GENETIC AND PATHOGENIC VARIABILITY AMONG THE INDIAN ISOLATES OF *SPORISORIUM SCITAMINEUM* CAUSING SUGARCANE SMUT

Leonard Barnabas[2], N. M. R. Ashwin[1], K. Nalayeni[1], A. Ramesh Sundar[1]*, P. Malathi[1] and R. Viswanathan[1]

Abstract

Sugarcane smut caused by *Sporisorium scitamineum* is one of the major diseases of sugarcane with global prevalence and economic significance. Over the recent years, smut incidence has been increasing in India, yet there is no much information available on smut pathogen variability of Indian isolates. Hence, in this study, genetic diversity and pathogenic variability among fifty *S. scitamineum* isolates representing seven major sugarcane growing regions of India were assessed by inter simple sequence repeats (ISSR) analysis and phenotyping of disease severity on differential hosts at field level. ISSR analysis indicated that there is a considerable genetic diversity among these isolates and majority of the isolates clustered based on their geographical origin. However, there was no variation in the *bE* mating type gene sequences of representative *S. scitamineum* isolates and also there were no observable differences in the morphology of teliospores, when examined under scanning electron microscope. In the field experiments, significant differences in the percentage of disease incidence were noticed when 30 representative *S. scitamineum* isolates were inoculated on Co 96007 (smut susceptible cultivar) and accordingly, the isolates were grouped into different virulence categories. A clear discrimination among representative *S. scitamineum* isolates was evident based on the differential responses of a set of sugarcane varieties. The results of this study suggests that there is a considerable genetic and pathogenic variation among these isolates representing major sugarcane growing regions of India, which could have been effected upon them by the prevailing environmental conditions and the varieties cultivated in the region.

Key words: Variability; molecular markers; smut; sugarcane; *Sporisorium scitamineum*; pathogenicity

Introduction

Sugarcane smut caused by *Sporisorium scitamineum* is one of the important diseases of sugarcane with global prevalence (Lee-Lovick 1978). The conspicuous symptom of sugarcane smut is the modification of the meristematic region into whip-shaped sorus bearing teliospores. The disease is primarily spread by infected seed material and by air borne teliospores. Infected canes are severely stunted with excessive tillering and the resulting ratoon crops are also unprofitable (Sundar et al. 2012). Sugarcane smut usually causes significant loss in stalk yield and sucrose recovery, whereas its severity is influenced by the variety cultivated and prevailing environmental conditions (Croft and Berding 2000). In India, yield losses were reported up to 75.3 % with about 3-7 % reduction of sucrose content in the infected stalks (Mohan Rao and Prakasam 1956; Sandhu et al. 1969, 1975). In Australia, Magarey et al. (2010) have reported an estimated yield loss of 62% due to the cultivation of the smut susceptible cultivar, Q157. Sometimes, total crop failure can also occur when susceptible varieties are cultivated and if the prevailing conditions are favourable for disease development.

Due to its increasing incidence, smut disease is becoming a serious threat to several sugarcane growing states of India and its impact is devastating in terms of economic significance. Several

Leonard Barnabas, N. M. R. Ashwin, K. Nalayeni, A. Ramesh Sundar, P. Malathi and R. Viswanathan

[1] Division of Crop Protection, ICAR-Sugarcane Breeding Institute, Coimbatore, India

[2] Present Address: Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova, Padua, Italy”

*Corresponding author: rameshsundar_sbi@yahoo.co.in

sugarcane varieties with superior commercial traits that are resistant to other important fungal diseases like red rot succumb to smut and hence, are withdrawn and are no longer cultivated due to their smut susceptibility. Although several methods are available for the management and control of smut, the only economical and practical strategy is the use of resistant cultivars (Ferreira and Comstock 1989). To strategize effective breeding programs for disease resistance, understanding the genetic variability and evolutionary potential of pathogens is indispensable (McDonald and Linde 2002). Smut being a disease of global importance, several reports addressing the genetic variability among *S. scitamineum* isolates have been accumulating. Amplified fragment length polymorphisms (AFLP) technique was used to assess the genetic diversity between 38 *S. scitamineum* isolates from 13 countries (Braithwaite et al. 2004). This study indicated that isolates from Southeast Asia were a divergent group and there was very low genetic diversity between isolates representing other countries. Xu et al. (2004) had analysed the genetic diversity among 18 *S. scitamineum* isolates representing 6 sugarcane growing regions in China using random amplified polymorphic DNA (RAPD) technique and suggested that molecular variation observed among these isolates was associated to an extent to the geographical origin. Subsequently, Singh et al. (2005) investigated the intra-species diversity within *S. scitamineum* populations from South Africa, Reunion Island, Hawaii and Guadeloupe using RAPDs, *bE* mating-type specific gene detection, rDNA sequence analysis, germination and morphological studies using microscopy and concluded that the *S. scitamineum* population could have a single lineage. Raboin et al. (2007) have analysed the genetic diversity and population structure of 142 *S. scitamineum* isolates representing 15 sugarcane growing countries from Asia to America for polymorphism at 17 microsatellite loci. Genetic diversity was lower among the *S. scitamineum* isolates representing America and Africa, however, the genetic diversity was consistently higher among the Asian populations.

RAPD and SRAP markers were used to analyse the molecular variation between 23 *S. scitamineum* isolates representing 6 major sugarcane growing regions of Mainland China, which indicated that the molecular variations were associated with their geographic origin (Que et al. 2012). However, the study did not yield any information about the race differentiation of *S. scitamineum* population. Xu et al. (2014) have investigated the biogeographic variation among 100 *S. scitamineum* isolates using inter simple sequence repeats (ISSR) and single primer-sequence related amplified polymorphism (SP-SRAP) markers and suggested that the considerable genetic variation observed among these populations could be attributed to the

Fig. 1. Geographical locations of the sampling of 50 S. *scitamineum* isolates

Table 1. Sampling information about the *S. scitamineum* isolates

Isolate No.	Host	Geographical location
1	Co 96007	Tamil Nadu
2	Co 97009	
3	Co 1287	
4	CoM 0254	
5	Co 06034	
6	CoV 05-356	
7	CoV 89101	
8	Co 86002	
9	GU-04-227	
10	GU-04-238	
11	GU-04-523	
12	GU-04-2360	
13	Co 86032	
14	Co Si 6	
15	Co 94050	
16	CoSe 98231	
17	CoA 92081	
18	Co 7201 x IND 90 810	Kerala
19	Co 7201 x IND 90 812	
20	Co 99004	
21	Co 0453	
22	NG 7728	
23	IND 90 810	
24	IJ 76551	
25	Co 7219	Andhra Pradesh
26	Co 6907	
27	CoS 1032	
28	CoC 03062	
29	Co 7527	
30	CoV 05-356	
31	Ugar 1	Karnataka
32	Ugar 2	
33	Ugar 3	
34	Ugar 108	
35	Co 8011	
36	CoA 92081	
37	Co 62175	
38	Co 8014	
39	Co 08371	
40	Co 2003-165	
41	VSI 515-15/46	Maharashtra
42	Co 2000-01	
43	CoSnk 030344	
44	LG 04603	Uttar Pradesh
45	UP 05233	
46	K-seedling 09	Haryana
47	K-seedling 10	
48	CoH 150	
49	CoS 96268	
50	Co 0238	

environmental heterogeneity. Genetic diversity analysis between 23 *S. scitamineum* isolates using internal transcribed spacers (ITS) indicated that ITS was only suitable for defining the genus and species differentiation and not for the molecular variation analysis among *S. scitamineum* isolates (Zhang et al. 2015). Shen et al. (2016) have used highly polymorphic Start Codon-Targeted (SCoT) markers to analyse the genetic diversity among 90 *S. scitamineum* isolates representing mainland China, which had grouped the isolates into 3 major clusters and the clustering was based on the geographical origin. Benevenuto et al. (2016) have investigated the genetic diversity among 41 *S. scitamineum* haploid sporidial strains representing Brazil and 6 *S. scitamineum* isolates representing Argentina using AFLP and telomere associated restricted fragment length polymorphism (tel-

RFLP) and ITS sequencing. Results of this study indicated the prevalence of significant genetic diversity among these *S. scitamineum* isolates, and suggested that the reason for this diversity could be due to the exchange of smut infected sugarcane material between regions of Brazil and between these neighbouring countries.

Comprehensively, it is evident from these reports that there is considerable genetic diversity between the *S. scitamineum* isolates, especially among the Asian populations. Despite increasing smut incidence in India, to the best of our knowledge, there are no reports or adequate information available, on the genetic diversity of *S. scitamineum* population representing India. In this study, we have used ISSR markers to assess the genetic diversity among 50 *S. scitamineum* isolates representing 7 major sugarcane growing regions of India and have attempted to correlate the genetic diversity with the results of field experiments to assess the relative virulence and differential host interactions. Further, the nucleotide sequences of *bE* (mating type) gene and the teliospore morphology of representative *S. scitamineum* isolates were analysed for possible variations.

Materials and methods

Sampling and isolation of *S. scitamineum* isolates

During the period 2011 to 2012, teliospores of 50 *S. scitamineum* isolates from different genotypes were sampled from 7 major sugarcane growing regions of India, representing diverse geographical region (Fig. 1; Table 1). Teliospores from 50 *S. scitamineum* isolates were surface sterilized with 500 ppm streptomycin sulphate, thoroughly rinsed with sterilized water and a dilute suspension of the teliospores was plated on potato dextrose agar (PDA) with 500 ppm streptomycin sulphate. Single haploid sporidial cultures were established by serial dilution of the resultant plate cultures and re-plating it on PDA. Random mating experiment was performed on PDA with 1% charcoal and 500 ppm streptomycin sulphate (Baunett and Herskowitz 1989) to arbitrarily designate the haploid sporidial cultures as same or opposite mating types (+ or -) based on the mating reaction

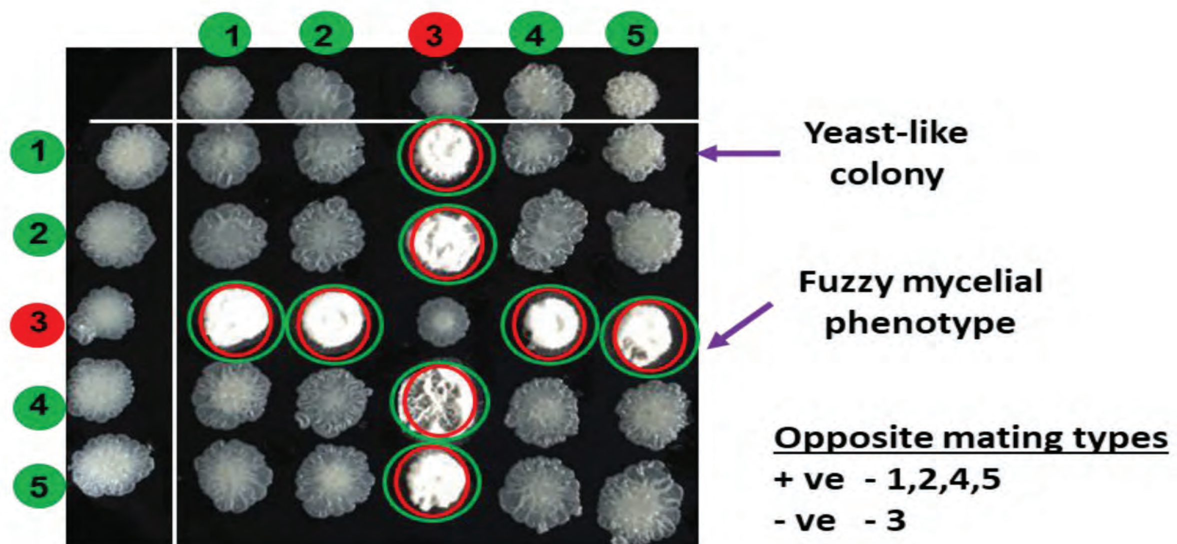


Fig. 2. Random mating experiment for identification of opposite mating type haploids of *S. scitamineum*

(‘Fuzzy mycelial’ phenotype or ‘yeast-like’ colony) observed (Fig. 2).

DNA extraction

Haploid sporidial cultures maintained on PDA were transferred to Czapek Dox media with 500 ppm streptomycin sulphate for mass multiplication. After incubation at 28 °C with shaking at 130 rpm for 48 to 72 h, cells were harvested by centrifugation at 10, 000 rpm for 15 min at 4 °C. Genomic DNA was extracted using CTAB method as described by Abu Almakarem et al. (2012). Briefly, the cells were pelleted and ground with CTAB extraction buffer (CTAB), followed by an incubation at 60°C for 1 h with periodical rocking and centrifuged at 12,000 rpm for 15 min at 4°C. After clarification of the supernatant with chloroform:isoamyl alcohol (24:1), the DNA was precipitated with 0.8 volume isopropanol. After washing with 70 % ethanol, the DNA pellets were dissolved in nuclease-free Milli Q water. Integrity and quality of the extracted DNA were verified on 0.8 % agarose gels stained with ethidium bromide. DNA samples were quantified using Nanodrop 2000C (Thermo Scientific) and the concentration of all the samples were adjusted to 30 ng/µl.

***bE* gene and ISSR PCR amplification and data analysis**

To ensure that all the extracted DNA samples were sourced from *S. scitamineum,* PCR amplification with *bE* gene - specific primers were carried out (Albert and Schenck 1996). Each 25 µl reaction mixture contained 30 ng of genomic DNA template, 30 micro moles of each primer *bE*-F (CGCTCTGGTTCATCAA) and *bE*-R (CTGCCGACCGTGCTGT), 1.6 µl of 2.5 mM dNTPs, 2.5 µl of 10X Taq buffer and 0.33 µl of Taq Polymerase. Milli Q water was used to make up the volume to 25 µl. PCR amplification was performed with an initial denaturation at 96 °C for 4 min, followed by 35 cycles each of denaturation at 94 °C for 15 s, annealing at 58 °C for 45 s and extension at 72 °C for 30 s. After a final extension at 72 °C for 10 min, samples were stored at 4 °C until electrophoresis. DNA sourced from dikaryotic mycelium of *S. scitamineum* and Milli Q water served as positive and negative controls, respectively. Electrophoresis of the PCR products was carried out in 1.5 % agarose gel stained with ethidium bromide. To analyse sequence level variation in *bE* gene among distinct and representative *S. scitamineum* isolates, PCR products obtained by *bE* primers were sequenced and multiple sequence alignment was performed with the resultant sequences using ClustalW.

PCR amplification of genomic DNA with ISSR primers (ISSR9, P5, P6, P10 and P25) was carried out in 20 µl reactions with each reaction mixture consisting of 30 ng of genomic DNA template, 40 micro moles of ISSR primer, 1.6 µl of 2.5 mM dNTPs, 2 µl of 10X Taq buffer and 0.33 µl of Taq Polymerase. The final volume was made up to 20 µl with Milli Q water. PCR conditions were initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 30s, annealing stage for 45s, extension at 72°C for 30s and final extension at 72°C for 10 min. PCR products were electrophoresed in 2% agarose gels stained with ethidium bromide in TAE buffer system and gel profiles were documented with Syngene (G: Box) gel documentation system.

From the ISSR gel profiles, a binary matrix was initially generated by scoring the presence or absence of each individual band in all lanes as 1 or 0, respectively. A similarity matrix generated using Jaccard coefficient was used for constructing unweighted pair-group method with arithmetic average (UPGMA) based dendrogram for cluster analysis using DARwin version 6.0.14.

Field experiments

Field experiments were set up in randomized

complete block design in duplicates, with at least 10 plants in each replication and were carried out for 3 crop seasons from 2014 to 2017. To ensure that the seed material used for the experiments is pathogen-free, sugarcane setts were treated with hot water at 52°C for 30 min (Srinivasan 1971; Bhuiyan et al. 2012). Prior to mock inoculation and pathogen challenge, setts treated with hot water were incubated in a moist chamber with appropriate humidity to facilitate germination. For the relative virulence experiment, teliospores of representative *S. scitamineum* isolates were mixed with a few drops of Tween 20 to improve adherence and were pasted on the sprouted buds of Co 96007 (smut susceptible standard cultivar). For the differential host experiments, teliospores suspension (with Tween 20) of distinct high and moderate virulent isolates were pasted on the sprouted buds of sugarcane varieties reported as host differentials along with resistant and susceptible standards. Mock inoculation was performed by pasting the suspension of Tween 20 and water on the sprouted buds. Following inoculation, mock inoculated and pathogen challenged setts were planted under ideal agronomical conditions in 20-feet rows at plant pathology farm, ICAR-Sugarcane Breeding Institute, India. Weekly observations were made after 4 weeks of planting and smut whips were removed as and when it emerged to avoid cross-contamination. The experiments were regularly observed and emergence of smut whips were recorded until 12 months post planting. Percentage of disease incidence (PDI) for each replication was calculated as PDI = Infected plants/Total plants x 100.

All statistical analyses were performed in IBM SPSS Statistics 21.0. The data from both the experiments were subjected to Kolmogorov-Smirnov test and Levene's test to verify the assumptions of normality and homogeneity of variance, respectively (Thode 2002). Interaction effects between the different factors including trials/years, genotypes and isolates were analysed using the general linear model of univariate analysis of variance (ANOVA). If the interaction effects were not significant, the data of individual years were combined and subjected to one way ANOVA. Post-hoc Tukey's test was used to test the differences between the means of PDI at the significance level of $p \leq 0.05$ (Hsu 1996; Sileshi 2012).

SEM analysis

For teliospore morphology analysis, fine dust of teliospores were collected by tapping the smut whips of representative *S. scitamineum* isolates and observed using scanning electron microscope (SEM) SEM Quanta 250FEG (FEI Co., Japan) without prior fixation, dehydration, embedding or sputter coating.

Results

bE gene amplification to confirm the identity

To confirm that the extracted DNA was sourced from the haploid sporidia of *S. scitamineum*, all the DNA samples were subjected to PCR amplification with *bE* - gene specific primers. Prior to which, the extracted DNA was quantified and the concentration was normalised to attain uniformity. The quality/integrity was assessed by electrophoresis. A specific amplicon of 459 bp was amplified in all the samples consistent with the DNA sourced from the *S. scitamineum* dikaryotic mycelium, which served as positive control (Fig. 3). This confirmed that all the DNA samples represent *S. scitamineum*.

ISSR analysis

Eight ISSR primers were used for assessing the genetic diversity among fifty *S. scitamineum* isolates representing seven major sugarcane growing regions in India. Of which, five primers (ISS9, P5, P6, P10 and P25) yielded highly

Table 2. ISSR primers and its PCR amplification results

S. No.	Primers	Sequence (5'-3')[a]	Total number of bands	Total number of polymorphic bands	Polymorphism %
1	ISSR9	$BDB(CCA)_5$	13	11	84.6
2	P5	$(AG)_8G$	10	7	70
3	P6	$(AG)_8C$	11	9	72.7
4	P10	$(AG)_8T$	7	4	57.1
5	P25	$(GA)_8DC$	12	8	66.7
		Total	53	38	

[a] Single letter abbreviations B and D denote mixed base positions: B = C, G, or T, not A; D = A, G, or T, not C; numbers in subscript indicate the number of repeats.

polymorphic and reproducible profiles (Table 2) (Fig. 4). In total, 53 bands were scored, of which 38 bands were polymorphic. Amplified bands ranged between 200 bp and 3000 bp. On an average, 7.6 bands were amplified per primer and the percentage of polymorphic bands was 70%. Highest dissimilarity coefficient (0.684) was between the isolates 48 (SsH150 HR) and 16 (SsSe98231 TN) sampled from Haryana and Tamil Nadu, respectively. Lowest genetic dissimilarity coefficient (0.003) was between isolates 31 (SsUgar2 KA) and 33 (SsUgar3 KA) both sampled from Karnataka. Cluster analysis of the UPGMA - based dendrogram data grouped the isolates into 3 major clusters encompassing multiple sub-clusters (Fig. 5). Cluster I comprised of *S. scitamineum* isolates representing Haryana, Uttar Pradesh, Maharashtra, Andhra Pradesh and

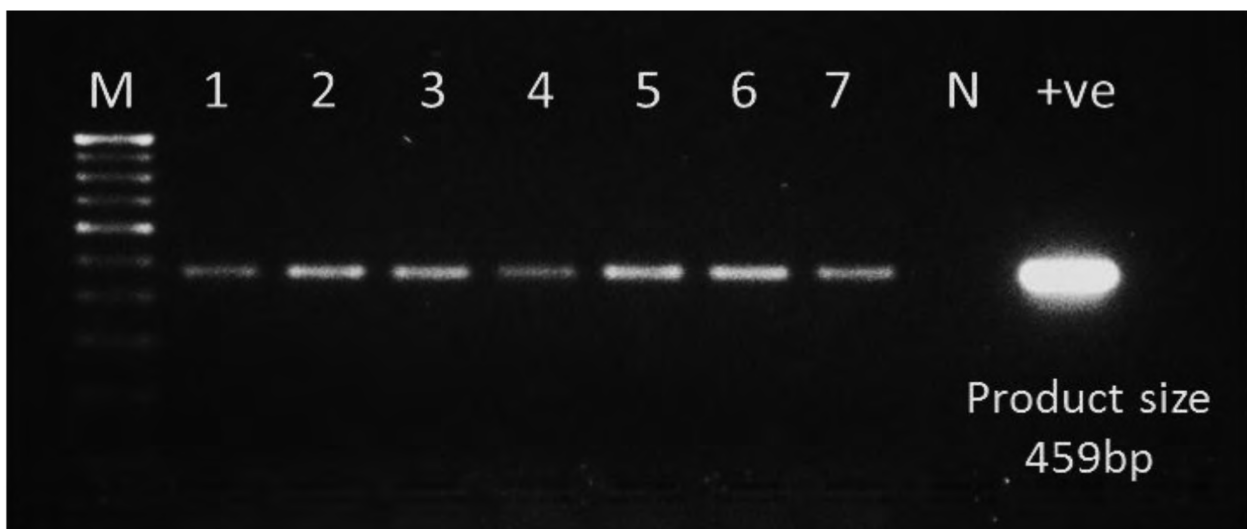


Fig. 3. Representative PCR amplification of the *bE* mating type gene to confirm the identity. Lanes M – 100 bp DNA ladder; 1,2,3,4,5,6,7 – DNA from haploid sporidial cultures; N – Negative control (Milli Q water); +ve – Positive control (DNA sourced from dikaryotic mycelium).

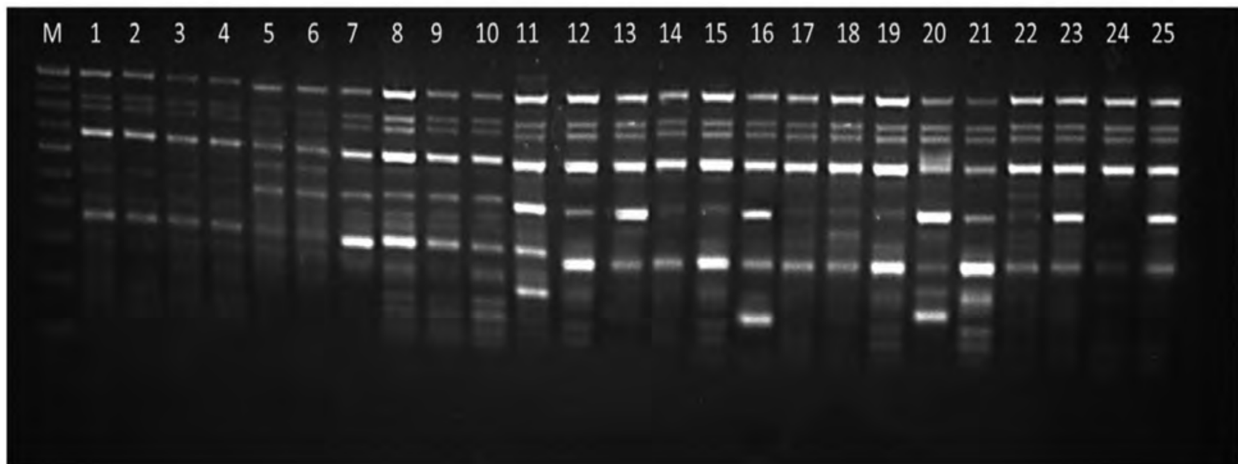


Fig. 4. Representative PCR profile obtained with ISSR primer (P6) on amplification of DNA sourced from isolates 1 to 25.

Karnataka. Isolates representing Karnataka were also grouped in separate sub-clades. Majority of the isolates representing Tamil Nadu and Kerala were grouped in cluster II and cluster III comprised isolates sampled from different regions of Tamil Nadu. Although, clustering of the isolates to a major extent was based on geographical region of sampling, some of the isolates representing the same geographical location clustered in separate sub-clusters.

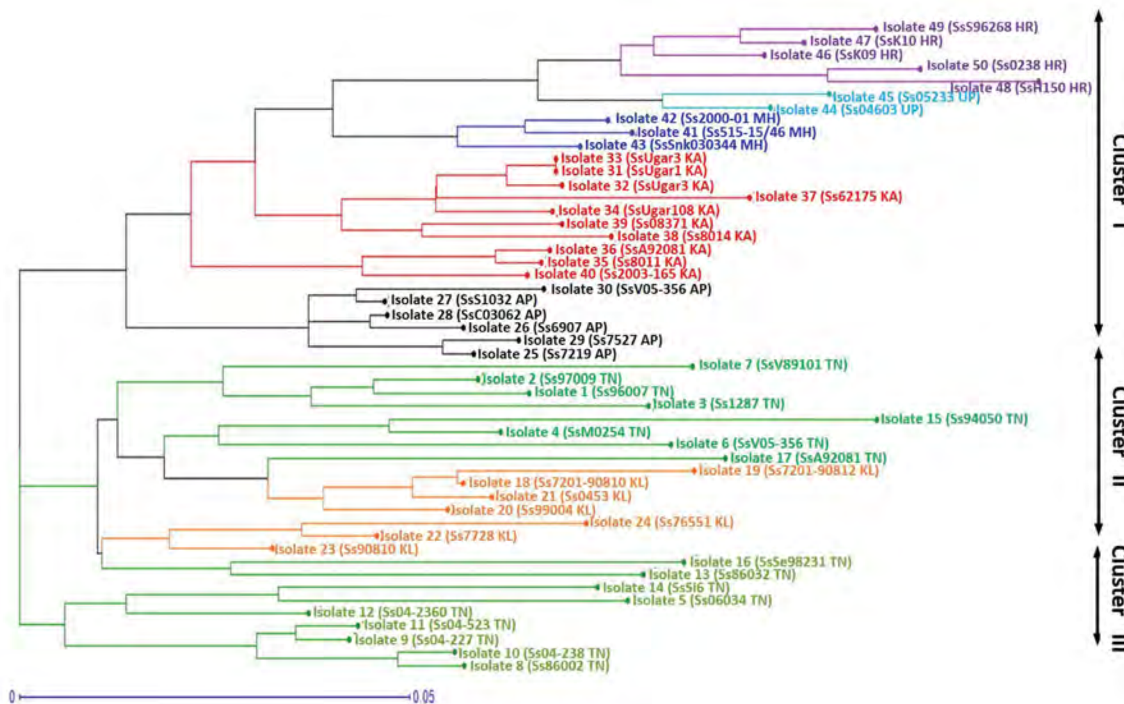


Fig. 5. Unweighted pair-group method with arithmetic average (UPGMA) based dendrogram constructed for 50 isolates from ISSR data using DARwin.

Table 3. Percentage disease incidence of representative *S. scitamineum* isolates in relative virulence experiment

S. No.	Isolate[a]	Cumulative mean of PDI[b] (%)	Category[c]
1	Isolate 1 (Ss96007 TN)	69.58[m] ±5.6	VH
2	Isolate 2 (Ss97009 TN)	59.32[k-m] ±4.1	VH
3	Isolate 41 (Ss515-15/46 MH)	58.60[kl] ±3.6	VH
4	Isolate 45 (Ss05233 UP)	53.49[kl] ±3.5	VH
5	Isolate 3 (Ss1287 TN)	45.92[i-j] ±2.2	H
6	Isolate 4 (Ss0254 TN)	30.79[f-i] ±2.3	H
7	Isolate 5 (Ss06034 TN)	32.42[f-i] ±1.5	H
8	Isolate 13 (Ss86032 TN)	43.01[g-j] ±1.2	H
9	Isolate 14 (SsSi6 TN)	36.26[f-i] ±1.7	H
10	Isolate 15 (Ss94050 TN)	32.81[f-i] ±3.2	H
11	Isolate 17 (SsA92081 TN)	32.32[f-i] ±3.3	H
12	Isolate 26 (Ss6907 AP)	47.24[i-j] ±0.9	H
13	Isolate 29 (Ss7527 AP)	44.76[h-j] ±4.2	H
14	Isolate 31 (SsUgar1 KA)	47.51[i-j] ±2.9	H
15	Isolate 32 (SsUgar2 KA)	39.15[f-h] ±2.9	H
16	Isolate 33 (SsUgar3 KA)	44.57[h-j] ±3.7	H
17	Isolate 34 (SsUgar108 KA)	32.86[f-i] ±2.0	H
18	Isolate 43 (Ss030344 MH)	34.30[f-i] ±2.1	H
19	Isolate 44 (Ss04603 UP)	36.96[f-i] ±7.8	H
20	Isolate 46 (SsK09 UP)	33.23[f-i] ±3.5	H
21	Isolate 47 (SsK10 UP)	34.58[f-h] ±3.2	H
22	Isolate 7 (SsV89101 TN)	11.48[a] ±4.3	M
23	Isolate 9 (Ss04227 TN)	12.58[ab] ±2.2	M
24	Isolate 16 (SsSe98231 TN)	26.10[a-e] ±4.5	M
25	Isolate 18 (Ss7201-90810 KL)	16.36[a-d] ±3.1	M
26	Isolate 19 (Ss7201-90812 KL)	14.70[abc] ±4.4	M
27	Isolate 22 (Ss7728 KL)	17.41[a-e] ±4.4	M
28	Isolate 23 (Ss90810 KL)	14.66[abc] ±2.2	M
29	Isolate 25 (Ss7219 AP)	26.30[b-e] ±4.1	M
30	Isolate 30 (SsV05-356 AP)	11.17[a] ±1.5	M

[a] 30 representative *S. scitamineum* isolates were used for this study;

[b] Cumulative mean of 2 replications of crop years 2012 to 2015 with ± standard errors; Means with the same letter are not significantly different (Tukey's test, $P \leq 0.05$);

[c] PDI values that shown statistically different groups were used to categorize the isolates into virulence categories. i.e. approximate PDI range of the groups are > 50 % - very high (VH); 30 – 50 % - High (H); 10 – 30 % Moderate (M); 1 – 10 % Low (L).

Relative virulence

Relative virulence experiment was carried out to assess the variations in the virulence/ pathogenicity of 30 representative *S. scitamineum* isolates on a susceptible variety (Co 96007). There were considerable differences noticed in the attributes like earliness and frequency of symptom emergence, and smut whip morphology (longer and shorter) including other unusual symptoms like galls and bud proliferation. Besides, some noticeable difference in the melanisation of the teliospores (black to brown) were also observed. There was a considerable variation in pathogenicity/virulence exhibited by *S. scitamineum* isolates on Co 96007 and the percentage disease incidence (PDI) was ranged from 69.6 (Isolate 1 – Ss96007 TN) to 11.2 % (Isolate 30 – SsV99v30 AP) (Table 3).

PDI data from all the crop season were tested by Kolmogorov-Smirnov test and Levene's test, which verified the compliance of the data to the assumptions of normality and homogeneity of variance were satisfied. There were no significant differences in the interaction effects between the data of experimental years and hence, the data of individual years were pooled and analysed by ANOVA. Post-hoc analysis by Tukey's test ($P \leq 0.05$) of PDI of these isolates indicated that there

Fig. 6. Multiple sequence alignment of *bE* mating gene amplified from DNA sourced from representative S. *scitamineum* isolates.

are statistically significant differences. Based on the PDI values that got statistically segregated into different groups, the isolates were categorized into different virulent groups. This categorization has indicated that majority of isolates represented high virulence category (57%), while 30% of the isolates were categorized under moderate virulence category and 13% of isolates represented very high virulence category. Categorization of isolates in terms of pathogenicity/virulence could not be correlated to its geographical region of sampling.

Differential host interaction

A clear discrimination of *S. scitamineum* isolates based on pathogenicity/virulence was evidenced in this differential host interaction study. All the isolates were able to produce symptoms in the susceptible sugarcane standards (Co 96007 and Co 97009) and not in the resistant standard Co 6806 (Table 4). Except isolates 1 (Ss96007 TN) and isolate 2 (Ss97009 TN), none of the *S. scitamineum* isolates caused smut symptoms in F134. However, smut incidence was not observed with these two isolates (isolates 1 and 2) in NCo 310, Co 94008, Co 92012 and NCo 376 cultivars inoculated. Notably, smut incidence was not observed in F134 inoculated with isolate 41 (Ss515-15/46 MH), but it has infected and caused smut symptoms in NCo 310 and NCo 376, indicating that this could be a very divergent strain than rest of the isolates. Further, the isolate 7 (SsV89101 TN) representing moderate virulence category produced smut symptoms only in Co 94008 and Co 92012, in addition to the susceptible standards. Intriguingly,

Table 4. Percentage disease incidence of representative *S. scitamineum* isolates in differential host interaction experiment

Genotypes[a]	Cumulative mean of the PDI[bc]					
	Isolate 1 (Ss96007 TN)	Isolate 2 (Ss97009 TN)	Isolate 41 (Ss515-15/46 MH)	Isolate 7 (SsV89101 TN)	Isolate 9 (Ss04-227 TN)	Isolate 30 (SsV05-356 AP)
F134	26.4^{cBC} ±0.9	10.0^{bB} ±1.2	*	*	*	*
NCo 310	*	*	34.0^{bC} ±2.3	*	*	*
Co 86249	23.5^{aA} ±1.1	*	40.8^{cD} ±1.7	*	*	*
Co Pant 03219	31.0^{bB} ±1.7	23.6^{bC} ±2.4	*	*	*	*
Co 94008	*	*	*	19.2^{bB} ±1.3	*	*
Co 92012	*	*	17.4^{bB} ±2.4	41.2^{cD} ±1.7	*	*
NCo 376	*	*	30.5^{bC} ±2.1	*	*	*
Co 96007	63.9^{dE} ±2.7	53.7^{cE} ±1.7	57.3^{dF} ±1.9	31.2^{bC} ±2.0	26.8^{bB} ±1.6	13.9^{aC} ±1.8
Co 97009	54.4^{cD} ±1.6	40.9^{bD} ±1.9	49.5^{cE} ±1.6	34.3^{bC} ±2.1	35.4^{bC} ±2.1	7.3^{aB} ±1.4
Co 6806	*	*	*	*	*	*

a - Genotypes reported as host differentials with susceptible and resistant standards; b - Values presented are cumulative mean of PDI of 2 replications of crop years 2012 to 2015. Mean values with same small letter are not significantly different (Tukey's test, $P \leq 0.05$); c - Mean values with same capital letter throughout the column between the isolates are not significantly different (Tukey's test, $P \leq 0.05$); * - indicate no disease incidence.

the isolates, isolate 9 (Ss04-227 TN) and isolate 30 (SsV05-356 AP), which grouped under the moderate virulent category did not cause any smut symptoms in any of the differential varieties, other than the two smut susceptible standards.

Variation in *bE* gene sequence

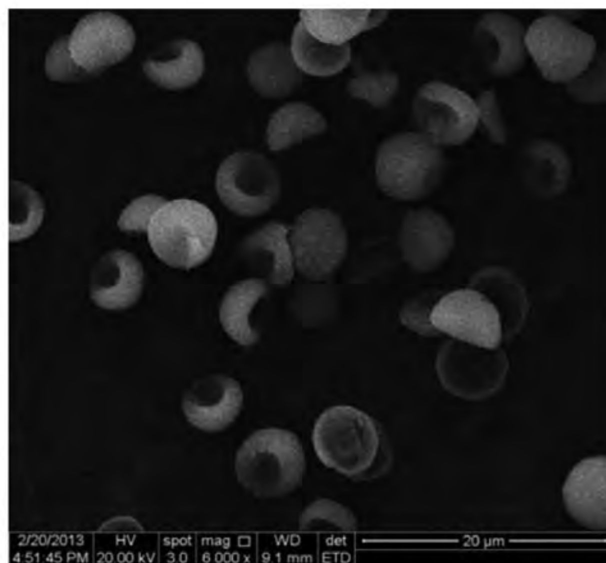
There was no noticeable difference in sequence similarity in the *bE* gene sequences of six representative *S. scitamineum* isolates representing different categories of virulence (Fig. 6).

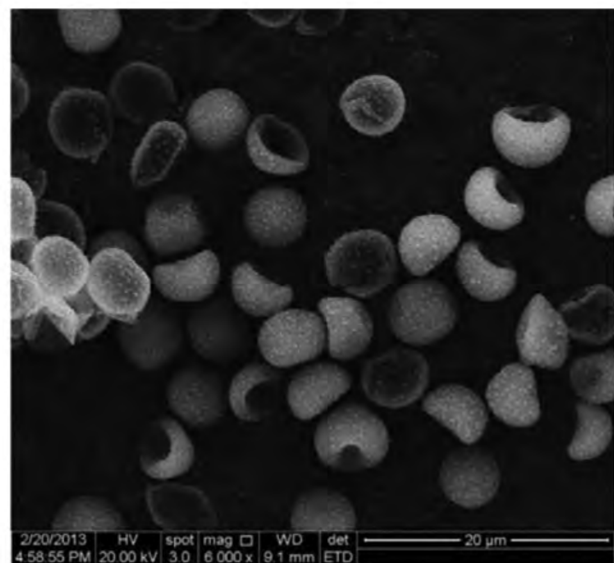
Teliospore morphology

Teliospores of *S. scitamineum* isolates representing different categories of virulence were morphologically similar when examined under SEM. Teliospores were globose to sub-globose, echinulate with marked concavity on one side and ranged from 4 to 5 µm in diameter (Fig. 7). There were no attributable differences noted in the teliospore morphology of *S. scitamineum* isolates that represented diverse geographical region and/or virulence category.

Discussion

Increasing trend of smut susceptibility has been an issue worldwide and is now becoming a serious concern for sugarcane breeders and growers. In the recent years, an upsurge in the incidence of sugarcane smut has been evidenced and has also been reported to cause havoc in different regions of India, hitherto it had not been a serious concern. This surge in smut incidence can possibly be attributed to emergence of new pathogenic variants of *S scitamineum*. It is evident from the previous reports (Braithwaite et al. 2004; Raboin et al. 2007), that the genetic diversity among the *S. scitamineum* populations representing Asia was comparably high. Although there are ample reports on the genetic diversity of *S. scitamineum* isolates representing Asian regions, especially China, there are no reports on the genetic diversity and pathogenic variability among *S. scitamineum* isolates representing India. In this context, the present study was focussed on analysing the genetic diversity among 50 *S. scitamineum* isolates representing 7 major sugarcane growing





Isolate 1 (Ss96007 TN) Isolate 41 (Ss515-15/46 MH)

Fig. 7. SEM images of teliospores of representative S. *scitamineum* isolates.

regions using ISSR markers and correlating it with the field phenotypic results. Incidentally, Que et al. (2012) and Xu et al. (2014) have also used *bE* gene - specific and ITS region amplification by PCR to ascertain the identity of the samples prior to genetic diversity analysis. Similarly, all our test samples were validated. In our study, genetic diversity analysis using ISSR markers indicated the existence of considerable variability among the isolates. Highest genetic dissimilarity (0.684) observed between isolates 48 (SsH150 HR) and isolate 16 (SsSe98231 TN) could possibly be attributed to its diverse geographical location as supported by previous reports by Que et al. (2012) and Xu et al. (2014), wherein the extreme values of genetic dissimilarity coefficients were attributed to the geographical location. Supporting it, the lowest dissimilarity coefficient (0.003) was observed between isolates 31 (SsUgar1 KA) and isolate 33 (SsUgar3 KA), which were sampled from infected plants at very close proximity from the same field in Karnataka. Cluster analysis revealed that majority of the isolates grouped based on its geographical region of origin. Isolates sampled from northern region and southern region of India were distinctly grouped into two distinct clusters. This indicated that these isolates could be very divergent, supposedly influenced by the contrasting agro-climatic conditions in both the regions. A few isolates representing different regions of Tamil Nadu and Karnataka were grouped in separate clades although were within the same major clusters. Intermingling of isolates representing Kerala with isolates representing Tamil Nadu could be due to frequent unscreened exchange of seed material between the farms in these region, without effecting the domestic quarantine measures. Evidences of such geographical region based clustering have been reported by Xu et al. (2004), Que et al. (2012) and Xu et al. (2014), wherein majority of *S. scitamineum* isolates were grouped based on its geographical region of sampling; however, this was not applicable for all the isolates. Similar trend in clustering was also observed in the closely related smut fungus – *S. reilianum* (Ma et al. 2008).

Although the true prevalence of *S. scitamineum* races is considered controversial (Ferreira and Comstock 1989), smut races have been reported worldwide (Comstock and Heinz 1977; Hsieh and Lee 1978; Leu, 1978; DaSilva and Sanguino 1978; Toffano 1976; Muhammad and Kausar 1962). Artificial inoculation of a set of cultivars that had been previously shown to have a differential response to *S. scitamineum* isolates can provide inferences on the existence of pathogenic variability and emergence of new pathogenic variants. Notably, Gillaspie et al. (1983) were able to distinguish 6 races on 5 sugarcane cultivars among a population of *S. scitamineum* representing Argentina, Florida, Hawaii, Taiwan and Zimbabwe. An international project to type the races of *S. scitamineum* at 14 locations in 10 countries using a set of 11 differential cultivars indicated that the existence of pathogenic variation in all these countries and a strong evidence of distinct races was observed in Taiwan (Grisham and Hogarth 2001). A new race of *S. scitamineum* was reported in Hawaii using 10 sugarcane varieties with differential response (Schenck 2003). Bhuiyan et al. (2015) have reported a differential hosts study with spore populations representing 2 regions of Australia. Differential response of the cultivars to the two isolates indicated that they were genetically diverse.

In our study, the results of the relative virulence experiment, wherein 30 representative *S. scitamineum* isolates were artificially inoculated on Co 96007 indicated that the isolates were genetically dissimilar, since they exhibited

significantly different PDI values. Categorization of the isolates based on pathogenicity/virulence with PDI values indicated that majority of the isolates represented highly virulent, virulent and few moderate virulent categories as expected. Representative isolates from high and moderate virulence category were carried forward to the differential host interaction experiment, wherein 6 isolates were screened against 10 varieties with reported differential response, including a susceptible and resistant standard. Based on the differential responses of varieties F134, NCo 310, Co 92012 and NCo 376 against isolate 1 (Ss96007 TN), isolate 2 (Ss97009 TN) and isolate 41 (Ss515-15/46), it is evident that these *S. scitamineum* isolates are genetically divergent and differ in its pathogenicity. However, distinguishing these isolates into races based on the noticed differential response would be inappropriate and controversial as other international races of *S. scitamineum* was not used in this study. It is noteworthy that there were differences in the % PDI on varieties inoculated with isolate 1 (Ss96007 TN) and 2 (Ss97009 TN). This could suggest that these isolates could also be genetically distinct and hence, the noticed difference in their virulence. Congruently, smut incidence was not noticed in most of the varieties inoculated with isolates 7 (SsV89101 TN), isolate 9 (Ss04-227 TN) and 30 (SsV05-356 AP), which repoints towards the moderate virulence observed in the relative virulence experiment. *bE* mating type gene sequences among the representative *S. scitamineum* isolates were identical and hence, could not be correlated to its observed virulence pattern (Fig. 6). This indicates that *bE* mating type gene could be one of the highly conserved genes in the *S. scitamineum* genome and might not be directly reflecting the variance in virulence as observed in field experiments. However, recently, it was reported that the fusion of opposite mating type bE gene products is essential for the activation of pathogenicity-related gene expression (Yan et al. 2016). There were also no striking differences in the teliospore morphology of isolates representing diverse geographical regions and different virulence/pathogenicity category, when examined under SEM (Fig. 7). Similarly, examination of teliospores morphology under SEM by Singh et al. (2005) did not indicate any difference in the morphology between geographically distinct *S. scitamineum* isolates. However, the possible differences between the *S. scitamineum* isolates in outer membrane thickness, density of echinulates, the melanin content and the variations in the composition of other structural components of the teliospores cannot be ruled out and hence, needs further detailed investigation.

Comprehensively, the present study has indicated that there is high genetic and pathogenic variability among the *S. scitamineum* isolates representing India. Observed genetic diversity and pathogenic variability could be associated to the geographical location and the varieties cultivated in the region. This points to the need of screening and evaluating the potential parents and breeding clones for smut resistance using a specific set of *S. scitamineum* isolates possibly prevalent in the region, wherein a divergent population of *S. scitamineum* could exist in other location. However, the cross hybridization ability of the compatible haploid sporidia of two distinct isolates that lead to emergence of newer pathogenic variants would be an ever prevailing challenge. Hence, stringent and periodical surveys need to be conducted to screen and identify newer pathotypes, against which the existing sources of smut resistance have to be screened prior to forwarding the clones for commercial breeding. One of the many reasons for long distance dispersal of *S. scitamineum* throughout the country could be due to unscreened exchange of stalk cuttings (seed material) between sugarcane farms. Hence, inclusion of stringent screening methods prior to seed material exchange also would strengthen

the existing smut management strategies. Conclusively, this study also highlights the need for a more concerted research involving larger *S. scitamineum* population worldwide and a wide variety of differentials hosts to better understand the existing race picture in India. The ongoing breeding programs to impart sugarcane cultivars with durable smut resistance would be greatly benefited with the information thus obtained on the prevailing genetic diversity and pathogenicity among the *S. scitamineum* population in India.

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