RESEARCH ARTICLE

MOLECULAR CYTOGENETIC CHARACTERIZATION OF *SORGHUM* CHROMOSOMES IN *SACCHARUM* BACKGROUND AND *VICE VERSA* USING GENOMIC *IN SITU* HYBRIDIZATION

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Abstract

Two intergeneric hybrids of sugarcane, Co 86032 (2n=112) x *Sorghum* (2n=20) and *Sorghum* (2n=20) x *S. officinarum* (2n= 112), were subjected to classical as well as molecular cytological analysis. The hybridity of these clones has been confirmed by using SSR markers and chloroplast gene. During genomic *in situ* hybridization (GISH) the genome composition of the hybrids studied and it has revealed that it follows n+n chromosome segregation. Introgression of 10 *Sorghum* chromosomes in both the hybrids was observed while using *Sorghum* genomic DNA as the labeled probe. We report for the first time the chromosome composition and condensation behavior of *Sorghum* chromosomes in hybrids involving *Saccharum* and *Sorghum*.

Key words: *Sorghum*, *Saccharum*, Sugarcane, Genomic *in situ* hybridization, Chromosome condensation, Hybridity, Chloroplast gene, Marker

Introduction

The commercial sugarcane varieties under cultivation are derived from complex interspecific hybridization performed a century ago between the sugar producing species, *Saccharum officinarum* L. and the wild species, *Saccharum spontaneum* L. Though *S. officinarum* contributed the high sugar content, the high productivity and high adaptability of the modern cultivars are attributable largely to *S. spontaneum*. By reviewing the parentage of present day cultivars it is clear that only a small number of original ancestral clones have contributed to the parental material in modern sugarcane breeding programmes. Therefore, there has been considerable interest worldwide to introgress genes from wild species and related genera of sugarcane to enhance the genetic base and to have better agronomic traits in this crop.

Several intergeneric hybrids of *Saccharum* with related genera like *Erianthus*, *Miscanthus*, *Narenga* and *Sclerostachya* had been produced in the past (Janaki Ammal 1941, Li *et al.,* 1948, Barber 1916, Parthasarathy 1948, Kandasami 1961). Hybrids of *Saccharum* with *Sorghum* and *Zea* also had been produced (Thomas and Venkataraman 1930, Janaki Ammal and Sing 1936, Janaki Ammal 1938). *Sorghum* had been crossed with *Saccharum* with the objective of producing hybrids with shorter crop duration. Though the desired level of earliness could be achieved in some of *Saccharum* x *Sorghum* hybrids, they lacked many of the agronomic traits of sugarcane (Thomas and Venkataraman 1930). *Sorghum* breeders also crossed *Sorghum* with *Saccharum*, for transferring some of pest resistance traits from *Saccharum* (de Wet *et al.,* 1976, Gupta *et al.,* 1978a, 1978b). In all these crosses *Saccharum* was used as female parent and the hybrids had *Saccharum* cytoplasm. A rare hybrid of *Sorghum* x sugarcane was developed at ICAR-Sugarcane Breeding Institute in which *Sorghum* was used as the female

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parent (Nair 1999). The somatic chromosome number of the hybrid was 2n=66. Another hybrid of *Saccharum* x *Sorghum* was developed by using modern sugarcane variety, i.e. Co 86032 as female parent. In this hybrid the somatic chromosome number was 2n= 66. All these hybrids have been matured in about 6-7 months. Due to male sterility in these hybrids they could be used as female parents only in further crosses.

In a detailed microsporogenesis study it has been revealed that syncyte formation and degeneration of chromatin content leads to complete pollen sterility in these hybrids (Sobhakumari and Nair 2014). Mitotic study in the somatic cells of these hybrids is necessary to understand the parental genome constitution, the organization of mitotic chromosomes and chromosome structure in different phases of cell division. As *Sorghum* shares high synteny with sugarcane genome it is necessary to study the hybrids between these two genera with modern cytogenetic tools rather than classical cytogenetic methods. Genomic *in situ* hybridization (GISH) is a powerful molecular cytogenetic tool to unravel the chromosome composition for the detection of different chromosome sets derived from two or more distinct species in allopolyploids. Wide hybridization is one of the stresses that might trigger reorganization of parental genomes. The potentiality of this molecular cytogenetic tool to infer the knowledge on chromosome transmission will make it possible to implement a strategy for developing useful varieties through breeding. Using GISH, much insight has been gained into sugarcane genomic constitution and chromosomal inheritance over the past several decades (D'Hont *et al.,* 1995, 1996, Piperidis *et al.,* 2000, 2001, 2010, Wu *et al.,* 2014, Huang *et al.,* 2015, Premachandran *et al.,* 2017, Yu *et al.,* 2018). So far molecular cytogenetic characterization has not been reported in sugarcane intergeneric hybrids having potential genome compliment from a genera which is not included in the '*Saccharum* complex'.

In this study two intergeneric hybrids of sugarcane having *Sorghum* as one of the parent, either female or male, were characterized by GISH. The objectives of the study were as follows (1) to understand the parental genome composition in the hybrid and (2) to study the mitotic chromosome behavior of the two distinct genomes during wide hybridization.

Materials and Methods

The plant materials used in this study consist of two intergeneric hybrids of *Saccharum* involved *Sorghum*. In one hybrid *Sorghum* used as male parent (Co 86032 x *Sorghum*) and in another hybrid *Sorghum* used as female parent (*Sorghum* x *S. officinarum*- IJ 76-316). Molecular study has been conducted to confirm the hybridity of these clones. *Sorghum* specific markers, SOMS 119 and SOMS 93, which were generated by sugarcane SSR was used to screen the hybrids. Primer pair sequence for SOMS 119 was F: 5' CAACATCTCACGAAACAATAC3' and R: 5' AACACCTCCTACACTGACACA 3' and primer pair sequence for SOMS 93 was F: 5' CCTGACCAACAGCGGCAC 3' and R: 5' CCCAACAAGACATCACCA 3'. Total genomic DNA has been isolated from young leaves using CTAB method (Doyle and Doyle, 1990). PCR was carried using the following profile: initial denaturation step at 94ºC for 5 min followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 56ºC for 1 min and primer extension at 72ºC for 1 min and stored at 4ºC. The amplified products were loaded on a polyacrylamide gel and the results were documented using a gel documentation system.

Presence of *Sorghum* cytoplasm in the selected hybrid clone, *Sorghum* x *Saccharum*, was determined using molecular marker for chloroplast

DNA polymorphism. Total DNA was extracted from young leaves of the hybrid using CTAB method (Doyle and Doyle, 1990). The chloroplast DNA marker was amplified using the primer pair F: 5' CTTCGTATTGTCGAGATCCT 3' and R: 5' CACCCAATGAAGTGTTAG 3'. Twenty micro liter of reaction mixture contains 30 ng DNA, 5 picomol each of forward and reverse primers, 200 mM dNTP mix, 1.2 U Taq polymerase in 2.5 µL 10x buffer using the following parameters: an initial denaturation step at 94ºC for 4 min, 32 cycles of denaturation at 92ºC for 20 s, annealing at 53ºC for 30 s and extension at 72ºC for 2 min, followed by final extension at 72ºC for 10 min. Amplified products were resolved in 1 % agarose gel. Later the amplified product of chloroplast rpl16Lregion was restricted with taq 1 enzyme.

Somatic chromosome number in the hybrids was determined by root tip squash technique (Sobhakumari and Asmita Dutta 2014). Mitotic chromosome preparation for GISH was performed as previously described (D'Hont *et al.,* 1996) with minor modifications. In brief, the hybrids and parents were grown in pots for root tip harvesting. Excised root tips about 1-2 cm were treated with 2mM 8-hydroxy quinolone at room temperature for 2h, rinsed in water and fixed in ethanol acetic acid (3:1) for about 16 h at 4ºC.The root tips were hydrolyzed in 0.25N HCl and digested in enzyme solution (2% cellulose ONOZUKA and 20% pectinase in citrate buffer) at 37ºC for 45 min. After washing in distilled water meristematic tissues were squashed in ethanol acetic acid (3:1) and put a cover slip over it. Cells can be separated by gentle pressing over the coverslip with a filter paper. Slides were then freezed on dry ice or dipping it in liquid nitrogen. The coverslip was removed and the frozen slide was immediately dehydrated in absolute ethanol and stored in moisture free slide box.

Freeze dried slides were treated with RNase (1µg/µl) at 37ºC for 45 min and washed 3 times in 2xSSC for 5 min each at room temperature. After drying, the slides were denatured in 70% formamide for 2 min at 72ºC and dehydrated in ice cold ethanol series. Genomic DNA of *Sorghum* labeled with biotin 11-duTP using random primed labeling method as described by the manufacturer (Thermo Scientific, USA) was used as probe. The hybridization mixture (30µl per slide) consisted of 50 ng of labeled probe (*Sorghum*), 50% deionized formamide, 10% dextran sulphate, 0.5µg of sheared salmon sperm DNA in 20x SSC. This mixture was denatured by treating for 10 min in water bath at 100ºC and then cooled down quickly on ice. Hybridization was performed overnight in a moist chamber at 37ºC. Post hybridization washes were carried out in 2xSSC at 42ºC. Biotin labeled probe was then detected using avidin-FITC (Fluorescein isothiocyanate) by 1h incubation at 37ºC. Post detection washes were carried out in 2xSSC, 1% triton-x in 4xSSC followed by 2xSSC for 5 min each at 37ºC followed by 5 min incubation in 2xSSC at room temperature. Slides were mounted in Vectasheild (Vector labs,UK) mounting medium with DAPI (4,6-diamidino-2-phenylindole). Mitotic cells were analyzed using a Carl Zeiss fluorescence microscope with appropriate filter sets. Images captured in different filters were photographed with Progress Capture Pro image capturing software and merged using Adobe Photoshop.

Results

Somatic chromosome number determination

The somatic chromosome number of Co 86032 (2n=112) x *Sorghum* (2n=20) was determined from the root tip cells and was found to be 2n=66. The somatic chromosome number of *Sorghum* x *Saccharum* was also 2n=66 which is the progeny of a *Sorghum* male sterile line ICSA 56 (2n=20) and an atypical *S. officinarum* clone IJ 76-316 (2n=112) (Fig.1).

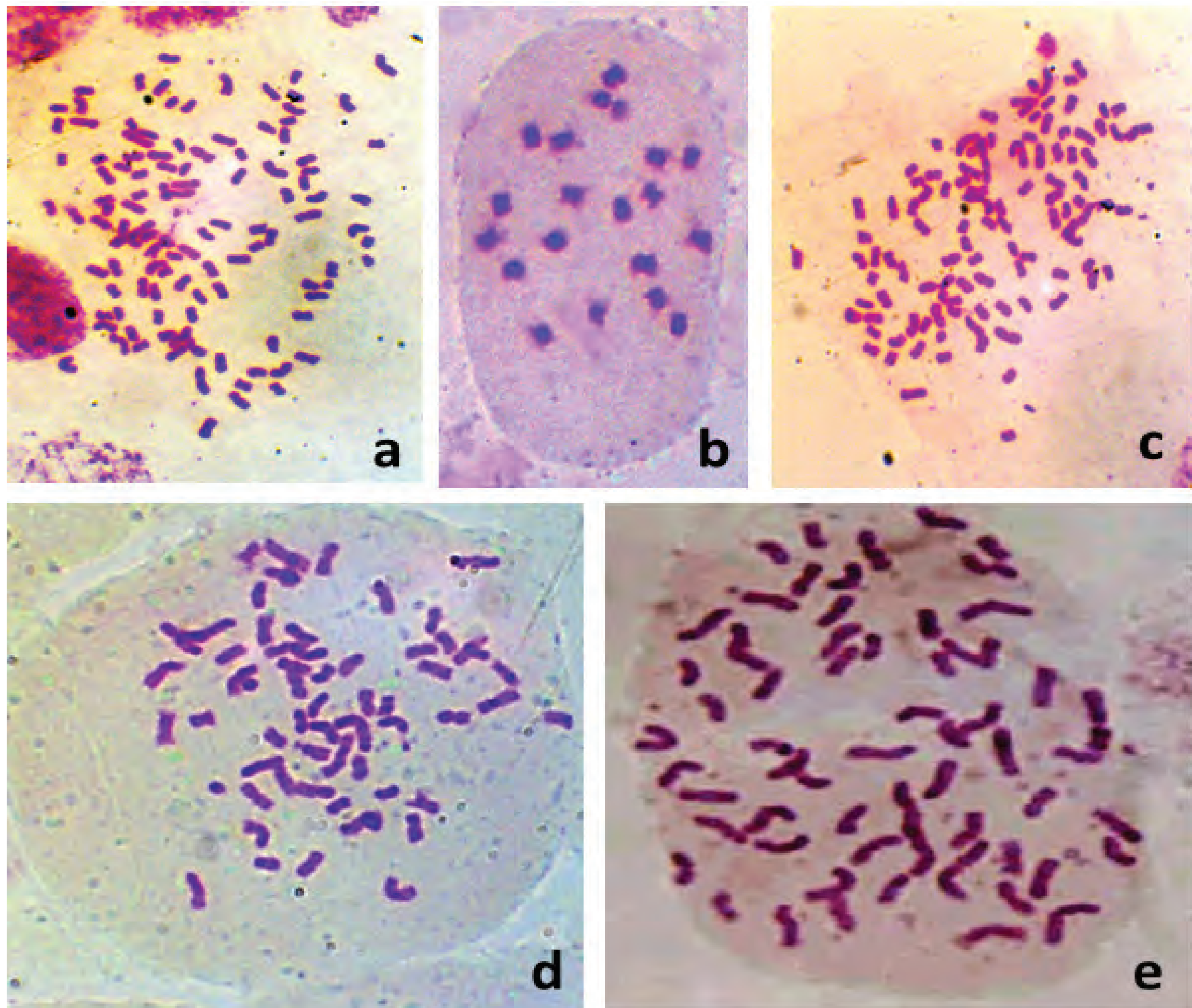


Fig. 1. Somatic chromosome number (a) Co 86032 (b) *Sorghum*, (c) IJ 76-316 (d) Co 86032 x *Sorghum* (e) *Sorghum* x IJ 76-316

Molecular study to confirm the hybridity

The hybridity of the progenies resulted from crosses involving *Sorghum* is determined largely based on the presence of distinct *Sorghum* traits, which is difficult or by the presence of *Sorghum* specific molecular markers. The sugarcane origin of the hybrids was not in question since the hybrids are resembled the sugarcane parent to a large extent especially in Co 86032 x Sorghum. We have selected the primers which showed polymorphic bands for the parents. While considering the first cross the SSR primer SOMS 119 developed sugarcane specific fragment in *Saccharum* parent (Co 86032) and *Sorghum* specific fragment in *Sorghum* parent (SSV 84) and both sugarcane and *Sorghum* specific fragments in Co 86032 x *Sorghum* hybrid. Similarly the primer SOMS 93 produced polymorphic bands for sugarcane and *Sorghum* and the hybrid *Sorghum* x *Saccharum* was having both these bands which were low in fragment size while compare to the other hybrid. The two sugarcane SSR primers, SOMS 119 and SOMS 93, used in our study were showing DNA fragments specific to both the parents. These primers which showed both parent specific bands

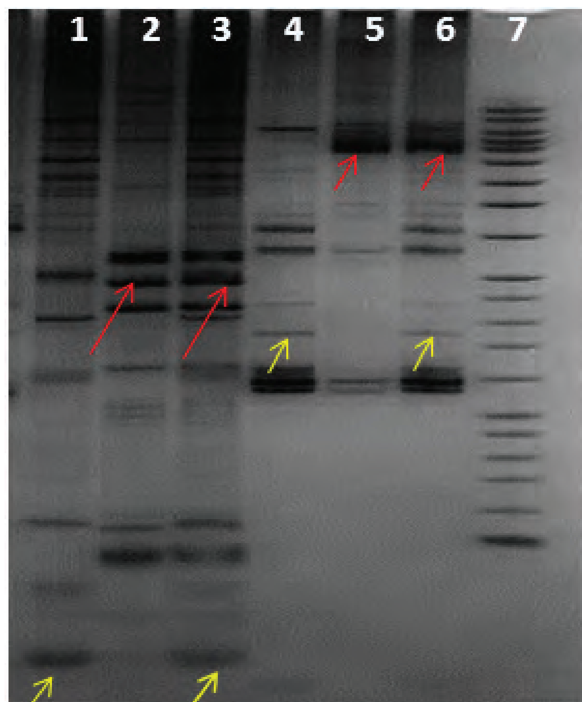


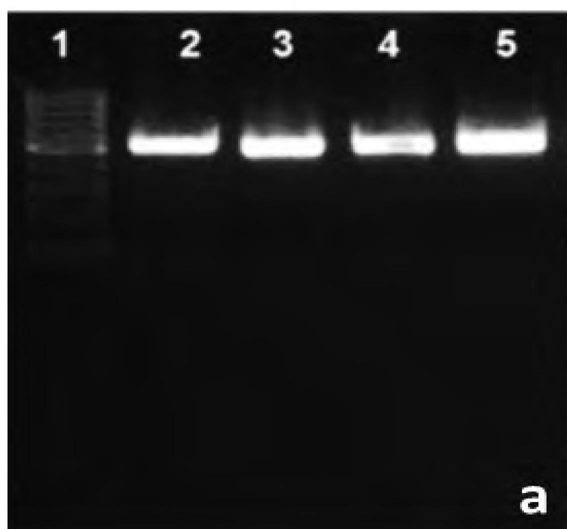
Fig. 2. Hybridity confirmation with sugarcane specific SSR markers. Lane 1-3 SOMS 119, Lane 4-6 SOMS 93. lane 1-Co 86032, lane 2-*Sorghum*, lane 3- Co 86032 x *Sorghum*, lane 4- IJ 76-316, lane 5 - *Sorghum*, lane 6- *Sorghum* x J 76-316 , lane 7- 20bp Marker

together in hybrids confirmed its hybridity (Fig. 2).

The cytoplasmic type of hybridity of *Sorghum* x *Saccharum* was confirmed with PCR amplification and restriction of a chloroplast gene, rplL16 (Fig. 3). The PCR amplification product of rpl L16 was approximately 500pb in all the samples. The amplicon when restricted using Taq 1 restriction enzyme gives a restriction pattern that distinguishing *Sorghum* and *Saccharum*. The Taq 1 digested restriction product of *Sorghum* and *Sorghum* x *Saccharum* hybrid remain undigested showing the lack of Taq 1 restriction site. Hence the fragment size remained 500bp even after the digestion. The *Saccharum* amplified product got restricted into two fragments of 300bp and 200bp indicating the presence of Taq 1 restriction site. This result confirmed that *Sorghum* x *Saccharum* hybrid is having *Sorghum* type cytoplasm which is inhired from its female parent.

Genome contribution of *Sorghum* in Sugarcane hybrids

The *Saccharum* x *Sorghum* (Co 86032 x SSV 84) and *Sorghum* x *Saccharum* (ICSA 56 x IJ 76-316) hybrids were subjected to GISH analysis by using *Sorghum* as the biotin labeled probe. In both the cases the somatic chromosome number was 2n=66 with ten chromosomes of *Sorghum* (Fig.4). The double genome structure of the hybrid was



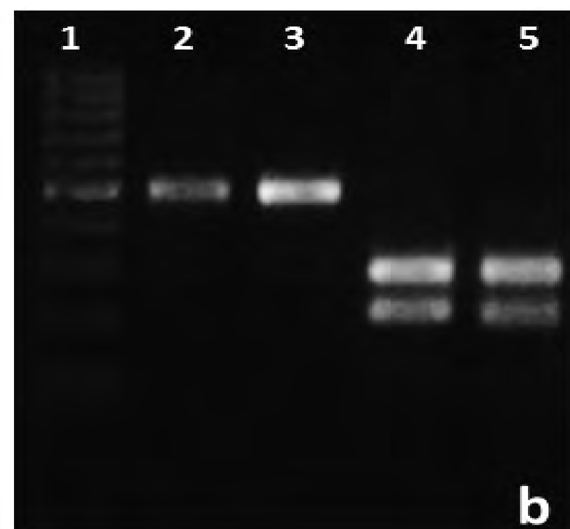


Fig. 3. (a) Amplification profile of chloroplast rpl L 16 intron **(b)** Chloroplast rpl L 16 intron restriction profile : lane 1- lOObp marker, lane 2- *Sorghum*, lane-3 *Sorghum x Saccharum*, lane 4-IJ76-316, lane 5-Co 86032

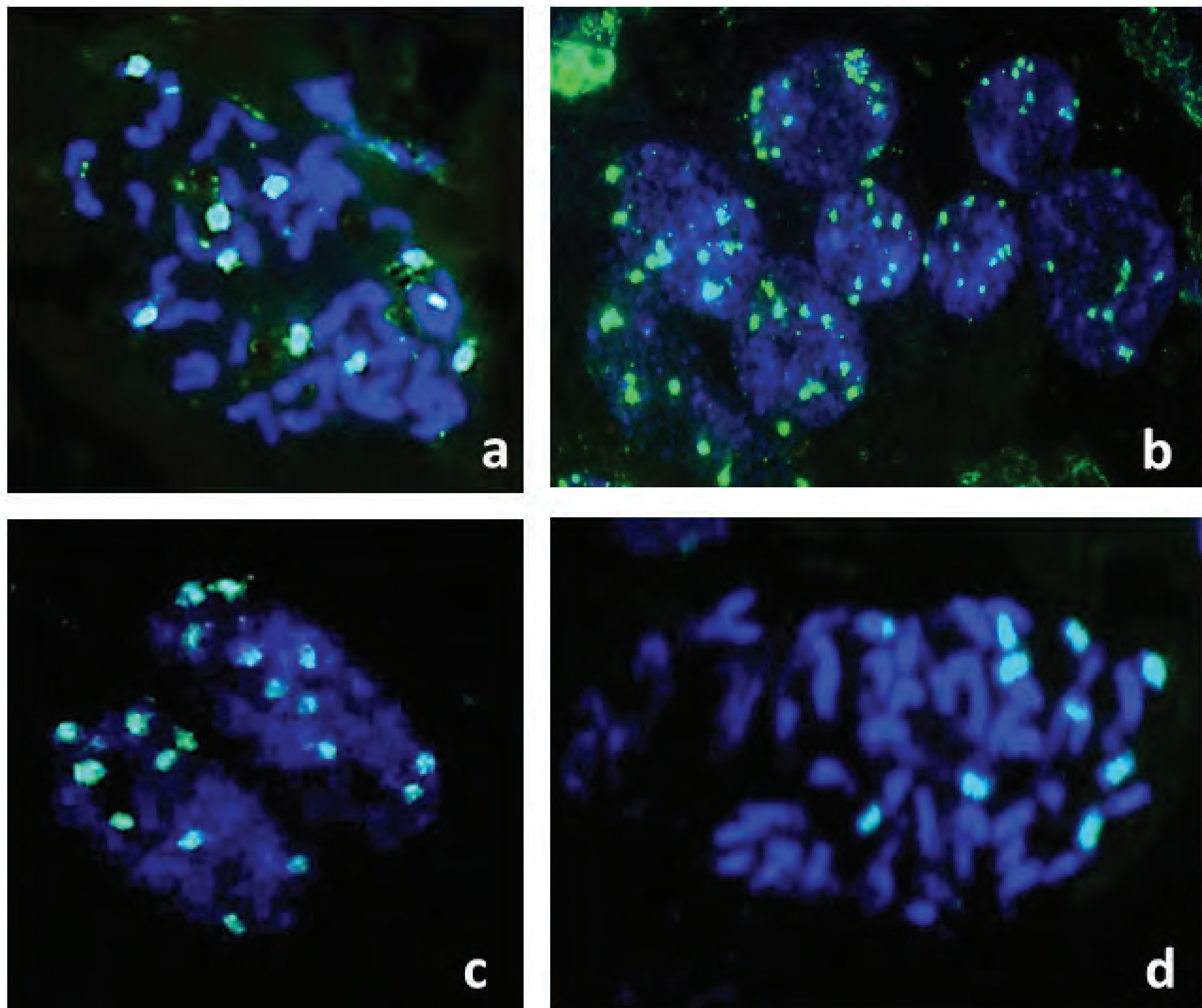


Fig. 4. GISH result of Co 86032 x *Sorghum* (a-b) and *Saccharum x Sorghum* (c-d) using biotin labeled Sorghum genomic DNA as probe: (a) Metaphase of Co 86032 x *Sorghum* (b) Interphase of Co 86032 x *Sorghum* (c) Anaphase of *Sorghum* x *Saccharum* (d) Metaphaseof *Sorghum x Saccharum*

clearly observed during GISH analysis with 10 well condensed green chromosomes of *Sorghum* in the hybrid genome. The interesting fact in these hybrids was the uniformity in the condensation of the *Sorghum* chromosomes irrespective of the mitotic stages. Even in the interphase stage the ten chromosomes of *Sorghum* were clearly visible in all cells. Early and stable condensation even in the prophase might be a peculiar character in *Sorghum* chromosomes in intergeneric background through folding of large scale chromatin fibers into condensed masses. The GISH analysis could not be extended to its meiosis due to the rare flowering pattern and severe meiotic abnormalities during microsporogenesis in these intergeneric hybrids.

Discussion

Sorghum and sugarcane genomes share more extensive genome wide colinearity and less chromosomal rearrangements than any other known grasses. Intergeneric hybrids of *Saccharum* involving *Sorghum* either as male or female has been made since 1930s by breeders to impart earliness in sugarcane varieties (Venkatraman and Thomas

1932, Janakiammal and Singh 1936, Nair 1999). In recent years a number of *Sorghum* x *Saccharum* and *Saccharum* x *Sorghum* hybrids have been developed at ICAR-Sugarcane Breeding Institute with *Sorghum* as well as *Saccharum* as female parents. Among these, two hybrids, Co 86032 x *Sorghum* and *Sorghum* x *Saccharum* were selected for the molecular cytogenetic characterization after confirming its hybridity by using molecular markers and chloroplast gene. Among the markers SSR markers have been found to be most suitable for such applications in view of its abundance in the genome, their co dominant nature and repeatability. In the present study SOMS 119 as well as SOMS 93 were found efficient to produce polymorphism between the parents in the case of Co 86032 x *Sorghum* and *Sorghum* x *Saccharum* respectively. The occurrence of parental specific bands in the hybrid genome DNA confirmed the hybridity of these clones. The organellar genomes like chloroplast DNA have been used as potential marker to asses maternal or paternal gene flow due to their uniparental mode of inheritance (Mc Cauley 1995). PCR amplification and restriction of a chloroplast gene, rpl L16 intron, confirmed the *Sorghum* cytoplasm in the hybrid evolved from *Sorghum* x *Saccharum* cross.

Though the classical cytology revealed that the chromosome number of both the hybrids as 2n=66, the chromosome composition of the hybrid genome was not known. In order to investigate the introgression of *Sorghum* genome into *Saccharum* while using it either male or female parent GISH analysis has been conducted. This is the first report in which introgression of *Sorghum* genome into Sugarcane has been confirmed by molecular cytogenetic technique. During the analysis biotin labeled *Sorghum* chromosomes were used as probe. The analysis revealed n+n chromosome segregation in these hybrids and also the introgression of 10 chromosomes of *Sorghum*. Earlier the same pattern of chromosome segregation has been reported in F1 intergeneric hybrids of *Saccharum* involving *Erianthus*, a wild related genera (Huang *et al.,* 2015, Piperidis *et al.,* 2010, Piperidis *et al.*, 2013, Wu *et al.,* 2014, Premachandran *et al.,* 2017).

During *in situ* hybridization an interesting fact noticed that the introgressed *Sorghum* showed stable condensation in interphase to anaphase stages of cell division. Generally the nuclear chromatin undergoes morphological changes with respect to degree of condensation and shape. It significantly compacted during interphase but later upon the entry into mitosis chromatin further condenses and individualizes to discrete chromosomes that are captured and moved independently by the mitosis spindle. The segregated chromosomes decondense to reestablish its interphase structure competent for DNA replication and transcription (Antonin and Newmann 2016). This type of hierarchical condensation and decondensation was not observed in *Sorghum* chromosomes in the intergeneric background. Though the underlying molecular mechanisms of mitotic chromatin condensation and decondensation are still ill defined, there is a possibility to study the impact of early condensation in further generations by molecular cytogenetic methods.

From the present study we could readily distinguished the chromosomes of *Sorghum* and *Saccharum* in the F1s of *Saccharum* x *Sorghum* and *Sorghum* x *Saccharum* by standard GISH conditions. In conclusion, the intergeneric or intertribal somatic hybrids and backcross progenies display some distinctive cytological features, such as differential chromatin condensation and spatial and temporal separation of chromosomes in mitotic and meiotic cells. Such differences may be attributed to the different structural characteristics of parental chromosomes and to differential expression of the parental alleles, such

as centromeric proteins. The results provide some new clues to the cytological mechanisms behind the phenotypic and genetic instability commonly displayed in hybrids with different genomes. These hybrids and progenies are ideal materials for studying the behavior of parental chromosomes in wide hybrids.

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