RESEARCH ARTICLE

STUDY OF GENETIC DIVERSITY AND EVALUATION OF INTERSPECIFIC HYBRIDS OF *SACCHARUM* SPP. USING SSR MARKERS

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Abstract

SSR marker analysis was done to assess the genetic diversity among 18 improved hybrids of *Saccharum officinarum* and 21 hybrids of *Saccharum robustum*. A total of 327 polymorphic loci were detected with 23 SSR primer pairs. The number of loci per marker ranged from five (NKS 8) to 22 (NKS 43). The primers NKS 34, NKS 43, NKS 33 and NKS 46 produced more number of polymorphic bands numbering 15, 22, 21 and 20, respectively. Marker size ranged from 103 bp to 1.3 Kb and 11 primers generated unique fingerprints for 10 genotypes. Four *S. robustum* specific markers, viz. SMC 477 GC $_{(279)}$, NKS 43 $_{(1049)\ \&\ (143)}$ and NKS 16 $_{(380)}$, and five *S. officinarum* specific markers, viz. NKS 6 $_{(873)}$, mSSCIR 54 $_{(1138)}$, NKS 42 $_{(179)}$, NKS 40 $_{(133)}$ and NKS 45 $_{(213)}$ were identified. The jaccard similarity coefficient ranged from 0.60 to 0.84 with a mean of 0.72. Most of the SSR based pair-wise comparisons exhibited least genetic similarities with coefficient values of less than 0.65. The molecular markers clearly grouped *S. officinarum* and *S. robustum* clones into two distinct clusters with moderate genetic diversity as indicated by Unweighted Pair Group Method. To investigate the introgression and stability of species specific alleles against the commercial genetic background, two generations of introgression breeding was done. First generation introgressed hybrids (IH_1) showed 15.1% improvement for HR brix % (300 days) over the *S. robustum* parents. Second generation introgressed hybrids (IH_2) showed 65.5% improvement for single cane weight and 24.6% for HR brix % at 300 days against *S. robustum*. Eighty six introgressed hybrids were screened with eight SSR primers which produced nine polymorphic markers between *S. officinarum* and *S. robustum* to identify the true hybrids. Among these markers, SMC 477 GC $_{(279)}$ and NKS 40 $_{(133)}$ were stably transmitted with maximum frequency in the introgressed hybrids. Significant correlations were identified with marker SMC 477 GC $_{(279)}$ for fibre % (r= 0.754; P<0.001) and NKS 40 $_{(133)}$ for sucrose % (r= 0.685; P<0.001). These markers also showed stability and strong correlation with the respective traits over successive generations of breeding and, hence, the marker trait association identified can be used in marker-assisted breeding programmes.

Key words: *Saccharum* spp., introgression, SSR markers, marker assisted evaluation

Introduction

Cultivated forms of sugarcane are heterozygous and aneupolyploid derivatives of interspecific hybrids involving *Saccharum officinarum* (noble cane) and *Saccharum spontaneum* (wild cane), with less contribution from *Saccharum barberi*, *Saccharum sinense* and *Saccharum robustum* (Dillon et al. 2007). The complex size and continued selection for recurrent parent genome enhanced sugar accumulation with only half as much increase in cane yield, the latter under non-additive genetic control (Hogarth 1987). Over the years, breeders have broadened the genetic base of commercial germplasm through introgression breeding involving related wild species (Burner and Legendre 1993). However, to obtain sufficient improvement in sugar content and stalk size in modern sugarcane development, they have reaped genetic gains only from the recurrent parent giving less importance to basic *Saccharum* species. This has resulted in narrow genetic base of elite cultivars due to the limited gene pool exploited in

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conventional breeding programs (Mariotti 2002). To sustain the genetic gains, novel and desired diversity should be introduced into elite parents. To achieve this, sugarcane breeders around the world have used basic germplasm in breeding programs (Miller and Tai 1992). Besides, since the modern sugarcane varieties are derived as interspecific hybrids of S. *officinarum* and S. *spontaneum* (Berding and Roach 1987) involving only a few parental clones (Arceneaux 1965; Price 1967), the genetic base of the modern varieties is apparently narrow, which results in slow progress in sugarcane breeding. To enlarge the genetic base, it is essential to utilize the variability found in other species and genera of the *Saccharum* complex.

Milligan et al. (1994) strengthened the implementation of an introgression program with successful transfer of mosaic virus resistance to BC4 progenies from cultivar x *S. spontaneum* crosses. In the present study, *S. robustum* derivatives, developed and maintained at the ICAR-Sugarcane Breeding Institute, Coimbatore, India, were used as parents to generate introgressed hybrids in two stages of breeding. It is hypothesized that *S. officinarum* originated from wild *S. robustum* populations in Papua New Guinea (Daniels and Roach 1987), where the sweet forms of *S. robustum* were selected and subjected to recombination and selection resulting in high sucrose and low fibre clones. As a consequence, there appears to be no clear discontinuity between *S. officinarum* and *S. robustum* in morphology or sugar content (Irvine 1999). Hence, DNA markers are used to detect the presence of allelic variation in the two species which helps in the selection of parents with maximum variability for introgression breeding. The use of DNA markers in plant breeding (marker-assisted selection) greatly improves the efficiency and probability of success in breeding programs (Ortiz 1998; Huang et al. 2002). Selection can be carried out at seedling stage with ensured diversity in basic breeding material along with the elimination of undesirable genotypes. Earlier, selfs were identified and removed based on vigor (Hogarth 1980) but in recent years, application of molecular markers has made it easy to determine the extent of selfing and identify true hybrids more accurately (McIntyre and Jackson 2001; Tew and Pan 2010). The present study was undertaken to assess the genetic diversity of improved clones of *S. officinarum* and *S. robustum* with identification of species specific alleles using SSRs; to characterize the hybrids generated over two generations (F_1 and BC_1) of introgression breeding for important traits and finally to select high value hybrids for marker assisted evaluation; to identify true hybrids and study the frequency of the species specific alleles transmitted in the introgressed population.

Materials and methods

Development of introgressed hybrids for marker assisted evaluation

Parents used

Improved clones of *S. robustum* and *S. officinarum* developed through intra-population improvement program are being maintained at ICAR-Sugarcane Breeding Institute, Coimbatore, Tamil Nadu State, India, for utilization in genetic base broadening programs (Anonymous 2000, 2003). The clones used for genetic characterization using SSR markers are given in Table 1. Crosses were made as *S. officinarum* x *S. robustum* and *S. robustum* x *S. officinarum* involving clones with least similarity index.

Table 1. Improved *Saccharum officinarum* and *Saccharum robustum* clones used as parents in introgressive hybridization

Improved *S. officinarum*		Improved *S. robustum*	
PIO 88 1704	PIO 00 581	PIR 93 317	PIR 96 285
PIO 96 428	PIO 00 845	PIR 03 107	PIR 00 1174
PIO 00 548	PIO 90 202	PIR 94 436	PIR 00 1176
PIO 88 332	PIO 96 436	PIR 00 15	PIR 96 475
PIO 96 446	PIO 00 741	PIR 00 1100	PIR 00 39
PIO 94 345	PIO 00 736	PIR 96 258	PIR 00 1157
PIO 96 441	PIO 00 846	PIR 00 1122	PIR 00 1057
PIO 00 1057	PIO 88 319	PIR 00 1062	PIR 00 1058
PIO 00 1188	PIO 88 79	PIR 96 305	PIR 00 1163
		PIR 00 34	PIR 00 1193
		PIR 03 149	

Crossing, seedling transplantation and phenotypic evaluation

Seventeen crosses were made during the crossing season of 2011 to raise first generation introgressed hybrids (IH_1). Four biparental crosses, viz. PIO 94 345 x PIR 96 475, PIO 00 1188 x PIR 96 258, PIR 00 1174 x PIO 00 581 and PIR 00 1163 x PIO 00 845 were selected for further studies based on the availability of sufficient population for evaluation. Seedlings grown under greenhouse conditions were transplanted to the field in April 2012. Seedlings from each of these four crosses were planted as two replicates in a plot of 6 m row with a spacing of 90 x 60 cm. Seedling evaluation was done at 300 days for major agronomic traits, viz. number of millable canes (NMC), cane diameter, single cane weight, clump yield and HR brix. From the original population of 457 seedlings, 225 top performing individuals combining good field stand and early vigor were selected and planted in a replicated field trial during the growing season of 2013 for evaluating cane yield and quality components. From this evaluation, 46 clones which showed significant improvement over parents for phenotypic traits were selected for marker assisted introgression study using the microsatellite (SSR) DNA markers SMC 1039 GC, NKS 43, NKS 16, NKS 40, NKS 6, mSSCIR 54, NKS 42, NKS 45 and SMC 477 GC, which produced the highest polymorphism between the *S. officinarum* and *S. robustum* clones. The individuals introgressed with species specific alleles were identified from which three individuals (IH_1 11-81, IH_1 11-66 and IH_1 11-15) were crossed with commercial types, viz. Co 0303, Co 0209 and Co 0240 to raise second generation introgressed hybrids (IH_2). Crossing was done during the flowering season of 2013 and seedlings were raised the same year and evaluated at 300 days after planting during the growing season of 2014; 40 progenies from second generation of introgression breeding were selected for marker assisted evaluation. Eighty six

clones selected from F_1 and BC_1 of introgression breeding were evaluated in a randomized complete block design with two replications during 2015 growing season. Observations were recorded on NMC, cane diameter, single cane weight, cane height, HR brix, sucrose %, pol %, CCS %, fibre % and clump yield.

Molecular characterization

Total genomic DNA was extracted from young shoots following CTAB method. Twenty three SSR primer pairs from Genbank database [www.nrcpb.org/STMS.html] (Table 2) were used for genetic diversity study. PCR reactions were carried out in 10 µl volume containing 25 ng of genomic DNA, 1x PCR buffer, 2.5 mM dNTPs, 2 µM of each primer and 1 unit of Taq polymerase. The PCR reaction was performed with the following cyclic condition: 95°C for 5 min (initial denaturation), 34 cycles of 94°C for 1 min, annealing for 40 sec, and 72°C for 50 sec, with a final extension at 72°C for 7 min. The amplified products were separated in non-denaturing 8% polyacrylamide gel electrophoresis. The SSR amplicons were visualized with silver staining. The genotypes were scored for the presence (1) or absence (0) of a band. Genetic similarity (GS) was calculated using the Jaccard similarity coefficient method. Based on the similarity matrices, cluster analysis was performed using UPGMA (Unweighted Pair Group method) with NTSYS v2.0 software. The primers SMC 477 GC, NKS 40, NKS 16, NKS 43, mSSCIR 54, NKS 42, NKS 45, NKS 6 and SMC 1039 GC, which generated significant allelic difference between *S. robustum* and *S. officinarum,* were used for the evaluation of introgressed hybrids. Details of procedure involved in the generation and evaluation of introgressed hybrids are shown in Fig. 1.

Results and discussion

Molecular characterization of intraspecific hybrids derived from S. officinarum and S. robustum

SSR genotyping

Twenty three SSR primers were used to assess the genetic diversity in 39 improved clones derived from *S. officinarum* and *S. robustum*. A total of 327 polymorphic loci were scored and the number of alleles per marker ranged from 5 to 22 with an average of 14. The band size ranged between 103 bp and 1.3 Kb (Table 2). The primers NKS 34, NKS 43, NKS 33 and NKS 46 generated more number of polymorphic markers, i.e. 15, 22, 21 and 20, respectively, than NKS 8. Thirteen alleles were identified as unique markers for 10 genotypes (Table 3) which will be useful to evaluate crosses involving the clones carrying the unique fingerprint. Out of 327 alleles scored, nine were

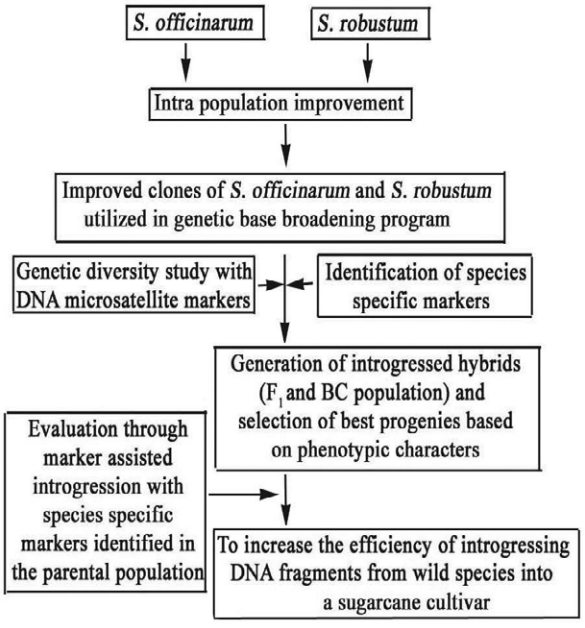

Fig. 1. Schematic representation of introgression breeding plan used in the exploitation of *Saccharum robustum*

Table 2. Sugarcane specific SSR primers used for genetic diversity analysis of *Saccharum* spp.

SSR primer	EST/ Genomic	Repeat motif	Forward primer sequence (5' to 3') Reverse primer sequence (5' to 3')	Annealing temp. (°C)	No of polymorphic bands	Allelic range (bp)	Percentage of polymorphic markers (%)
SMC 1039 GC	Genomic	(TG)17	AGGTGAGAGTTCCTGGCTTTCCA TGTGCTGGCAAGCCCCTACTT	46	12	158-373	92.31
SMC 477 GC	Genomic	(CA)31	CCAACAACGAATTGTGCATGT CCTGGTTGGCTACCTGTCTTCA	46	15	151-707	93.75
mSSCIR 54	Genomic	(GA)23	CGAAGGACCAGTTGAAAG CGAAGGACCAGTTGA AAG	55	7	168-834	87.50
NKS 1	EST	(GAA)6	TGGCATGTGTCATAGCCAAT CCCCAACTGGGACTTTTACA	58	9	216-554	69.23
NKS 2	EST	(GA)13	GCTGTCCCGTTCCAAGTTAC GCGACCGGATTATGATGATT	58	16	172-558	94.12
NKS 3	EST	(TGC)5	CGTGTTCCTCTTCAACAACG TGCTTCGCTATATATGGGTTCA	58	7	254-568	87.50
NKS 6	Genomic	(TG)32	TCCAAATTGCCTGTTGTTTTC CTTACACATGCACAGGCACA	58	18	139-873	94.74
NKS 7	Genomic	(CGG)9	TTACAGCCTGGAGCTCGTTT CGAAGCCTCTCCTCTCCTC	58	10	189-367	90.91
NKS 8	Genomic	(CGG)6	GTGACAGCGGCTTGTTCAG TTAAACACGCAGCCATTCAG	58	5	182-236	83.33
NKS 9	Genomic	(CGC)6	CTTTCAGTGGCCATCTCCAT GAATGCGCAGGGATAGGATA	58	14	155-1322	93.33

Table 2. Contd...

NKS 14	Genomic	(GA)22	TTCCACCAGTGACATTCAGC CCAACAGCAGCTTCTTCCTT	49	17	115-1008	94.44
NKS 16	Genomic	(AG)23	GACAGAATATGCCATGGATAACAA CGTTCTCTGGTCCTATTGAGC	49	16	176-465	94.12
NKS 17	Genomic	(AG)24	GCTCGCCATGAATAGAAAGG ACCGAGGTAGGAGGGAGTGT	58	18	124-601	94.74
NKS 28	Genomic	(AG)27	GTGCTGGGATTCTGAGCTTC GCAAGTTCTTGGCCTTTGTT	49	16	131-1136	88.89
NKS 30	Genomic	(CGG)7	CTCCTTCTCCTTCGCATCCT CACCTTTCTGGAGCACGTTA	58	11	190-466	91.67
NKS 31	Genomic	(CGG)8	AACCACCACTCATCGTCCTC CACCGAGTTCCCATTGTTCT	49	15	103-999	88.24
NKS 33	Genomic	(TGT)6	ACAGGAGCGCTTGGAGATTA GAGCAGAAGGGCTAGAAGCA	57	21	117-1318	95.45
NKS 34	Genomic	(GT)18 (GA)31	CGTCTTGTGGATTGGATTGG TGGATTGCTCAGGTGTTTCA	58	15	112-331	100.00
NKS 40	Genomic	(TG)36	GATGGAGGCTTTGCAATGAT GCATGTCCCACTGAACTGAA	55	15	122-481	93.75
NKS 42	Genomic	(TG)35	ACCGATTGTTCAGTGGGAAG AACCTAGCAATTTACAAGAGAATTAGA	57	11	138-389	91.67
NKS 43	Genomic	(TG)20	CTGATGGGAGGTTGAAGGAA ATAAGCACCAAAAGCGTGGT	57	22	131-1087	95.65
NKS 45	Genomic	(TG)35	GTCGGTCGTGAGAAGGAAAG CACGTATAAAGGCCCTGTGG	49	17	133-971	85.00
NKS 46	Genomic	(TG)24	ACAATAACCCCGCAGACATC TAATGCGTCATTTGGAGCAG	49	20	115-1078	95.24

Table 3. Unique SSR markers identified in *Saccharum robustum* and *Saccharum officinarum* clones

S. officinarum / *S. robustum*	Primers with unique SSR fragment
PIR 00 1157	NKS 1(525)
PIR 93 317	NKS 2(252), NKS 17(601) & NKS 33(1006)
PIR 94 436	NKS 6(597)
PIR 03 149	NKS 40(275)
PIR 00 1193	NKS 31(128)
PIR 96 305	NKS 45(142)
PIR 00 15	NKS 46(125)
PIO 00 736	NKS 33(1110)
PIO 88 79	NKS 8(162) & (236)
PIO 88 319	NKS 2(558)

significantly polymorphic between the genotypes of *S. officinarum* and *S. robustum*. Alleles detected were converted into binary matrix and data were used for genetic similarity estimation using Jaccard similarity coefficient method with NTSYSpc v2.0 software. The similarity coefficient ranged from 0.60 to 0.84 with an average of 0.72. Table 4 shows SSR-based pair-wise comparisons (having both pollen donor and pollen receptor to perform hybridization) with genetic similarity below 0.65 for further exploration in genetic base broadening programs. Two combinations were identified with maximum genetic similarity, viz. PIR 00 1100 & PIO 96 446 (0.82) and PI0 96 446 & PIR 00 1193 (0.83).

Polymorphic loci between *S. officinarum* and *S. robustum*

Eight SSR primers produced informative profiles

Table 4. Pair-wise combinations of improved clones of *Saccharum officinarum* and *Saccharum robustum* with least similarity index based on SSR marker data

Pollen receptor x pollen donor[#]	
PIO x PIR[@]	
PIO 94 345 x PIR 00 1122	PIO 00 1188 x PIR 00 1163
PIO 94 345 x PIR 96 258	PIO 90 202 x PIR 96 475
PIO 94 345 x PIR 00 1163	PIO 90 202 x PIR 00 34
PIO 94 345 x PIR 00 34	PIO 90 202 x PIR 00 1176
PIO 94 345 x PIR 96 475	PIO 00 1057 x PIR 00 34
PIO 00 1188 x PIR 00 1122	PIO 00 846 x PIR 00 34
PIO 00 1188 x PIR 96 258	PIO 00 846 x PIR 00 1163
PIO 00 1188 x PIR 00 34	
PIR x PIO[@]	
PIR 03 107 x PIO 00 845	PIR 00 15 x PIO 00 548
PIR 03 107 x PIO 00 548	PIR 00 1163 x PIO 00 845
PIR 00 15 x PIO 00 581	PIR 00 1163 x PIO 00 548
PIR 00 15 x PIO 00 845	

[#] Combinations with Jaccard similarity coefficient ≤ 0.65

[@] PIR - Improved *Saccharum robustum*; PIO - Improved *Saccharum officinarum*

and were able to discriminate *S. officinarum* and *S. robustum* parents. The primers SMC 477 GC, NKS 43 and NKS 16 generated four *S. robustum* specific markers and five alleles from NKS 6, mSSCIR 54, NKS 42, NKS 40 and NKS 45 were specific to *S. officinarum* (Table 5). Primer SMC 1039 GC produced three species specific loci of which SMC 1039 GC (187) & (356) were specific to 12 clones of *S. officinarum* and SMC 1039 GC (452) was found specific to 16 clones of *S. robustum*. This showed the primer SMC 1039 GC will also be advantageous in evaluation of progenies. The primers SMC 477 GC, NKS 40, NKS 43, NKS 42, NKS 45, NKS 16, mSSCIR 54, NKS 6 and SMC 1039 GC were used in marker assisted evaluation of introgressed hybrids raised over two successive crop cycles. The species specific polymorphism of alleles NKS 40 (133) and SMC 477 GC (279) between *S. officinarum* and *S. robustum* is shown in Fig. 2 and Fig. 3 respectively.

Cluster analysis

Dendrogram (Fig. 4) constructed from similarity matrices using UPGMA method with NTSYSpc v2.0 software grouped the 39 genotypes studied in to two major clusters 'A' and 'B'. All the improved clones derived from *S. officinarum* were grouped into cluster 'B' and the improved clones of *S. robustum*, except PIR 00 1193, PIR 00 39, PIR 00 1100 and PIR 93 317, were grouped into cluster 'A'. *Saccharum robustum* clones that recorded highest NMC and cane yield/clump were grouped in sub-cluster 'AI' and individuals with maximum brix % of above 14.00 were grouped in sub cluster 'AII'.

In cluster 'B', the sub-cluster 'BI' had most of the *S. officinarum* clones identified as parents with high heritability for sucrose and cane thickness. On the other hand, sub-cluster 'BII' had genotypes which recorded highest clump yield among *S. officinarum* clones. The results suggested that intercrossing of genotypes from different clusters

Table 5. Distribution of polymorphic alleles in *Saccharum officinarum* and *Saccharum robustum* clones and their frequency in the introgressed hybrids

Parents / introgressed hybrids population	SSR markers (polymorphic alleles)							
	SMC 477 $GC_{(279)}$	NKS $40_{(133)}$	NKS $16_{(380)}$	NKS $43_{(1049)\ \&\ (143)}$	mSSCIR $54_{(1138)}$	NKS $42_{(179)}$	NKS $45_{(213)}$	NKS $6_{(873)}$
*S. officinarum**	0	1	0	0	1	1	1	1
*S. robustum**	1	0	1	1	0	0	0	0
First generation introgressed hybrids (F_1)#	6	17	7	4	8	6	5	4
Second generation introgressed hybrids (BC_1)#	7	16	2	3	2	4	1	2

* First two rows show genotype data for the parents (presence of an allele is coded by "1")

Last two rows show the number of progenies out of the 86 tested possessing the polymorphic allele specific to the parents

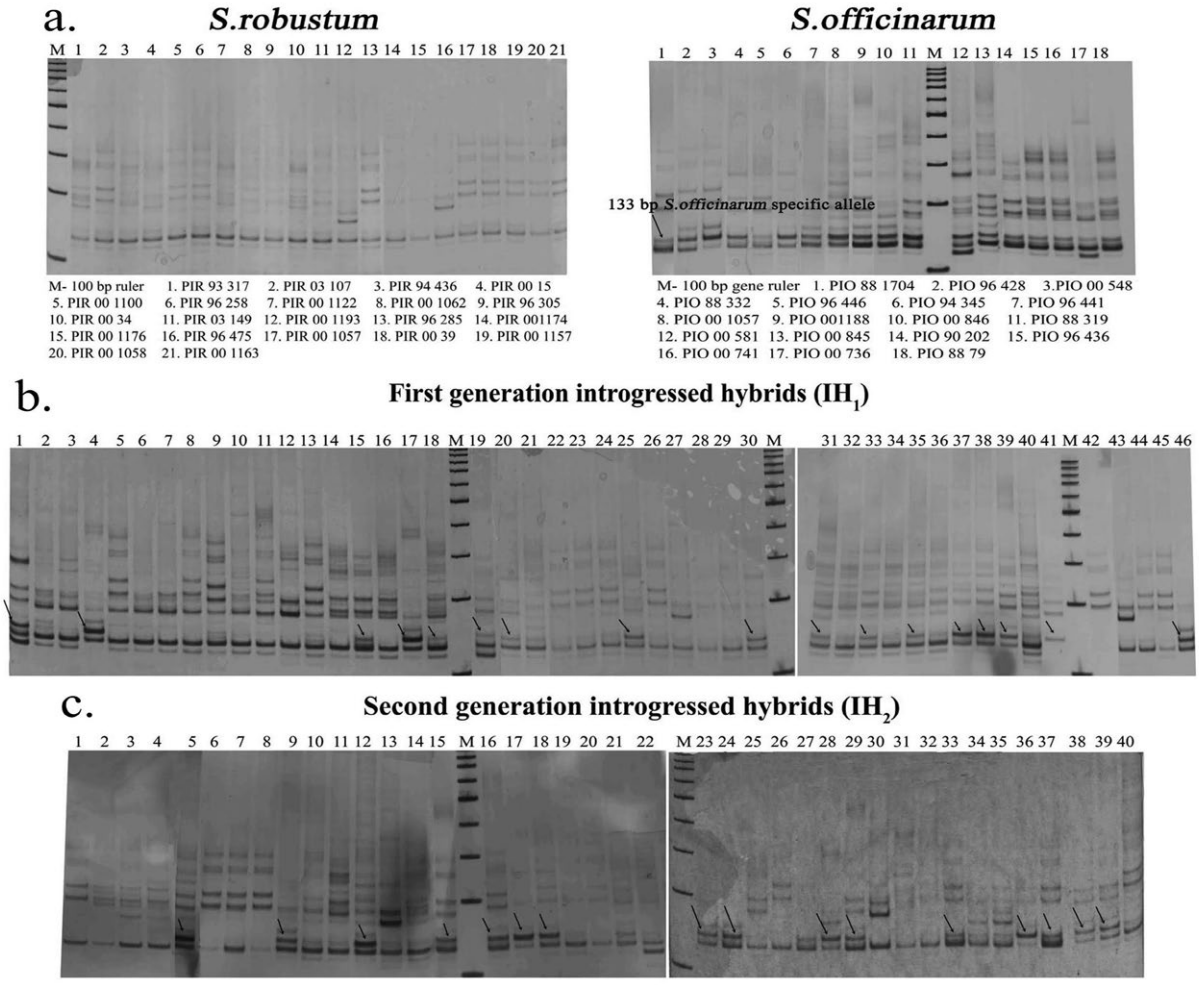

Fig. 2. SSR primer NKS 40 profile for *Saccharum officinarum*, *Saccharum robustum* and introgressed hybrids. (a) allele 133 bp specific to *S. officinarum*, which is absent in all the *S. robustum* clones (b) introgressed hybrids inherited with *S. officinarum* specific allele are indicated (c) genotypes transmitted with species specific allele over two generations of hybridization

will increase heterosis with broad spectrum of variability.

Marker assisted introgression breeding

Vigorous, low sucrose and high yielding *S. robustum* (PIR 96 475, PIR 00 1163, PIR 00 1174, PIR 96 258) was crossed with high sucrose *S. officinarum* (PIO 00 581, PIO 00 845, PIO 94 345, PIO 00 1188) to select novel sources of segregation for genetic base broadening of sugarcane breeding population. Four crosses were studied from first generation introgression breeding. Three individuals selected, viz. IH_1 11-66, IH_1 11-81, IH_1 11-15, identified as true hybrids with molecular markers, were crossed with commercial types (Co 0240, Co 0303 and Co 0209) to raise second generation of introgressed hybrids.

Phenotypic evaluation of introgressed hybrids

Phenotypic evaluation of progenies from two generations of introgression breeding displayed

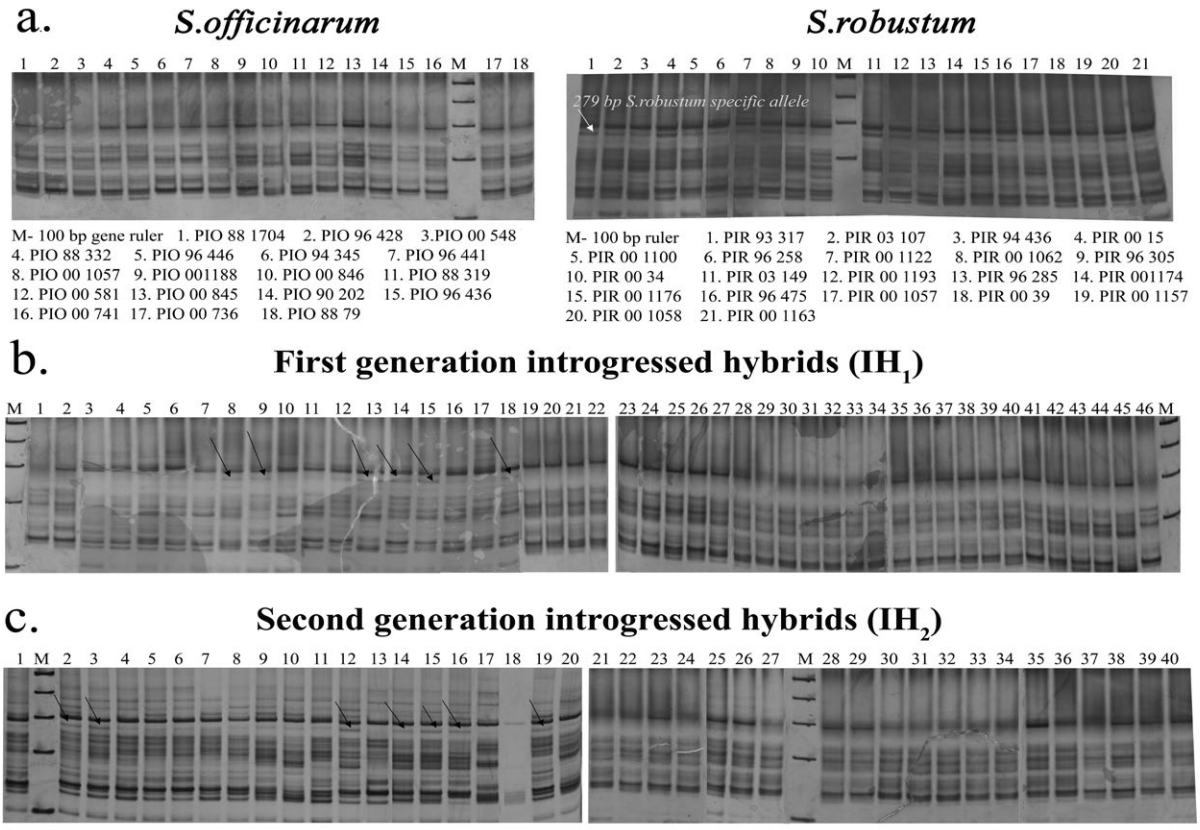

Fig. 3. SSR primer - SMC 477 GC profile for *Saccharum officinarum, Saccharum robustum* and introgressed hybrids. (a) allele 279 bp is specific only to *S. robustum* clones, which is absent in all the *S. officinarum* clones (b) introgressed hybrids in lanes 8, 9, 12, 13, 14, 15 and 18 are inherited with *S. robustum* specific allele (c) genotypes with species specific allele transmitted through two generations of introgression breeding

an increasing trend for cane thickness, single cane weight and brix % with significant difference among the genotypes and crosses, and between the generations of introgression breeding for quality and yield traits. In the first generation of introgressed hybrids (F_1), cross mean values ranged 9.9 - 17.6 for canes/clump, 1.83 - 2.45 cm for cane thickness, 0.50 - 0.92 kg for single cane weight and 15.05 - 16.50 for brix % (Table 6). The mean NMC was comparatively higher in crosses PIR 00 1163 x PIO 00 845 (17.6) and PIR 00 1174 x PIO 00 581 (16.5), which may be due to the maternal influence of *S. robustum*. Earlier studies on relative effects of parents on economic characters indicated similar maternal influence as evidenced by an increase in NMC in progenies when Co 527 with high NMC was used as a pistil parent (Natarajan et al. 1967). Mean values of the crosses PIO 94 345 x PIR 96 475 and PIO 00 1188 x PIR 96 258 exhibited 19.49% improvement in brix % and 43.11% higher cane thickness than *S. robustum* parents (Table 7) and this infers that the hybrids generated are good segregants of brix % and cane thickness. Lower cross mean values were recorded for NMC and clump yield in comparison to parental clone mean values (Table 6) but single cane weight showed a mean value of 0.73 kg with 25.86% improvement over

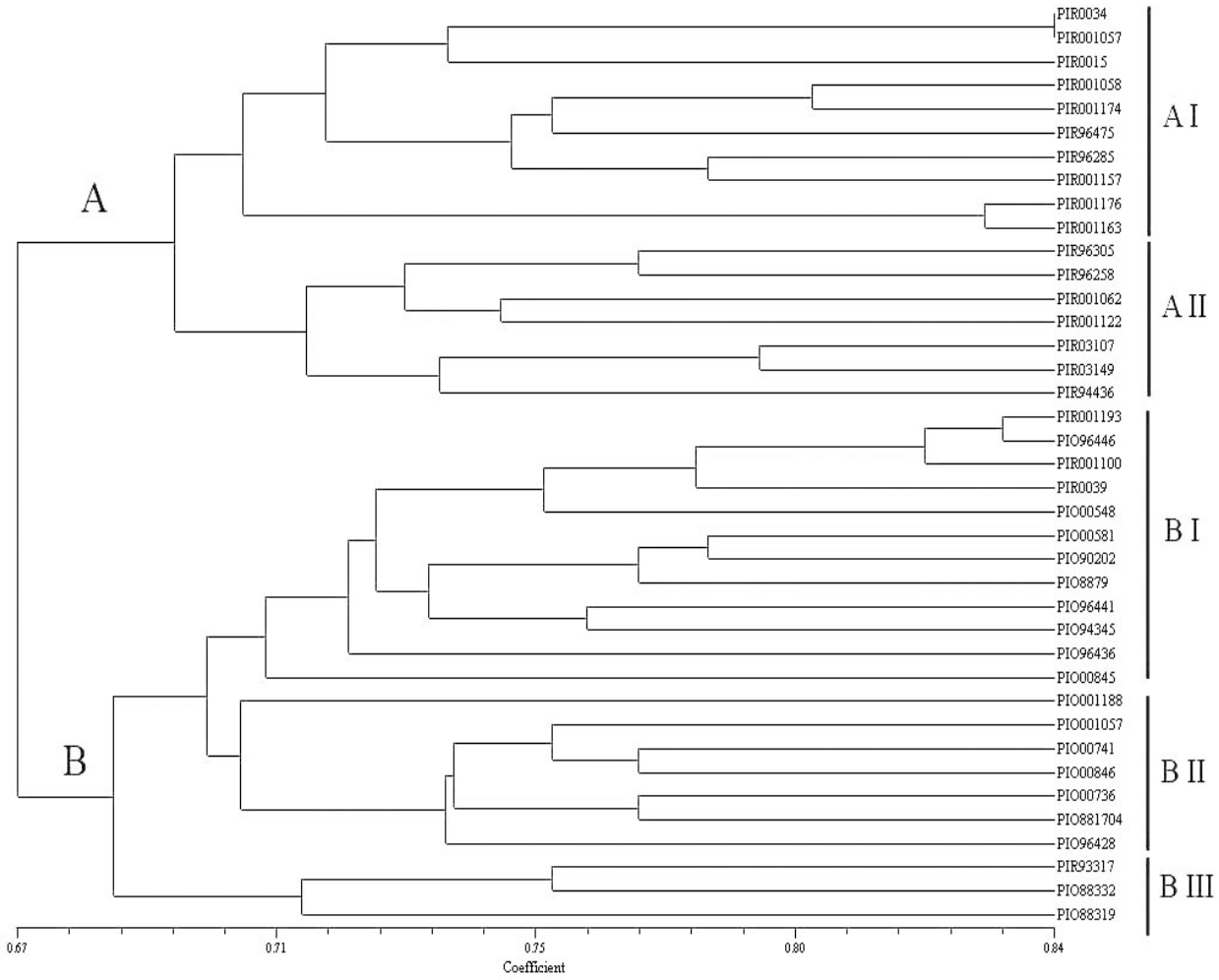

Fig. 4. Dendrogram depicting the genetic relationship between *Saccharum officinarum* and *Saccharum robustum*

S. robustum parent (Table 7). Fourteen introgressed hybrids with NMC of 23-31 canes/ clump and 32 hybrids with cane thickness of 2.42 - 2.86 cm and brix % of 17.4 - 18.89 were identified as high value hybrids. Forty six genotypes which performed superior to the cross mean values were further advanced to clonal trials for the marker assisted evaluation. Crosses were performed involving commercial types in the clonal trial to raise second generation introgressed hybrids. With brix % and cane thickness as selection criteria, three crosses were selected for further evaluation.

Three crosses evaluated in second generation (BC_1) introgression breeding were Co 0240 x IH_1 11-15, IH_1 11-81 x Co 0303 and IH_1 11-66 x Co 0209. Introgressed hybrids showed improved hybrid vigor with a wide range of variation for cane thickness (2.09-2.68 cm), single cane weight (0.79-1.06 kg) and brix % (16.30 - 18.07%) (Table 6). Clump yield was low, though a few individuals recorded around 7.95 - 8.58 kg/clump, and did not exceed both the parents' mean value. Mean value of NMC showed a 52.05% decrease over that of *S. robustum* parent (Table 7). The overall mean value of 723 introgressed hybrids developed

Table 6. Mean values for quality and yield traits of hybrid derivatives generated in two generations (F_1 and BC_1) of introgression breeding involving *Saccharum* spp.

Crosses	Total no. of progenies evaluated	Cross mean value				
		NMC / clump	Cane diameter (cm)	Single cane weight (kg)	Clump yield (kg)	HR brix (300 days)
PIO 94 345 x PIR 96 475	182	9.9	2.45	0.92	8.75	16.48
PIO 00 1188 x PIR 96 258	92	10.0	2.33	0.92	8.86	16.50
PIR 00 1174 x PIO 00 581	68	16.5	1.87	0.58	9.20	15.54
PIR 00 1163 x PIO 00 845	116	17.6	1.83	0.5	8.51	15.05
IH_1 11-66 x Co 0209	78	8.0	2.59	1.05	8.01	18.07
IH_1 11-81 x Co 0303	82	8.9	2.68	1.06	6.13	17.25
Co 0240 x IH_1 11-15	106	11.6	2.09	0.79	7.51	16.30

over two generations showed an improvement of 36.41%, 46.55% and 20.65% for cane diameter, single cane weight and brix %, respectively against the *S. robustum* parent mean value. From second generation introgression breeding, superior genotypes having brix % > 17.20 (overall cross mean value) were selected for marker assisted evaluation. Eighty six individuals selected from stage I (F_1) and II (BC_1) introgression breeding showed 60.00% and 24.32% improvement for CCS % in comparison with *S. robustum* and *S. officinarum* parents, respectively. Improvement for cane thickness in best performed introgressed hybrids generated over two cycles of hybridization is shown in Fig. 5. Crosses PIO 94 345 x PIR 96 475 and IH_1 11-66 x Co 0209 generated the highest percentage of elite progenies followed by PIO 00 1188 x PIR 96 258.

Marker assisted evaluation of progenies developed from two generations of introgression breeding

Eighty six hybrids selected from introgression breeding were screened with a subset of markers which produced species specific polymorphism. DNA marker-assisted selection is carried out to ensure introgression of species specific alleles in the interspecific hybrids. Frequency of allelic distribution in the introgressed hybrids is given in Table 5. Among 40 progenies evaluated from second generation of introgression breeding, only six individuals retained the maximum of five specific alleles against the commercial genetic background. Polymorphic allele NKS 40 (133) specific to *S. officinarum* has occurred with

Table 7. Improvement for yield and quality traits over two generations of introgression breeding against *Saccharum robustum* parents

Generation	NMC	Cane diameter (cm)	Single cane weight (kg)	Clump yield (kg)	HR brix (300 days)
First generation introgressed hybrids (IH_1)	13.5	2.12	0.73	8.83	15.89
Second generation introgressed hybrids (IH_2)	9.5	2.45	0.96	7.21	17.20
Improvement (%) in first generation of introgression breeding over *S. robustum* parent	-31.5	26.94	25.86	-23.21	15.14
Improvement (%) in second generation of introgression breeding over *S. robustum* parent	-52.1	46.70	65.51	-37.30	24.63
S. robustum parent	19.8	1.67	0.58	11.50	13.8
S. officinarum parent	11.8	2.29	0.69	8.515	15.34

maximum frequency in the introgressed hybrids (Table 5). The introgressed hybrids IH_1 11-66 and IH_1 11-15 carried the highest proportion of *S. robustum* specific allele SMC 477 GC (279). SSR marker profiles indicated the presence of unique polymorphic loci in *S. officinarum, S. robustum* and hybrids (Figs. 2 and 3).

McIntyre and Jackson (2001) on evaluating the interspecific hybrids of sugarcane with molecular markers found that 17.60% of the offspring were not true hybrids. Molecular characterization of 609 sugarcane progenies by Tew and Pan (2010) indicated a selfing percentage of 0-45%; also, 6.89% of offspring with foreign pollen. In the present study, 38.3% of genotypes selected from F_1 and BC_1 were identified as possessing either male or female parent specific alleles. These clones (from F_1 and BC_1 generation) identified as selfs can be maintained as semi-hybrids and after sufficient incorporation of new alleles from related species can be included in genetic base broadening gene pool. Beginning with an introgression breeding population of 723 individuals and continuing with 86 selected progenies, 53 individuals carrying the unique alleles from less utilized germplasm were identified through marker assisted selection (with nine SSR markers, viz. SMC 477 GC, NKS 40, NKS 16, NKS 43, mSSCIR 54, NKS 42, NKS 45, NKS 6 and SMC 1039 GC) to disrupt the elite genetic background of commercial cultivars.

Genetic analysis of improved clones of *S. officinarum* and *S. robustum* grouped them into separate clusters which are in close correspondence with the results of phylogenetic study of *Saccharum* complex based on RAPD markers (Vijayan Nair et al. 1999). While the cross combination *S. officinarum* x *S. robustum* showed high heterosis for sucrose %, *S. robustum*

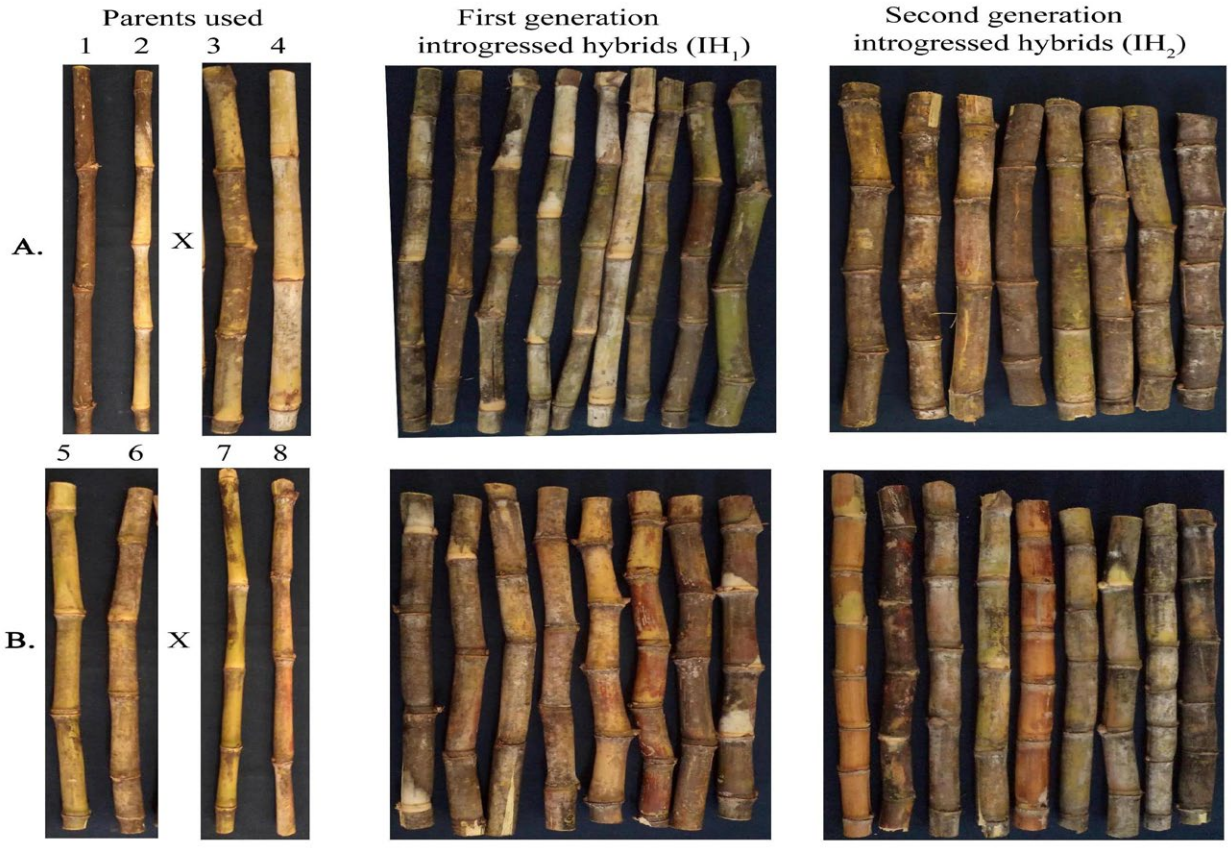

Fig. 5. Improvement in cane thickness over two generations of introgression breeding. (A) 1 - PIR 00 1174; 2 - PIR 00 1163; 3 - PIO 00 581; 4 - PIO 00 845 (*Saccharum robustum* x *Saccharum officinarum*); (B) 1 - PIO 94 345; 6 - PIO 00 1188; 7 - PIR 96 475; 8 - PIR 96 258 (*Saccharum officinarum* x *Saccharum robustum*)

x *S. officinarum* crosses had the highest range of variation for yield contributing traits. Eight genotypes of *S. officinarum* and seven from *S. robustum*, viz. PIO 94 345, PIO 00 1188, PIO 90 202, PIO 00 1057, PIO 00 845, PIO 00 548, PIO 00 581, PIO 00 846, PIR 00 1122, PIR 96 258, PIR 00 34, PIR 96 475, PIR 00 1176, PIR 03 107 and PIR 00 15 were identified as the most diverse clones with least genetic similarity to ensure high genetic variability in future breeding. The *S. robustum* clones PIR 93 317, PIR 00 1193, PIR 00 1100 and PIR 00 39 showed maximum similarity with *S. officinarum* genotypes. The genetic variability study would help in the selection of variable clones/parents for breeding and thus reduce the chances of low variability in the basic breeding material. Vijayan Nair et al. (2006) reported *S. robustum* specific allele with the primer mSSCIR 66, used to screen the interspecific hybrids developed from a cross between *S. robustum* and *S. spontaneum* (PIR 00 1188 x IND 99 904). *Saccharum* species specific DNA fragments were effectively used in the evaluation of interspecific and intergeneric hybrids (Selvi et al. 2003; Vijayan Nair et al. 2006) and in varietal identification (Govindaraj et al. 2011; Hemaprabha et al. 2006). Molecular characterization of introgressed hybrids showed that the allele NKS 40 (133) was segregated with 64.45% of hybrids with sucrose % above 17.0 and SMC 477 GC (279) was segregated with 60% of hybrids with fibre

% above 22 in two generations of introgression breeding. The correlation analysis of phenotypic traits and marker segregation showed significant correlations between NKS 40 (133) and sucrose content in IH_1 (r = 0.685; P<0.001) and IH_2 (r = 0.752; P<0.001). *Saccharum robustum* specific SMC 477 GC (279) was found to be significantly correlated with fibre % in both the generations, viz. IH_1 (r = 0.754; P <0.001) and IH_2 (r = 0.877; P<0.001). Correlation between the markers SMC 477 GC (279) and NKS 40 (133) was found to be negative in both IH_1 (r= -0.104; P>0.05) and IH_2 (r = -0.091; P>0.05). The markers SMC 477 GC (279) and NKS 40 (133) were significantly associated with the phenotype traits fibre % and sucrose content, respectively. Thus, when measured under similar environmental conditions for all genotypes and if stably inherited over successive crop cycles, these can be used as optimal primer combinations for early generation selection. Similarly, Nivetha et al. (2013) identified effective microsatellite markers that are stably associated with high fibre content in the interspecific hybrids generated involving PIO-00-513 *(S. officinarum)* x IND99-904 (*S. spontaneum*). Species specific alleles identified in the present study will also be useful in the selection of superior genotypes with wild alleles to complement the adapted germplasm for vigor, ratooning ability and disease reaction, and to identify the paternity information in polycrosses. This study also suggests further validation of the markers SMC 477 GC (279) and NKS 40 (133) on different genetic background involving *S. spontaneum* or with other members of *Saccharum* complex.

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