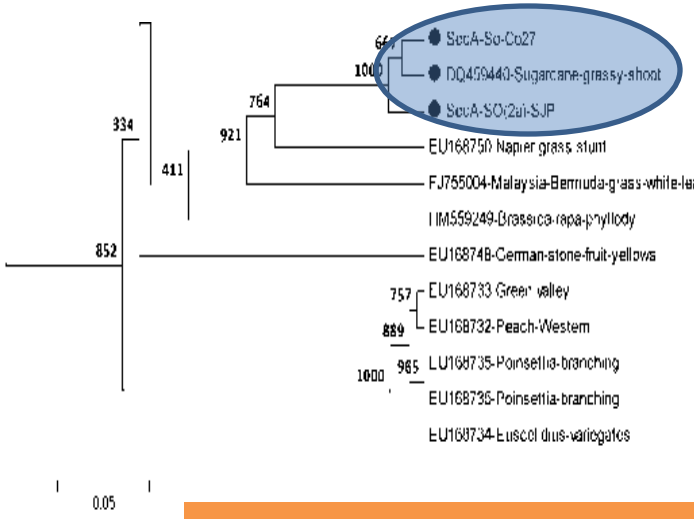
Population genetic studies should be extended to several isolates

Haplotype Diversity (Hd): 1.00

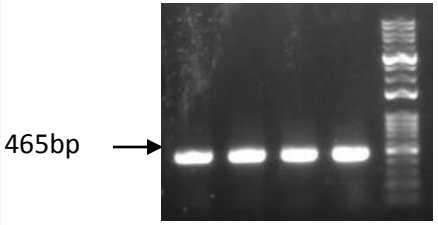
Gene function: housekeeping

secA- *secretion* dependent pathway genes as a candidate gene for phylogenetic analysis





*sec*A sequence clustered SCGS with other isolates of SCGS



Haplotype Diversity (Hd): 0.975

Gene function: Virulence

Selection of gene candidate for phylogenetic analysis

Gene Parameters	16S rDNA	*sec*A	*gyr*A	*gyr*B
No of isolates	31	53	6	6
Gene size (bp)	1127	251	557	1439
% G+C	0.454	0.267	0.271	0.319
Polymorphic sites (%)	4.4	68.1	45.6	25.4
Number of polymorphic sites, S	50	171	254	366
Total number of mutations, Eta	51	291	313	392
Recombination events, Rm	1	31	20	5
Codon Bias Index, CBI	0.205	0.436	0.723	0.423
Average number of nucleotide differences, k	6.011	62.70	125.4	133.87
Nucleotide diversity, Pi	0.0055	0.251	0.227	0.0951
Number of Haplotypes, h	9	40	5	6
Haplotypes (gene) diversity, Hd	0.712	0.975	0.933	1.000
Tajima's D	-1.964	-0.080	-0.554	-1.433
Fu and Li's D	-2.902	0.1502	-0.231	-1.381
Fu and Li's F	-3.062	0.0738	-0.330	-1.533

Genes with high haplotype diversity are suitable for genotyping of phytoplasma

Sugarcane Leaf Yellows

- Recently reported disease
- The prevalent agent is a member of the X-disease group which showed a sequence similarity of 98.8% with the western X-disease phytoplasma.
- Detection and molecular characterization of phytoplasmas from SCYLS-diseased sugarcane plants have also been reported from Cuba (Arocha *et al.* 1999). In this case, an organism of the AY group, subgroup 16SrI-A, was identified on the basis sequence analysis of 16S/23S rDNA spacer region and RFLP analysis of PCR-amplified 16S rDNA sequences using *Alu*I, *Rsa*I and *Hae*III.
- In Australia, a great genetic diversity among phytoplasmas associated with SLY disease has been evidenced by RFLP and sequence analyses of PCR-amplified rDNA (Tran-Nguyen *et al.* 2000).
- SCYLV reported from many sugarcane cvs showing YLS in India (Rao,2001; Viswanathan et al.,2009;Rao et al.,2011)

- **But no information was available until 2010 about phytoplasma association from India**

Infected clumps of Sugarcane by Sugarcane yellow leaf syndrome (SCYLV)








CoSe05459


Sh709/01


CoSe05451



Mixed infection of SCYLV+ SCYLP+RSD
(Crop age 5 months)
(Rao et al., 2010)
Yield loss up to 40%

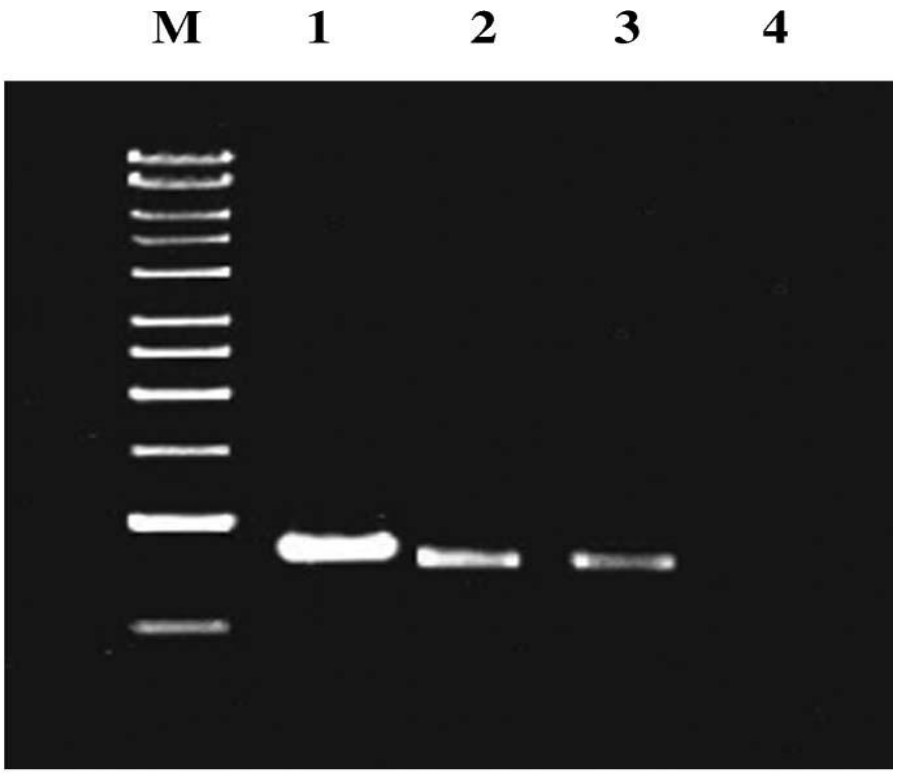
Characterization of SCLY-Indian isolate

- Phytoplasma strain was detected in leaves of sugarcane in India exhibiting symptoms of yellowing of midribs.
- A phytoplasma characteristic 1.2 kb rDNA PCR product was amplified from diseased samples using universal primer pairs P1/P7 and f5U/r3U.
- Restriction fragment length polymorphism (RFLP) analysis of amplified 16S rDNA indicated that diseased sugarcane was infected by phytoplasma.
- The 16S rDNA sequence of the Indian sugarcane yellow leaf phytoplasma (SCYLP) showed the closest identity (99%) to that of SCYLP in Cuba identified as *Macroptilium lathyroides* (AY725233), which belongs to 16SrXII (Stolbur group). This is the first record of the detection of SCYLP, and identification of the 16SrXII group of phytoplasma associated with yellow leaf syndrome (YLS) in India.



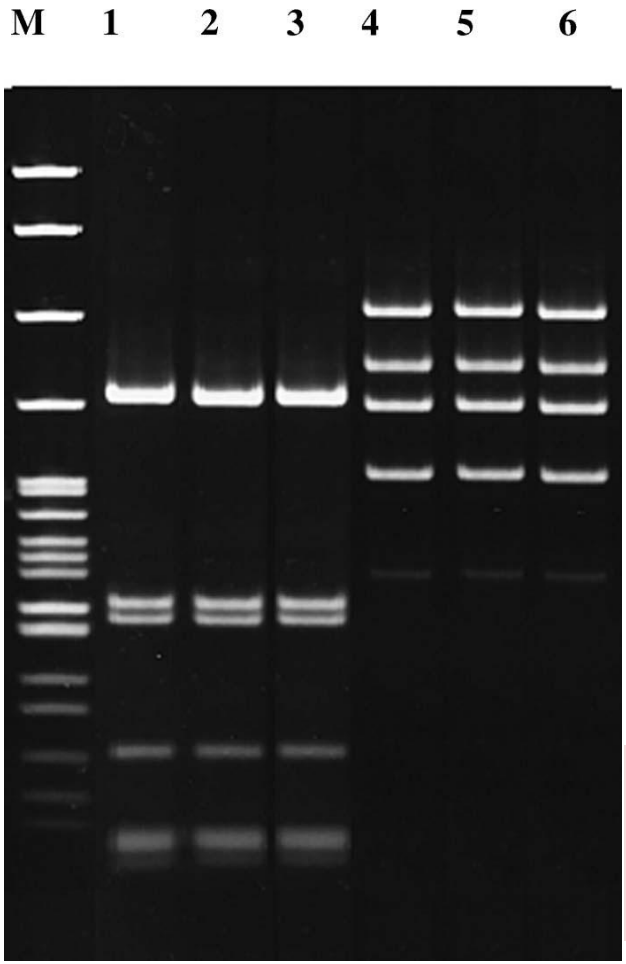
(a)

P1/P7 Lane 1–2 infected leaf sample and Lane 3 _-ve control



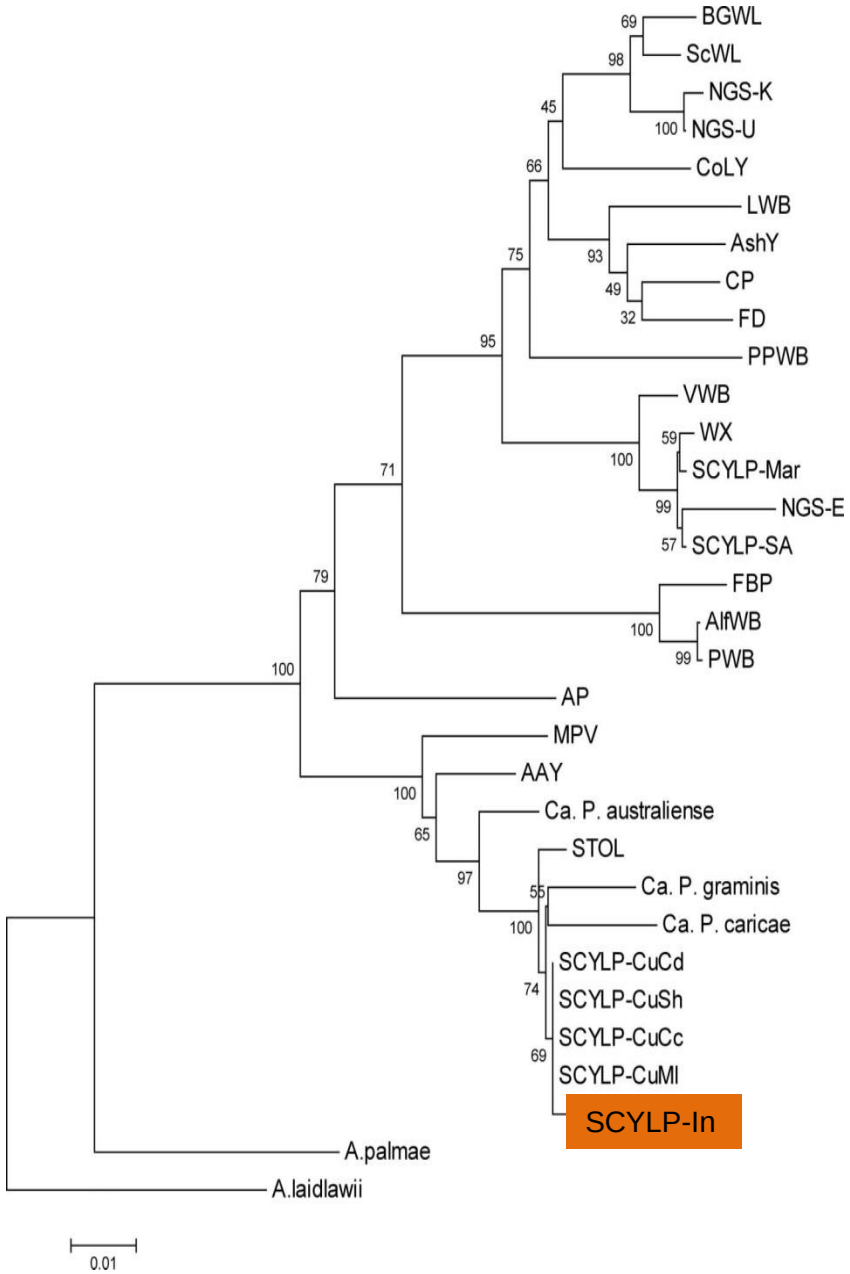
(b)

) f5U/r3U Lane 1–3 infected sample and 4 –ve control



RFLP pattern of 16S rDNA amplified by nested PCR with primer f5U/r3U. Restriction enzymes 1–3 Hae III and 4–6 Hha I

Gaur and Rao (2010)
Archives of Phytopathology and Plant Protection, 43:1132–1137



- The 16S rDNA sequence of the Indian sugarcane yellow leaf phytoplasma (SCYLP) (EU170474) showed the closest identity (99%) with that of SCYLP in Cuba identified in *Macroptilium lathyroides* (AY725233), which belongs to 16SrXII (Stolbur group)

- Phylogeny results clearly support that it is a member of16SrXII group

SCYLP Diversity

- 16SrIgroup: Cuba
- 16SrII group : Australia
- 16Sr III group: South Africa, Barbados
- 16SrXII (Stolbur group): India

- Insect: Saccarosydne saccarivora from Cuba
  - Arocha et al(2005).Pl pathol,54:634-642.

Future Areas

- Studies on genetic variability of SCGS phytoplasmas isolates by utilizing novel genes
- Identification of vector species
- Other epidemiological significance
- Identification of alternative/collateral hosts
- Develop resistance screening techniques
- Identification of virulence related genes
- Full genome sequencing

Sugarcane Phytoplasmas

- Grassy Shoot
- Green Grassy Shoot
- White leaf disease



Are they same or different ??????

Come forward to solve this issue……..



Thanks for your kind attention.....

