Journal of Sugarcane Research
https://doi.org/10.37580/JSR.2019.2.9.130-137

RESEARCH ARTICLE

PARENTAL GENOME DIFFERENTIATION BY GENOMIC *IN SITU* HYBRIDIZATION (GISH) IN INTERGENERIC HYBRID OF *SACCHARUM* X *ZEA*

V. P. Sobhakumari

Abstract

Tissue culture plants were developed from a rare non flowering hybrid of *Saccharum officinarum* (Vellai, 2n=80) x *Zea mays* (Golden Beauty 2n= 20+2B). Flowering was induced and the cytogenetic studies revealed that the number of chromosomes varies in root tip cells of the tissue cultured plants form 2n=52-56. The increase in chromosome number was due to the accumulation of B chromosomes. Genomic *in situ* hybridization (GISH) with Zea probe could reveal only repetitive sequences in the hybrid genome but not the whole chromosomes of Zea and this may be due to the rearrangements and loss of genome sequences especially in the maize which is rich in transposons and retrotransposons in its genome. GISH with *Saccharum* probe identifies 40 chromosomes of *S. officinarum* and 10+2B chromosomes of *Zea mays* in the hybrid. *In situ* hybridization with *Saccharum* probe was also revealed the homeology between the two genomes.

Keywords : *Saccharum, Zea*, Cytology, Tissue culture, Genomic *in situ* hybridization

Introduction

In sugarcane, wide crosses were conducted to develop karyotypically stable hybrid plants which have been used as starting materials to widen the genetic base of sugarcane and to develop genetic stocks for further analysis and utilization. Intergeneric hybridization is frequently being used in sugarcane and a number of hybrids of *Saccharum* with related genera like *Erianthus* (Janaki Ammal 1941), *Miscanthus* (Li et al. 1948), *Narenga* and *Sclerostachya* (Barber 1916, Parthasarathy 1948; Kandasami 1961) had been produced in the past. Hybrids of *Saccharum* with distantly related genera like *Sorghum* and *Zea* were also had been produced in the past (Thomas and Venkataraman 1930; Janaki Ammal and Singh 1936; Janaki Ammal 1938; Nair 1999). While *Erianthus* and *Miscanthus* introgression had been pursued rigorously in few countries with some success, but the utilization of other genera had not yielded any fruitful results. Earlier *Sorghum* had been crossed with *Saccharum* with the objective of reducing the crop duration (Thomas and Venkataraman 1930). Though some of these *Saccharum* x *Sorghum* hybrids attained maturity in about 6-7 months, they lacked many of the agronomic traits of sugarcane (Thomas and Venkataraman 1930). Four *Sorghum* x *Saccharum* hybrids were produced at Sugarcane Breeding Institute, wherein *Sorghum* was used as the female parent and flowering was induced through tissue culture (Nair 1999, Sobhakumari and Nair, 2005). Most of the intergeneric hybrids were completely or partially male or female sterile and

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Submitted: 17 October 2019; Accepted: 25 February 2020

in Sorghum x *Saccharum* complete male sterility has been reported due to the occurrence of syncyte formation and subsequent chromatin degeneration during microsporogenesis (Sobhakumari and Nair 2014).

The readiness of both *Saccharum* and Zea to cross with species of related genera is well known. In 1938 after several attempts using many thousands of flowers of a male sterile accession of *S. officinarum* (Vellai, 2n=80) as female parent and *Zea mays* (Golden Beauty, 2n=20+2B) as the male parent Dr. E. K. Janaki Ammal obtained a single seedling (Janaki Ammal 1938a). Its hybridity was confirmed with cytological analysis. This hybrid had 52 chromosomes being the sum of haploid component of *Saccharum* (n=40) and of Zea (n=10) with two B chromosomes of Zea (Janaki Ammal et al. 1972). This plant was clonally propagated for several years but normal flowering was not obtained in this hybrid. Once the induction of flowering was done by the application of gibberelic acid and found to be similar to that of *Saccharum* (Naidu and Ramakrishnan 1970).

In this paper we discussed the cytogenetic analysis of the sub clones of *Saccharum* x Zea hybrid which has derived from the original hybrid via tissue culture. The results of comparative GISH experiments, using genomic DNA of Zea as well as *Saccharum* as probe on the hybrid chromosomes are also discussed. These data will provide new clues about the genomic affinity among these distantly related genera and instability in the genome organization in the hybrids resulted by over the period of vegetative propagation and tissue culture.

Materials and Methods

Tissue cultured pants developed from *Saccharum officinarum* (clone 'Vellai' 2n=80) x *Zea mays* (variety 'Golden Beauty') hybrid is used for the study. These plants were cultivated and maintained in big cement pots in the glass house at ICAR-Sugarcane Breeding Institute, Coimbatore.

Actively growing shoots of about 2-3 months old were collected from the potted plants. After sterilization the developing leaves encircling the growing apex were dissected out and bits of about 0.5 x 0.5 cm were inoculated in 250 ml bottle with 20 ml MS basal medium (Murashige and Skoog, 1962) supplemented with 100 mg/l meso inositol, 2 mg/l 2,4 D, 10% by volume coconut milk and 20 gm/l sucrose. The medium was solidified with 0.8 % agar (Hi media). The pH of the medium was adjusted to 5.8.

For the induction of callus the bottles were incubated in dark at 25 ± 1°C. The callus was subcultured three to four times. The friable calli were transferred to solid differentiation medium and kept in light for 8 hours. For plantlet development and growth MS medium with the following additions were used: sucrose 20 gm/l, glycine 2 mg/l, naphthalene acetic acid 2 mg/l, kinetin 2 mg/l, meso-inositol 100 mg/l, nicotinic acid 0.5 mg/l, pyridoxine-HCl 0.1 mg/l. The pH was adjusted to 5.8. The plantlets, which differentiated in clumps initially, were separated into smaller groups and subcultured in fresh medium. The shoots were separated when they were of few inches height and kept for rooting in ½ MS medium. Rooted plants were transplanted in polythene bags and later to pots.

Somatic chromosome number of the clones selected for the study was determined by root tip squash technique as reported by Sobhakumari and Asmita Dutta 2014.

Genomic *in situ* Hybridization (GISH)

For GISH analysis the cytological slides were prepared separately from the root tips fixed in 2mM 8-hydroxy quinolone at room temperature for 2h. Then the root tips of the *Saccharum* x *Zea* hybrids were rinsed in water and fixed in ethanol

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https://doi.org/10.37580/JSR.2019.2.9.130-137

acetic acid (3:1) for about 16 h at 4°C.The root tips were hydrolyzed in 0.25N HCl and digested in enzyme solution (2% cellulase ONOZUKA R-10 and 20% pectinase in citrate buffer) at 37°C for 1h. After washing in distilled water meristematic tissues were squashed in 45% acetic acid and put a cover slip over it. Cells can be separated by gentle pressing over the coverslip with a filter paper. Slides with well spreaded mitotic chromosomes were then freezed by 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.

Total genomic DNA was isolated from leaves of *Zea maize* and *S. officinarum* by CTAB method (Doyle and Doyle 1990). The quality and quantity of the total genomic DNA has been confirmed and fragmented to the size of 1000bp – 500bp by sonication. Genomic DNA labeled with biotin 11-duTP using random primed labeling method as described by the manufacturer (Thermo Scientific, USA) was used as probe. Different concentrations of probe (40ng – 150ng) have been tried and a concentration of 50ng/slide was used both in the case of maize and *Saccharum*.

Genomic *In situ* hybridization was conducted as the protocol reported by Sobhakumari et al. (2018) with minor modifications. After *in situ* hybridization the slides were counter stained with DAPI (4,6-diamidino-2-phenylindole) (Vector laboratories, UK) and 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 and Discussion

Tissue culture plantlets were developed from the calli that initiated from young leaf bits of the

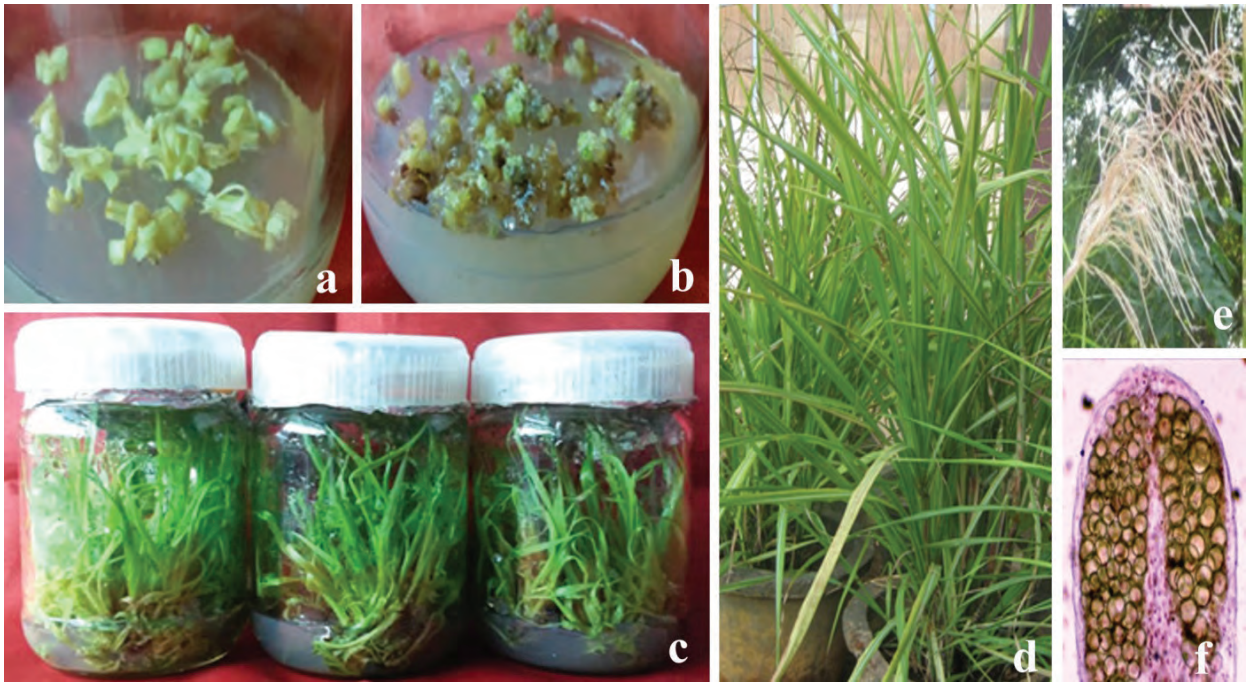

Fig. 1. Callus culture of *Saccharum* x *Zea* hybrid (a) Explant- young leaf bits (b) Callus induction and regeneration (c) Plantlets multiplication (d) Tissue culture developed plants (e) inflorescence of *Saccharum* x *Zea* hybrid (f) LS of anther sac showing sterile pollen

Saccharum x *Zea* hybrid (Fig. 1 a & b). Phenol extrusion from the explant was the problem faced during tissue culture. This was overcome by frequent subculturing of the explants. Within two weeks the cell proliferation was initiated in the culture. After 2-3 subculture in the callus induction medium the friable calli were transferred to solid differentiation medium for multiplication (Fig. 1c). The multiplied shoots were rooted, hardened and maintained in the glass house (Fig. 1d).

Six months old plants were subjected to cytological analysis. Root tip mitosis of the subclones indicated plant-to-plant chromosome number variation. Even in the same plant the number varies in different root tip cells. The chromosome number varies from 52-56 (Table 1 and Fig. 2). From the structure and staining intensity we could identify that the extra chromosomes present in the mitotic cells were the B chromosomes from *Zea mays*. It varies from 2-6 in numbers in different cells. In all hybrid cells, one pair of satellite chromosomes was observed. Normal flowering was observed in three clones (Fig. 1e). The flower was similar to *Saccharum*. The female reproductive structures like stigma, style and ovary were found to be normal. While analyzing the pollen fertility it was found that the anther sac was filled with sterile pollen (Fig. 1f) and the plant was showing 100% male sterility.

Table 1. Frequency of B chromosomes in 25 root tip cells of *Saccharum* x *Zea* hybrid

No. of cells	Somatic Chromosome number (2n)	No. of B chromosomes
13	52	2
4	53	3
5	54	4
3	56	6

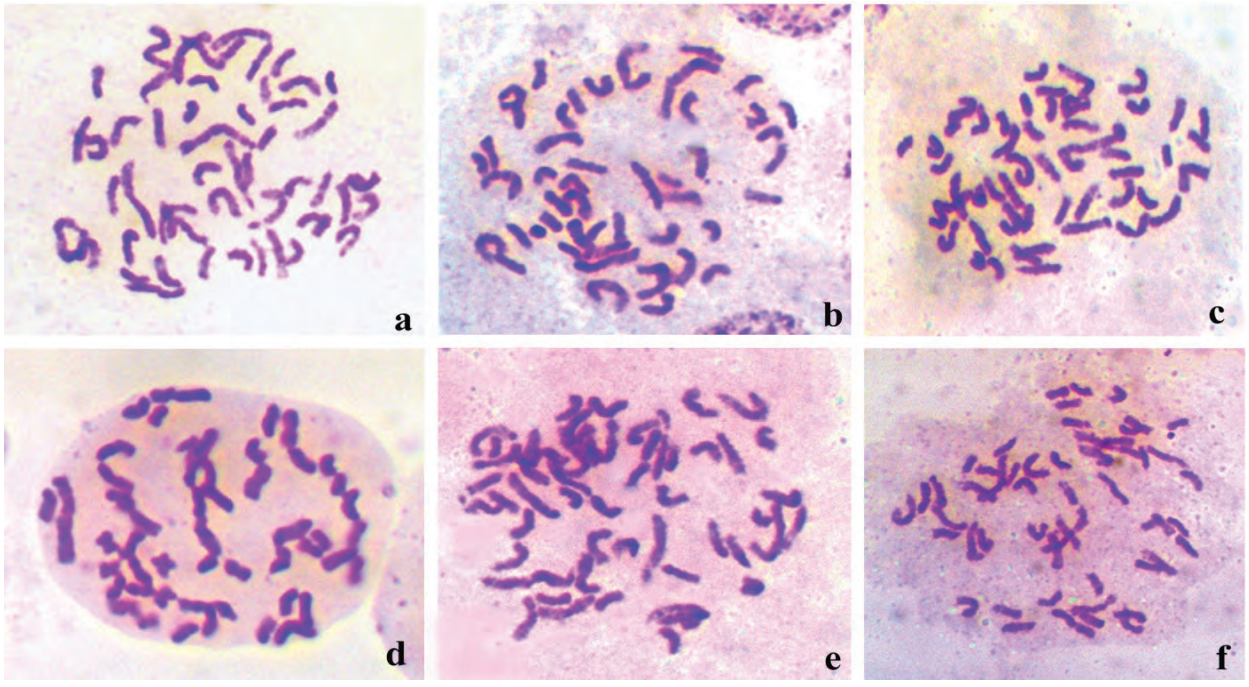

Fig. 2. Root tip squash (mitosis) of hybrid showing different chromosome numbers (a – c) Cells with 2n=52 chromosomes (d) Somatic cell with 53 chromosomes (e) Somatic cell with 54 chromosomes (f) Somatic cell with 56 chromosomes

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In GISH experiments, total genomic DNA of *Zea mays* were biotin labeled and used as probe on mitotic metaphases of *Zea mays* to check the efficiency of the probe. Strong signals of hybridization were observed along all maize chromosomes. During GISH analysis with maize probe the interphase was showing hybridized signals as bluish green spheres whereas in metaphase only 5-6 strong signal spots were observed (Fig. 3 a-c). While using *Saccharum* as the labeled probe all the 40 chromosomes from *S. officinarum* was showing strong signals of hybridization in the hybrid. In some hybridized cells apart from the strong signals on the *Saccharum* chromosomes the maize chromosomes were also showing some weak patches of hybridization (Fig. 3 d-f). The observed homeology could not be quantified due to the difficulty in measuring the small dispersed marks. Though the *Saccharum* probe was hybridized with 40 chromosomes in the hybrid, some regions especially telomeric regions of 4-6 chromosomes were showing weak signals (Fig. 3 g-i).

The intergeneric hybrid cross involving sugarcane was made by Janaki Ammal (1938a) between *Saccharum* x maize. The hybrid had 52 chromosomes being the sum of the haploid complements of *Saccharum* (n=40) and of *Zea* (n=10) with two B chromosomes of *Zea* (Janaki Ammal 1941). The hybrid which is maintained through vegetative propagation was not flowered normally. Some of the stalks showed symptoms of flowering during one season, but the panicle could not emerge through the tight wrap of leaf sheaths enclosing the panicle, resulting in the drying of the panicle within the sheath. These plants were subjected to tissue culture for improvement and multiplication. Out of 25 plants hardened and planted in pots, three plants flowered normally and it was completely male sterile. Earlier the complete male sterility in Sorghum x *Saccharum* intergeneric hybrid has been reported due to abnormalities in the microsporogenesis which leads to complete chromatin degeneration (Sobhakumari and Nair 2014). Here in this hybrid degeneration of chromatin content was not observed whereas the anther sac was filled with complete sterile pollen.

The mitotic study in a particular hybrid through root tip squash technique has been showed that the number of chromosomes varies in the cells. In 25 metaphase plates the number varied from 52-56. The frequency of the distribution of extra chromosome (B chromosome) was 2-6. By structure and intensity of staining the B chromosomes could identify in the somatic cells and it has been found that the variation in chromosome number is believed to be, in part, derived from the parent plant itself from which the explant has been taken, where deletion and duplication of B chromosomes have been reported. The variation in the chromosome number in the somatic cells of *Saccharum* x *Zea* has already reported by Janaki Ammal et al. (1972). The reason behind the variation in chromosome number has been justified by the course of many years (more than 30) of vegetative propagation which leads to the multiplication of B chromosomes. Reports also say that B chromosomes show lots of instability during mitosis in somatic cells and therefore they are present or absent in variable numbers in specific tissue and organs. In plants, only one-third of the investigated species display constancy of B Chromosomes in different tissues of the plant (Jones and Rees 1982). Absence of B chromosomes from roots has been observed in *Erianthus munja* and *Erianthus ravennae* (Datta et al. 2016). In a few species, the instability of the B chromosomes is partial and strictly defined, e.g., in *Sorghum stipoideum*, mosaicism of B chromosomes has been reported in tapetal cells and microsporocytes, while they were totally

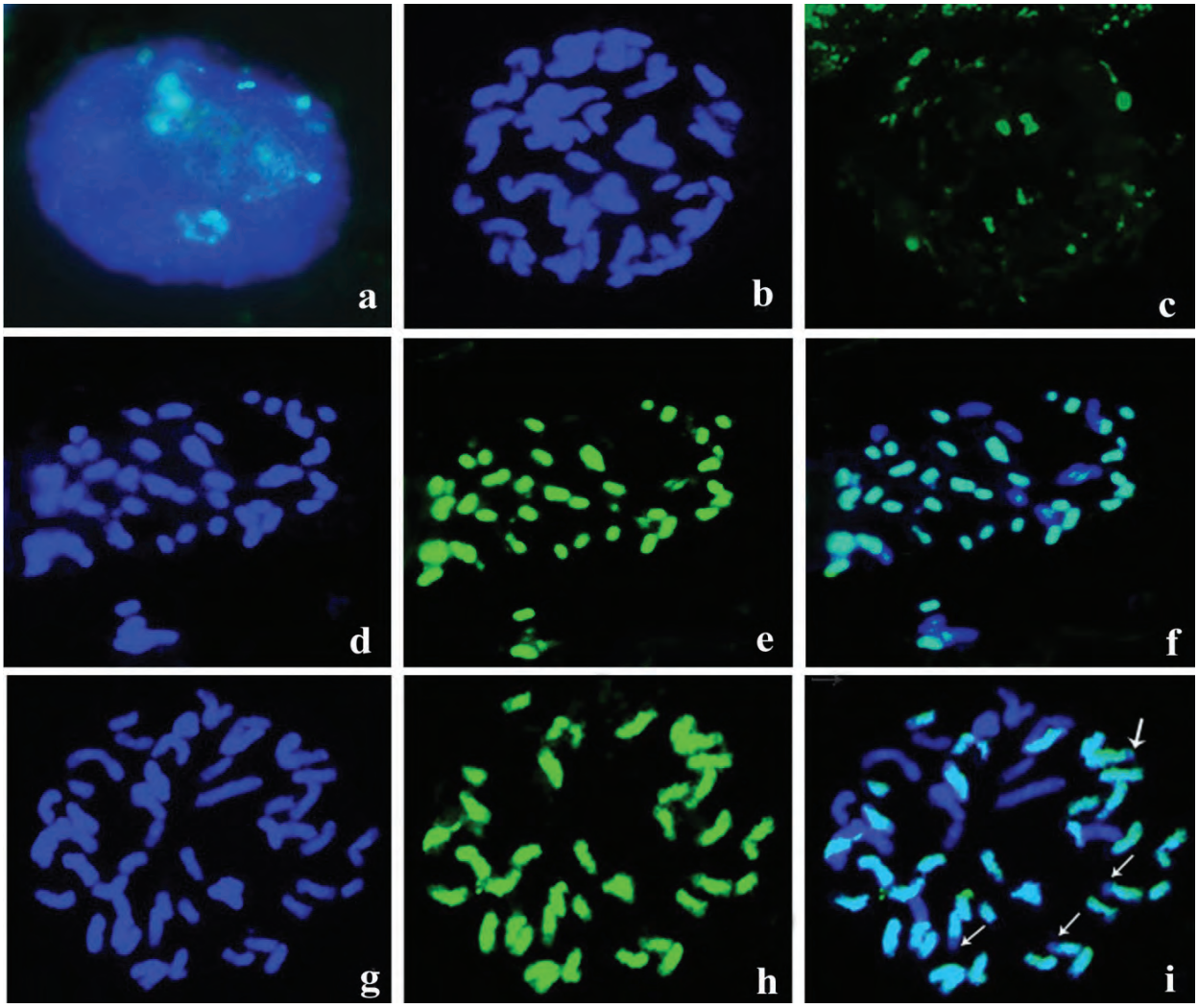

Fig. 3. GISH analysis in *Saccharum* x *Zea* hybrid (a) Interphase nuclei of the hybrid probed with labeled *Zea* showing heterochromatic knobes as bluish green spheres (b-c) GISH analysis with *Zea* probe: (b) Chromosomes of hybrid stained with DAPI (c) Chromosomes of hybrid hybridized with *Zea* probe(d-i) GISH analysis with *Saccharum* probe: (d,g) Chromosomes of hybrid with DAPI (e,h) Chromosomes of hybrid hybridized with *Saccharum* probe (f,i) merged images

eliminated from the leaves and stem (Dhar et al. 2019). In the present study the hybrid in which chromosome mosaicism has been reported was used as explant for tissue culture propagation. Hence it is obvious to expect the same kind of variation in the regenerated plantlets.

GISH using total genomic DNA as a probe to identify alien chromosomes or chromosome segments is well-established in many plant species (Garriga-caldere et al. 1997; Tang et al. 1997; Petroski and Armstrong, 2000). As we know, no confident results for GISH characterization of the distant hybrids of *Saccharum* x maize has been obtained so far. In GISH experiment while using maize as the labeled probe only signal spots were observed. These may be the repeated sequences

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https://doi.org/10.37580/JSR.2019.2.9.130-137

available in the maize chromosomes. The intensity of spot signals varies accordingly to the length and frequency of the repeated sequences. The reason for the absence of whole chromosome hybridization in maize may be the presence of transposons or jumping genes. These transposable elements are found in most of organisms, making up to 90% of the maize genome (SanMiguel et al. 1996). Hence there is chance for continuous rearrangements taking place in the genome of maize from generation to generation. These changes in the gene sequences blocking the whole chromosome hybridization in maize. While using *Saccharum* as the labeled probe, it has been found that strong hybridization in whole chromosomes of 40 *Saccharum* chromosomes (green). DAPI stained 10+2 B chromosomes (blue) of maize were clearly visible in merged images. Some chromosomes of maize showed bluish green weak patches on them. These may be the homeologous sequences among *Saccharum* and maize. In some *Saccharum* chromosomes, telomere region was not showing hybridization. In spite of their occurrence in distinct genic groups and difference in ploidy level the genetic proximity between these two species become evident by its breeding compatibility. This type of genome synteny has been reported in many members of grass family (Poggio et al. 1999). GISH predominantly detects repetitive sequences and in maize these are known to be composed mainly of retrotransposons (SanMiguel and Bennetzen 1998). It is therefore reasonable to suggest that most of the dispersed signal observed using *Zea* and *Saccharum* as probes represents hybridization between retrotransposons that are common between *Saccharum* and *Zea*. These regions which possess good *Saccharum*-maize homeology are for potential occurrence of recombination-translocation events. These kinds of genetic interchanges could be important for the successful transfer of genes with important value from distantly related species.

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