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**Research Article**

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# Meiotic abnormalities in sugarcane (*Saccharum* spp.): Evidence for peri- and paracentric inversions

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Short running head:

Meiotic abnormalities in sugarcane

**Abstract**

The modern cultivars of sugarcane (*Saccharum* spp.) are highly polyploid and accumulate aneuploidies due to their history of domestication, genetic improvement and interspecific hybrid origin involving the domesticated sweet species *S. officinarum* ('noble cane') and the wild *S. spontaneum*, both with an evolutionary history of polyploidy. The first hybrids were backcrossed with *S. officinarum*, and selection from progenies in subsequent generations established the genetic basis of modern cultivars. *Saccharum* genome complexity has inspired several molecular studies that have elucidated aspects of sugarcane genome constitution, architecture and cytogenetics. Herein, we conducted a comparative analysis of the meiotic behavior of representatives of the parentals *S. officinarum* and *S. spontaneum*, and the commercial variety, SP80-3280. *S. officinarum*, an octoploid species, exhibited regular meiotic behavior. In contrast, *S. spontaneum* and SP80-3280 exhibited several abnormalities from metaphase I to the end of division. We reported and typified, for the first time, the occurrence of peri- and paracentric inversions. Using *in-situ* hybridization techniques, we were able to determine how pairing association occurred at diakinesis and, in particular, the chromosome composition of SP80-3280. Our findings have implications for sugarcane genetic mapping, genomics, and for studies on resynthesized polyploids.

**Keywords:** *Saccharum*; meiotic behavior; chromosome associations; chromosome inversions, *in situ* hybridization

## Introduction

The sugarcane (*Saccharum* spp.) crop is of considerable industrial importance, accounting for nearly 80% of global sugar production (see https://www.isosugar.org/sugarsector/sugar). Sugarcane is generally regarded as the most sustainable source of biomass for producing biofuels, with high potential for mitigating the effects of climate change without affecting food security (Long et al. 2015; Kline et al. 2017). Crops and by-products can be developed for producing bioelectricity, bioplastics and fertilizers, in addition to cellulosic ethanol. Importantly, the energy contained in sugarcane-derived ethanol and in the electricity generated from burning sugarcane bagasse accounts for 17.5% of the Brazilian energy matrix (see http://www.mme.gov.br).

*Saccharum* species originated in New Guinea, where sugar canes have been grown for millennia. The earliest record of domestication dates back to around 8,000 BCE, and cultivation gradually spread across human migration routes to Southeast Asia and India. This long history of cultivation has facilitated the generation of a diversified germplasm which includes species of the *Saccharum* complex (two wild, *S. spontaneum* and *S. robustum*, and four cultivated species, *S. officinarum*, *S. sinense*, *S. barberi*, and *S. edule*) and four interbreeding genera (*Erianthus*, *Miscanthus*, *Narenga*, and *Sclerostachya*). Collections currently include interspecific hybrids, commercial cultivars and elite clones (see Barreto et al. 2021; Cursi et al. 2021).

Selection practices in former times resulted in *Saccharum officinarum* clones with a higher sugar content and fewer fibers. These are known as 'noble canes' (Simmonds 1975). Subsequently, in the late 19$^{th}$ century, new varieties emerged from interspecific hybridization of the formerly cultivated species (*Saccharum barberi* and *S. officinarum*) and wild *Saccharum spontaneum*. These hybrids were then successively crossed with *S. officinarum* in order to recover the sucrose content. *S. spontaneum* was chosen due to its peculiar attributes, especially hardiness, resistance to diseases, tillering and ratooning ability, which are of remarkable value in the profitability of the crop everywhere (see Grivet et al. 2004; Cheavegatti-Gianotto et al. 2011; Matsuoka and Stolf 2012; Barreto et al. 2021). Importantly, due to a mechanism known as meiotic restitution, unreduced gametes were transmitted by *S. officinarum* (i.e., $2n$, its somatic chromosome number) to its progenies (Bremer, 1961a, 1961b; Price, 1963a, 1963b), which accounts for the overrepresentation of the *S. officinarum* genome in subsequent generations. Overall, the origin of modern cultivars is well documented (see Pompidor 2021).

The genus *Saccharum* includes diverse forms of polyploids and exclusively higher order polyploid species (>4$x$), such as *S. officinarum*, a typical octoploid ($2n = 8\times = 80$, $x = 10$), *S. robustum* ($2n = 60$, 80 to 200), and its presumed natural mutant clone, *Saccharum edule* ($2n = 60$ to 122) (Grivet et al. 2006). *S. spontaneum* is an autopolyploid with variable chromosome number and aneuploid accessions (Panje and Babu 1960). It is considered a mixed ploidy species, with

chromosome numbers higher than expected for species in multiples of 8 (*2n* = 40 to 128). The basic number of *S. spontaneum* (*x* = 8) was supposedly reached in two steps by rearrangements from *x* = 10 leading to *x* = 9 and then *x* = 8 (Piperidis and D'Hont 2020). Current sequencing results for a typical contemporary cultivar (12 homoeologous haplotypes of the R570 cultivar) suggest the existence of three founding genomes in modern sugarcanes, two contributed by *S. officinarum* and also found in its presumed ancestor, *S. robustum*, and one contributed by *S. spontaneum* (Pompidor et al. 2021).

According to pioneering molecular cytogenetic studies, *S. officinarum* and *S. spontaneum* account respectively for 75 to 85% and 15 to 25% of sugarcane chromosomes. The remaining chromosomes are recombinant from both origins (D'Hont et al. 1996; Cuadrado et al. 2004; Piperidis et al. 2010; Piperidis and D´Hont 2020), due to pairing and recombination between homoeologous chromosomes. In addition, the incorporation of other germplasm into cultivated backgrounds has so far stymied attempts to decipher the genetic architecture and genomic organization of modern sugarcane cultivars. Importantly, due to the geographical locations of experimental stations (US, India, Brazil, Australia, etc.) and agricultural requirements, each sugarcane pedigree has particular features. There are differences in the contributions of each ancestral species and hybrid genotypes within the pedigrees.

As a result of all these processes, sugarcane has an 'artificial' genome of interspecific constitution (polyploid and aneuploid), produced by human intervention, and a complexity that exceeds that of most crops (Gouy et al. 2013). Despite its redundant origin (all modern varieties have primarily the same origin) and genome complexity, including a variable number of chromosomes (*2n* = 110 to 130), from a meiotic point of view several classic studies have suggested that both parental species and interspecific hybrids predominantly form bivalents at meiosis, as well as the contemporary cultivars (Nair 1975; Price 1963a, 1963b; Suzuki 1941; Pagliarini et al. 1990; Burner 1991; Bielig et al. 2003). Recently, our group has confirmed a bivalent association in the IACSP93-3046 variety (*2n* = 112). This was done using FISH (fluorescent *in situ* hybridization) with labeled probes targeting the centromeric regions at diakinesis. These probes allowed us to enumerate the number of centromeres (i.e., 56 bivalents), although in some cells 1 or 2 univalents were also found (Vieira et al. 2018).

Herein, our aim was to investigate the meiotic behavior of representatives of the parental species (*S. officinarum* and *S. spontaneum*) and the SP80-3280 commercial variety. We examined in detail the frequency and types of meiotic irregularities, such as the incidence of both peri- and paracentric inversions. Using *in situ* hybridization techniques, we were able to determine chromosome composition and how pairing association occurs in SP80-3280 prophase cells, especially at diakinesis.

## Material and methods

### Plant material

The following plant material was investigated: (*i*) sugarcane clone Caiana Fita ($2n$ = 80), representative of *S. officinarum*; (*ii*) accession SES205 ($2n$ = 64), representative of *S. spontaneum*, and (*iii*) the commercial variety, SP80-3280, for which genetic and genomic data is available (Garcia et al. 2013; Balsalobre et al. 2017; Souza et al. 2019). The pedigree of SP80-3280 is shown in Supplementary Fig. 1.

Plant material was kindly provided by the Universidade Federal de São Carlos (UFSCar) (http://pmgca.dbv.cca.ufscar.br), a member of the Inter-university Network for the Development of the Sugar and Ethanol Industry (RIDESA); and the IAC Sugarcane Center (https://www.iac.sp.gov.br/areasdepesquisa/cana/index.php?lang=en) run by the Agronomic Institute of Campinas (IAC), both Brazilian public institutions located in Southeastern Brazil.

Immature panicles (pre-emerged inflorescences still wrapped in the flag leaf sheath) were collected from Caiana Fita and SES205 at the Sugarcane Hybridization Station (IAC) in Uruçuca (14°35′34″ S, 3917′2″ W, Bahia State), and from the SP80-3280 variety at UFSCar in Araras (22º21'25" S, 47º23'03" W, São Paulo State).

### Meiotic chromosome behavior

Immature inflorescences of Caiana Fita, SES205 and SP80-3280 were collected and fixed in Carnoy (3 acetic acid: 1 etanol) solution at room temperature. After 24 h, the fixative solution was replaced with 70% absolute ethanol and flasks stored at 4ºC.

Following conventional protocols (Sharma and Sharma 1980), flower buds were carefully dissected and anthers placed on a slide in a drop of 2% acetic carmine. After cross-sectioning with a scalpel blade, anthers were lightly crushed to expel the microsporocytes on the slide covered with a 20 × 20 coverslip. Slides were visualized under the microscope and selected for examination.

Meiotic cells from metaphase I to telophase II (including tetrads) were analyzed and images captured using an OPTIKAM B3 camera (Optika) and Adobe Photoshop CS5 (Adobe Systems). The percentage of cells with chromosome irregularities was estimated at each stage for a total of 200 cells each of Caiana Fita and SES205, and 850 cells of SP80-3280.

### Pairing investigation using fluorescent *in situ* hybridization (FISH) with centromeric probes

Anther cells at diakinesis were previously selected to prepare a cell suspension according to Murata and Motoyoshi (1995) and Vieira et al. (2018), with modifications. First, the anthers were

144 washed in distilled water and placed in a microcentrifuge tube containing an enzyme mixture
145 consisting of 2% cellulase (Onozuka), 20% pectinase (Sigma) and 1% macerozyme (Sigma), and
146 kept at 37°C for 2 to 3 h. Microsporocytes were carefully separated using a micropipette in order
147 to obtain a cellular suspension that was then centrifuged (13,000 rpm for 5 min). The pellet was
148 washed in distilled water (50 µl), centrifuged as above, and fixed in Carnoy solution (50 µl) for 5
149 min. Cells were then resuspended in a new fixative solution (30 µl) and 10 µl of the suspension
150 was dropped on a clean slide and dried at room temperature. High-contrast images were examined
151 under a microscope (Nikon E200) and selected for hybridization.
152 To investigate chromosome pairing, we used *in situ* hybridization with fluorescent probes
153 to detect centromere sequences. First, genomic DNA was extracted from Caiana Fita, SES205
154 and SP80-3280 using the CTAB method as described in Vieira et al. (2018). Next, a primer pair
155 previously designed to amplify sugarcane CENT repeats was used (Nagaki et al. 1988; Vieira et
156 al. 2018). The amplification reaction consisted of 1 × buffer solution, 1.5 mM $MgCl_2$, 0.2 mM of
157 dNTP, 0.3 µM of each primer, 1 U of GoTaq Flexi DNA Polymerase (Promega), 40 ng of genomic
158 DNA, and ultrapure water for a final volume of 20 µl. The amplifications were performed in a
159 GeneAmp PCR System 9700 thermocycler (Applied Biosystems) with an initial denaturation step
160 (95°C, 5 min), followed by 35 amplification cycles (95 °C for 40 s, 60 °C for 50 s, 72 °C for 1
161 min 30 s) and a final extension at 72 °C for 10 min.
162 A standard gel electrophoresis was run to check the size of the PCR products, which were
163 then purified using a Wizard SV Gel and PCR Clean-Up System kit (Promega). Purified DNAs
164 were labeled using the DIG-nick translation labeling kit (Roche) with digoxigenin-11-dUTP,
165 following the manufacturer's instructions.
166 FISH procedures were carried out according to Schwarzacher and Heslop-Harrison
167 (2000) and Vieira et al. (2018), with modifications. Slides were treated with RNase (100 µg /ml
168 for 1 h, 37 °C), fixed in paraformaldehyde (4%, w/v) for 10 min and dehydrated in an ethanol
169 series (70, 90 and 100%, 5 min each). The hybridization mixture consisted of formamide (50%,
170 v/v), dextran sulfate (10%, w/v), saline sodium citrate (2 × SSC), sodium dodecyl sulfate (0.13%,
171 w/v SDS) and 3 ng/µl of DNA probe. The hybridization mixture was previously denatured (10
172 min, 90 °C) and applied to chromosomal preparations. Slides were denatured and hybridized for
173 10 min at 90 °C and 37 °C respectively, in a GeneAmp PCR System 9700 thermocycler (Applied
174 Biosystems) and then incubated in a humidity chamber overnight at 37 °C.
175 The CENT probe was detected with anti-digoxigenin conjugated to rhodamine (Roche).
176 Slides were mounted in DAPI-Vectashield (Vector Labs). Images at diakinesis were captured
177 using a DFC365 FX digital camera (Leica) coupled to a DM 4000B fluorescence microscope

(Leica). The selected images were processed using Adobe Photoshop CS5 (Adobe Systems). The hybridization sites of the top 10 cells were analyzed to determine chromosome pairing.

Chromosome associations were also investigated in early prophase I cells, specifically at pachytene using DAPI, a blue fluorescing DNA-specific stain to reveal chromosome details (see Ahmad et al. 2021) and FISH using telomeric probes. At this stage, the telomeres were labeled using rhodamine-labeled synthetic oligonucleotides (5' TELO1F – FLUORO- CCC TAA ACC CCT AAA CCC TAA ACC CTA AAC CCT AAA 3' and 3' TELO1R – RHOD – CCC TAA ACC CTA CCT AAA CCC TAA ACC CTA AAC CCT AAA 5', Life Technologies).

**Mitotic chromosome counting**

Sugarcane stalks were collected from SP80-3280 field plants and cut into pieces ~8 cm in length. In the laboratory, the cuttings were placed on trays containing sphagnum moss watered daily and kept at 28°C±3°C to induce bud rooting. Roots ~2 cm long were excised and pre-treated with a blocking solution of 8-hydroxyquinoline (0.03% w/v; Sigma) and cyclohexamide (25 ppm; Cayaman Chemical Company) for 4h 30min at room temperature. The roots were then fixed in Carnoy solution (3:1 v/v ethanol: acetic acid) for 24 h, transferred to a 70% ethanol solution, and stored at 4°C.

For slide preparation, the roots were washed twice in distilled water, hydrolyzed in 1 N HCl at 60°C for 8 min, washed again and stained as usual using the Schiff's reagent for 45 min in the dark. Digestion was performed using an enzymatic solution of 2% cellulase (Onozuka), 20% pectinase (Sigma) and 1% macerozyme (Sigma) at 37°C for approximately 90 min. Then the roots were washed twice in distilled water, immersed in 45% acetic acid for 2 min, and the root tips squashed in a drop of 1% acetic carmine. Slides were mounted in Entellan embedding agent (Merck) and examined under an Olympus BX50 microscope.

Metaphase images were captured using an OPTIKAM B3 camera (Optika) and Adobe Photoshop CS5 (Adobe Systems). Twenty-three intact cells showing well-spread chromosomes were selected for chromosome counting.

**Genomic *in situ* hybridization (GISH)**

Roots were collected, pretreated, fixed and digested as described above. Root tips were immersed in Carnoy solution to prepare slides by the flame-drying technique (Dong et al. 2000).

For GISH analysis, slightly modified previous protocols optimized for sugarcane (D'Hont et al. 1996; Piperidis et al. 2010) were used. Genomic DNA from Caiana Fita and SES205 was extracted as described above and accurately quantified with a Qubit4 Fluorometer (Invitrogen). DNA integrity was checked by agarose gel (1.2% w/v) standard electrophoresis. To do this, DNA

bands were stained with SYBR Safe 0.5 × (Invitrogen) and visualized under a UV transilluminator.

Genomic DNA probes were labeled by nick translation with digoxigenin-11-dUTP (Roche Biochemicals) for Caiana Fita and the biotin14-dATP kit (Invitrogen) for SES205.

Slides were treated with RNAse (100 µg/ml; Sigma-Aldrich) at 37°C for 45 min, fixed in formaldehyde (4%, w/v), denatured in 70% formamide in 2 × SSC at 80°C for 1 min 45 s and then dehydrated in an ethanol series (70, 90 and 100%, 5 min each) at –20°C. The hybridization mixture (50 µl per slide) consisted of 50% formamide, 10% dextran sulfate, 2 × SSC, 1.5 µl Salmon sperm DNA and 100 ng of each probe. The hybridization mixture was denatured at 97°C for 10 min and stored on ice for 15 min. Hybridization was performed in a humidity chamber for 48 h at 37°C.

*S. officinarum* labeled probes were detected with rabbit anti-mouse FITC (Roche Biochemicals) and *S. spontaneum* probes with sheep anti-digoxigenin TRITC (Roche Biochemicals). Slides were mounted using Vectashield with 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs). Mitotic chromosome images were captured by a DFC365 FX digital camera (Leica) coupled to a DM 4000B fluorescence microscope (Leica).

## Results

### Microsporogenesis in *Saccharum officinarum* and *S. spontaneum* representatives

As expected, the octoploid *S. officinarum* exhibited regular microsporogenesis: for both divisions, abnormalities were visualized in only ~6% of the cells (12/209), some at metaphase I (2/34) (e.g., chromosomes not lined up at the equatorial plate) and some at anaphase I (4/23) (e.g., lagging chromosomes). In the second division, the percentage of cells with chromosomes not lined up at the equatorial plate was ~13% (5/39, metaphase II) and those with laggards, 11% (1/9, anaphase II), both acceptable percentages in autopolyploids (Table 1).

In contrast, ~52% (111/214) of *S. spontaneum* cells exhibited irregularities, 42% (35/84) in the first and 58% (76/130) in the second division (Table 1). Thirty-seven percent (11/30) of metaphase I cells exhibited chromosomes not lined up at the equatorial plate and ~43% (17/40) of anaphase I cells exhibited lagging chromosomes (up to 9 chromosomes, although predominantly 1 or 2). Only 2 cells were found to exhibit chromosome bridges. Lagging chromosomes were observed in up to 36% (5/14) of telophase I cells, although usually only 1 or 2.

During meiosis II, a regular pattern was found in 42% (54/130) of the cells. At prophase II, ~54% (28/52) of the cells exhibited up to 2 chromosomes not incorporated into the nucleus. At

metaphase II, up to 6 chromosomes were found not lined up at the equatorial plate (more frequently 2 chromosomes). Lagging chromosomes were observed in only one cell (1/4) at anaphase II. Meiosis II is known to be a faster division, and this may account for the low number of cells observed at anaphase II. Nevertheless, a high number of cells was found at telophase II, 67% (16/24) of them exhibiting lagging chromosomes not incorporated into the nuclei.

Remarkably, we visualized asynchronous cells in *S. spontaneum* but not in *S. officinarum*. *S. spontaneum* is a mixed-ploidy species, which may explain the incidence of asynchronous cells. Furthermore, approximately 40% of tetrad cells exhibited four nuclei with no micronuclei, but in the remaining cells (20/33) up to 5 micronuclei were observed. This leads to the conclusion that a very low number of irregularities occurs in *S. officinarum*, in contrast to *S. spontaneum*, in which 52% of meiotic cells exhibited abnormalities from metaphase I up to the subsequent phases.

**Table 1.** Meiotic abnormalities in pollen mother cells of Caiana Fita ($2n = 80$) and SES205 ($2n = 64$). Numbers in brackets are percentages

| Meiotic phase | Caiana Fita | | SES205 | | Abnormality |
|---|---|---|---|---|---|
| | No. of cells examined | No. of cells with abnormalities | No. of cells examined | No. of cells with abnormalities | |
| Metaphase I | 34 | 2 (5.9) | 30 | 11 (36.7) | Chromosomes not lined up at the equatorial plate |
| Anaphase I | 23 | 4 (17) | 40 | 17 (42.5) | Lagging chromosomes |
| | | 0 | | 2 (5) | Chromosome bridges |
| Telophase I | 35 | 0 | 14 | 5 (35.7) | Lagging chromosomes |
| Subtotal | 92 | 6 (6.6) | 84 | 35 (41.7) | |
| Prophase II | 23 | 0 | 52 | 28 (53.8) | Chromosomes outside the nucleus |
| Metaphase II | 39 | 5 (12.8) | 14 | 8 (57.1) | Chromosomes not lined up at the equatorial plate |
| Anaphase II | 9 | 1 (11.1) | 4 | 1 (25) | Lagging chromosomes |
| Telophase II | 15 | 0 | 24 | 16 (66.7) | Lagging chromosomes |
| Metaphase/ Anaphase | 0 | 0 | 1 | 1 | Asynchrony |
| Anaphase/ Telophase | 0 | 0 | 2 | 2 | Asynchrony |
| Tetrad | 31 | 0 | 33 | 20 (60.6) | Micronucleus |
| Subtotal | 117 | 6 (5.1) | 130 | 76 (58.4) | |
| Total | 209 | 12 (5.7) | 214 | 111 (51.8) | |

**Meiotic chromosome behavior in SP80-3280**

Of a total of 850 pollen mother cells assessed, only 194 (~23%) exhibited regular behavior in both meiosis I (excepting prophase I, not analyzed herein) and II. The remaining cells (656/850, ~77%) exhibited irregularities that varied in type and frequency depending on the meiotic phase (Table 2). A similar rate of irregularities was found in cells undergoing the first and second division 77.4% (295/381) and 76.9% (361/469) respectively.

Approximately half of metaphase I cells exhibited irregular behavior (89/160), with chromosomes (predominantly 1 or 2) not lined up at the equatorial plate (Fig. 1 a-c). In both anaphase I (Fig. 1 d-g) and telophase I (Fig. 1 h-i), the percentage of cells with irregularities was notably high, with respective values of ~92% (70/76); and ~94% (136/145). In addition, up to 8 not-aligned chromosomes were frequently visualized. Because of this lagging tendency, bivalents were visualized as rod-shaped chromosomes (~27%, predominantly 1 or 2) (Fig. 1 e). Chromosome bridges were found at a percentage of 6.6% (5/76) and 2.7% (4/145) respectively at anaphase I and telophase I.

Almost all prophase II cells (~96%) exhibited up to 8 chromosomes not incorporated into the nuclei (Fig. 2 a-b). Of the total of metaphase II cells (= 100), chromosomes non-aligned at the equatorial plate were visualized in 38% (Fig. 2 c). In the remaining cells (49%), chromosomes seemed to migrate early to the poles (Fig. 2 d). A tiny chromatid bridge is shown at the bottom cell of Fig. 2 e. Several lagging chromosomes were identified in anaphase II (Fig. 2 f) and may be the remnants not incorporated into telophase I nuclei (Fig. 2 g-h); the most frequent values were from 2 to 5 chromosomes. Only 11% (7/59) of telophase II cells exhibited complete nuclei with no micronuclei.

We also visualized ~8% of cells with asynchronous behavior, (including metaphase/anaphase and anaphase/telophase) (Fig. 2 i-j). At the end of meiosis, approximately half of the resulting daughter cells (80/147) exhibited four normal nuclei, but in the remaining cells (67/147) there were up to four chromosomes (or fragments) entrapped in micronuclei (Fig. 2 k-l).

293 **Table 2** Meiotic abnormalities in pollen mother cells of SP80-3280 (*2n* = 112). Numbers in
294 brackets are percentages

| **Meiotic phase** | **No. of cells examined** | **No. of cells with abnormalities** | **Abnormality** |
|---|---|---|---|
| Metaphase I | 160 | 89 (55.6) | Chromosomes not lined up at the equatorial plate |
| Anaphase I | 76 | 65 (85.5) | Lagging chromosomes |
| | | 5 (6.6) | Chromosome bridges |
| Telophase I | 145 | 132 (91.0) | Lagging chromosomes |
| | | 4 (2.7) | Chromosome bridges |
| Subtotal | 381 | 295 (77.4) | |
| Prophase II | 120 | 115 (95.8) | Chromosomes outside the nucleus |
| Metaphase II | 100 | 49 (49) | Chromosomes migrating precociously to poles |
| | | 38 (38) | Chromosomes not lined up at the equatorial plate |
| Anaphase II | 12 | 8 (66.6) | Lagging chromosomes |
| | | 4 (33.3) | Chromatid bridges |
| Telophase II | 59 | 50 (84.7) | Lagging chromosomes |
| | | 2 (3.4) | Chromatid bridges |
| Metaphase/Anaphase | 15 | 15 | Asynchrony |
| Metaphase/Telophase | 6 | 6 | Asynchrony |
| Anaphase/Telophase | 10 | 10 | Asynchrony |
| Tetrad | 147 | 67 (43.5) | Micronucleus |
| Subtotal | 469 | 361 (76.9) | |
| Total | 850 | 656 (77.1) | |

295
296

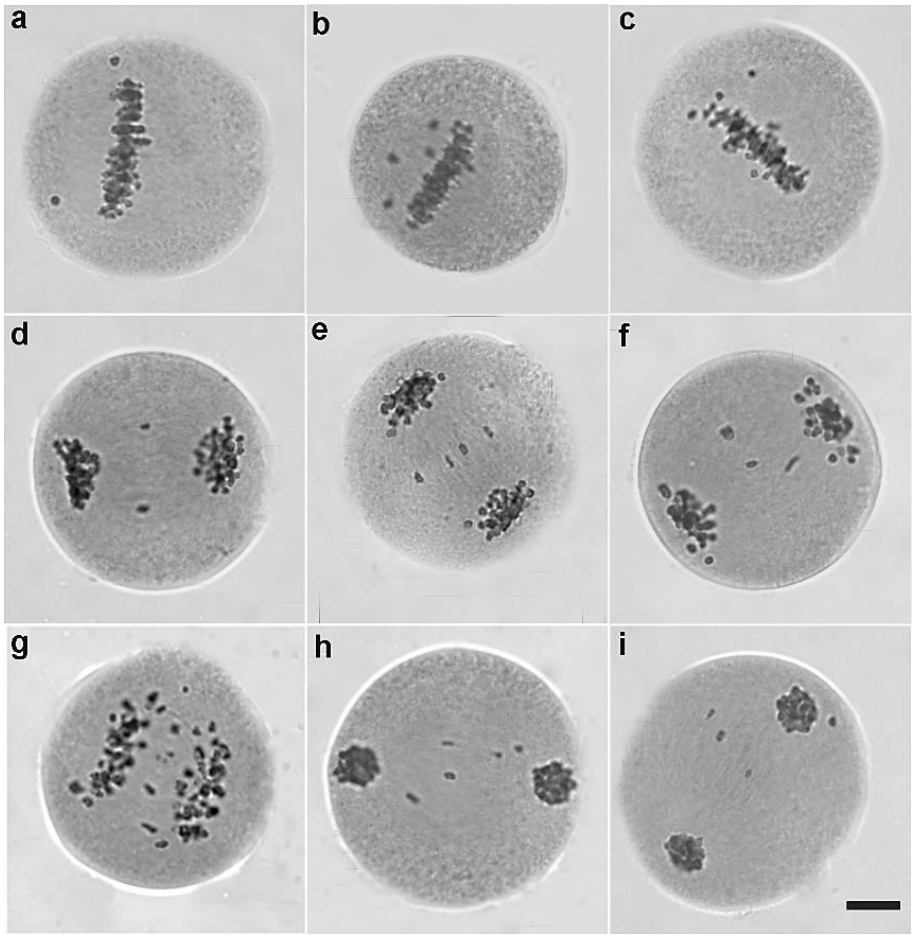


**Fig. 1** Microsporocytes of the SP80-3280 variety (*2n* = 112) during meiosis I exhibiting chromosomes not lined up at the equatorial plate (a-c), some exhibiting early segregation (b). Lagging chromosomes at anaphase I (d–g). Bridges at anaphase I support the existence of paracentric inversions (g). A telophase I showing lagging chromosomes (h). The absence of a bridge is a result of the position and number of crossovers taken place during the pachytene (i). Bar, 10 µm.

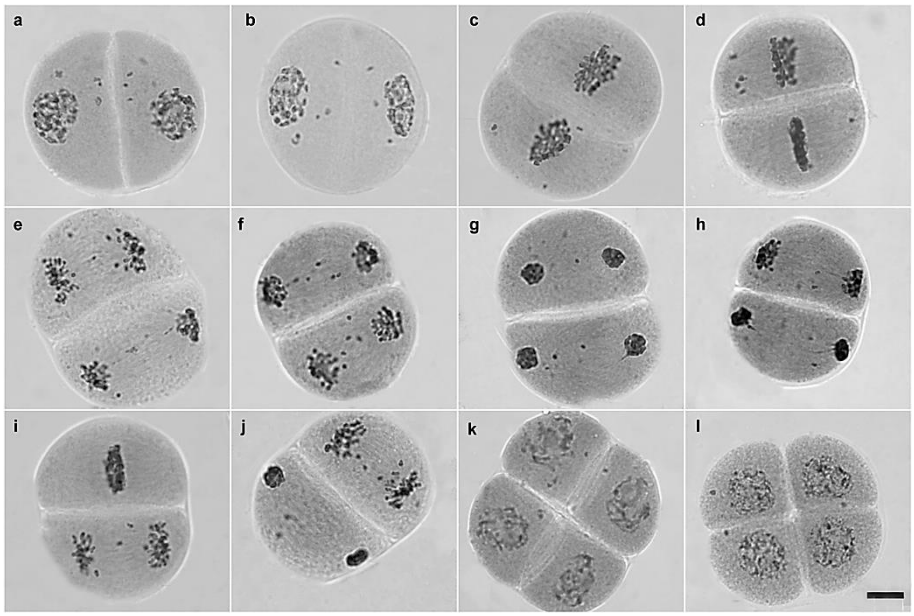


**Fig. 2** Microsporocytes of SP80-3280 (*2n* = 112) during meiosis II. Prophase cells exhibiting from 2 to 8 chromosomes not incorporated into the nuclei, randomly distributed throughout the cytoplasm (a-b). Chromosomes exhibiting early segregation (c-d). A tiny chromatid bridge at the bottom cell (e) and several lagging chromosomes at late anaphase II (f). Telophase II cells exhibiting evidence of resolved bridges g, h). Asynchronously dividing cells with lagging chromosomes (i-j). Tetrad cells exhibiting possible fragments not forming micronucleus (k). Typical micronucleus, two of them closer or inside the cell wall, indicating its non-inclusion in the final microspore (l). Bar, 10 µm.

**Chromosome association analysis using FISH and centromeric sequence validation**

With the aim of identifying chromosome associations at prophase I, we used FISH with centromeric probes. Intense fluorescent signals were observed in centromeric regions at diakinesis in Caiana Fita, SES205 and SP80-3280 (Table 3).

Ten cells were analyzed for each genotype. Most of the Caiana Fita cells exhibited 40 bivalents (Fig. 3 a-c); although bivalents (II) were also prevalent in SES205, one or two univalents (I) were observed in half the cells (Table 3; Fig. 3 d-f). With regard to SP80-3280, 56 bivalents were observed in just one cell, with bivalent chromosome associations prevalent at diakinesis, together with two to four univalents (Fig. 3, g-i), and even lagging chromosomes associated as bivalents (Fig. 3 j-l).

The centromeric-specific probes were also used to *in situ* hybridize anaphase I cells. For the first time in sugarcane, dicentric bridges have been identified (Fig. 4 a-f).

329 **Table 3** Chromosomal associations at diakinesis in cells of Caiana Fita, SES205 and SP80-3280

| **Cell** | **Caiana Fita (*2n* = 80)** | **SES205 (*2n* = 64)** | **SP80-3280 (*2n* = 112)** |
|---|---|---|---|
| 1 | 38II+2I | 30II+2I | 53II+3I |
| 2 | 40II | 30II+1I | 53II+3I |
| 3 | 40II | 32II | 54II+4I |
| 4 | 40II | 31II | 55II+2I |
| 5 | 40II | 31II+1I | 56II |
| 6 | 38II+2I | 21II+1I | 54II+3I |
| 7 | 39II+2I | 32II | 55II |
| 8 | 40II | 31II+2I | 52II+4I |
| 9 | 40II | 32II | 52II+2I |
| 10 | 40II | 32II | 55II+2I |

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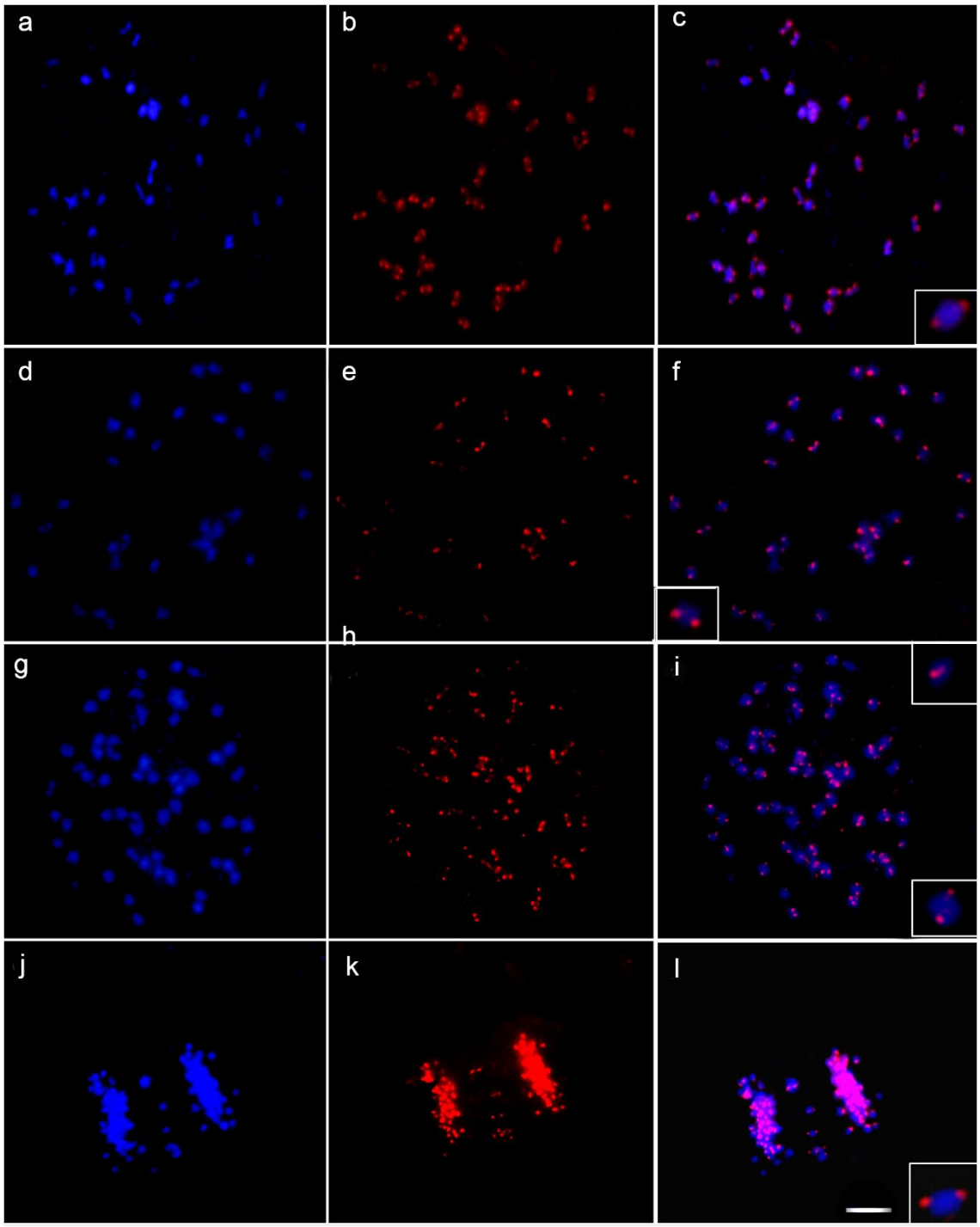


**Fig. 3** Fluorescent *in situ* hybridization of centromeric probes hybridized at diakinesis: Chromosomes of Caiana Fita stained with DAPI (blue) (a); Centromeric sites hybridized with the CENT probe detected with anti-DIG-rhodamine (red) (b); Merged images (a/b) showing 40 bivalents; the inset shows a typical bivalent (c); Chromosomes of SES205 stained with DAPI (blue) (d); Centromeric sites hybridized with the CENT probe detected with anti-DIG-rhodamine (red) (e); Merged images (d/e) showing 31II + 2I; (f) Chromosomes of SP80-3280 stained with DAPI (blue) (g); Centromeric sites hybridized with the CENT probe detected with anti-DIG-rhodamine (red) (h); Merged images (g/h) showing 56II + 1I chromosomes; the insets show a typical bivalent and univalent (i); Chromosomes of SP80-3280 at anaphase I stained with DAPI (blue) (j); Centromeric sites hybridized with the CENT probe detected with anti-DIG-rhodamine (red) (k); Merged images (j/k) showing lagging chromosomes at anaphase I (l). Bar, 10 µm.

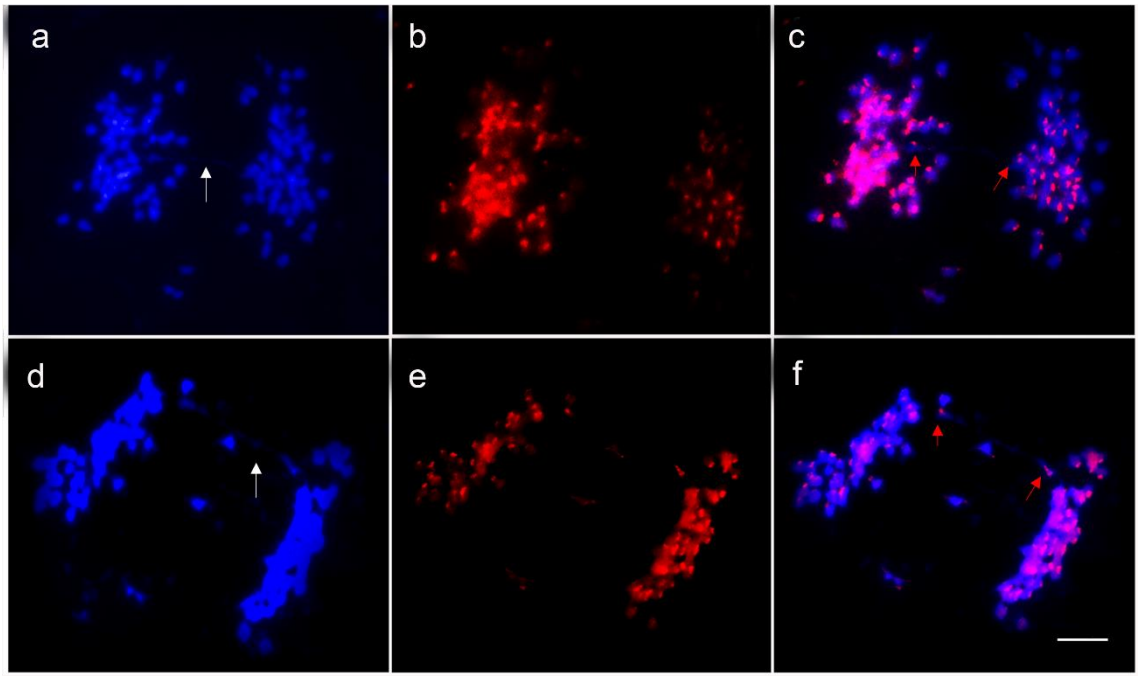


**Fig. 4**. Chromosome bridges (arrowed) in meiotic cells of the SP80-3280 variety: Anaphase I-cells stained with DAPI (a, d); Centromeric sites hybridized with the CENT probe detected with anti-DIG-rhodamine (red) (b, e); Merged images (a/b and d/e) exhibiting chromosome bridges; the arrows (in red) show dicentric chromosomes (c, f). Bar, 10 µm.

**Chromosome association in early prophase I cells of SP80-3280**

In order to enrich our analysis of chromosomal association, pachytene cells were also examined (Fig. 5 a-i). Due to the high number of chromosomes, it was not possible to trace with any certainty the individual chromosomes along their length. Sites of possible pairing partner switching were observed in pachytene cells, in which a chromosome may synapse with more than one partner simultaneously (Fig. 5 b, e-f). However, it was possible to identify some unpaired chromosomal segments (Fig. 5 c) suggesting a lack of homology. Additionally, centromeric and telomeric probes were used to hybridize pachytene chromosomes, allowing us to observe fluorescent signals consistently with $n = 56$ (Fig. 5 g-i).

We created a diagram to explain the inversion loop visualized (Fig. 6 a-c). For a pericentric inversion to occur, two breaks arise on opposite arms or sides of the centromere; the region between the breaks is inverted, and the ends are rejoined to the rest of the chromosome. The presence of a heterozygous inversion involves forming a loop to pair during meiosis (Fig. 6 d), so that the homologs can line up along their lengths (Fig. 6 e).

If just one crossover occurs within the inverted region, one chromatid will end up with the inverted region and the other will be normal. The two others will be unbalanced products.

Thus, the larger the inverted region, the greater the chance of producing aneuploid gametes, which may not be seen in offspring.

We also visualized chromosome dicentric bridges in some anaphase I and II cells. We created schemes to explain their origin (Supplementary Fig. 2; Supplementary Fig. 3). Undoubtedly, the presence of paracentric inversions is supported by the results. When two breaks in one chromosome arm rejoin after the excised piece has inverted, not including the centromere, this results in a paracentric inversion, and the incidence of two crossovers, one within and another outside the inverted segment, this results in a dicentric chromosome and an acentric fragment, which are not transmitted normally. Ultimately, the dicentric bridge will fragment somewhere along its length The existence of bridges (together with or without chromosome fragments) in the first division is a consequence of this type of rearrangement (Supplementary Fig. 2).

Bridges at anaphase II also confirm that a paracentric inversion did occur. A bridge in only one cell of the dyad indicates, as mentioned above, that two crossovers have happened, one inside and another outside the inverted segment, adjacent (Supplementary Fig. 2). Bridges in both cells of the dyad indicate that three crossovers occurred in the pachytene stage. All the four chromatids are involved, and two crossovers occur inside the inversion loop and one outside, adjacent. Consequently, two dicentric chromatids are formed. The presence of two acentric fragments can be observed, but not obligatorily (Supplementary Fig. 3). During the anaphase II both dicentric chromatids should be resolved, and bridges become evident in both dyad cells.

In both cases, the acentric fragments are not necessarily present. The fragment size depends on the segment length involved in the inversion and the position of the crossover inside the loop.

Alternatively, the presence of inverted duplicated segments and the formation of one crossover inside the loops could explain the excess of bridges at anaphase I. This type of rearrangement results in dicentrics that are resolved during anaphase I. No acentric fragments are formed in this case (Supplementary Fig.4).

In both peri- and paracentric inversions, crossovers taking place within the inversion loop generate duplicated/deficient gametes that may result in zygotic lethality. The presence of duplicated/deficient gametes is expected to translate into a reduction in fertility in inversion heterozygotes. This assumes that the inversion is sufficiently large to induce a probability of a crossover close to unity.

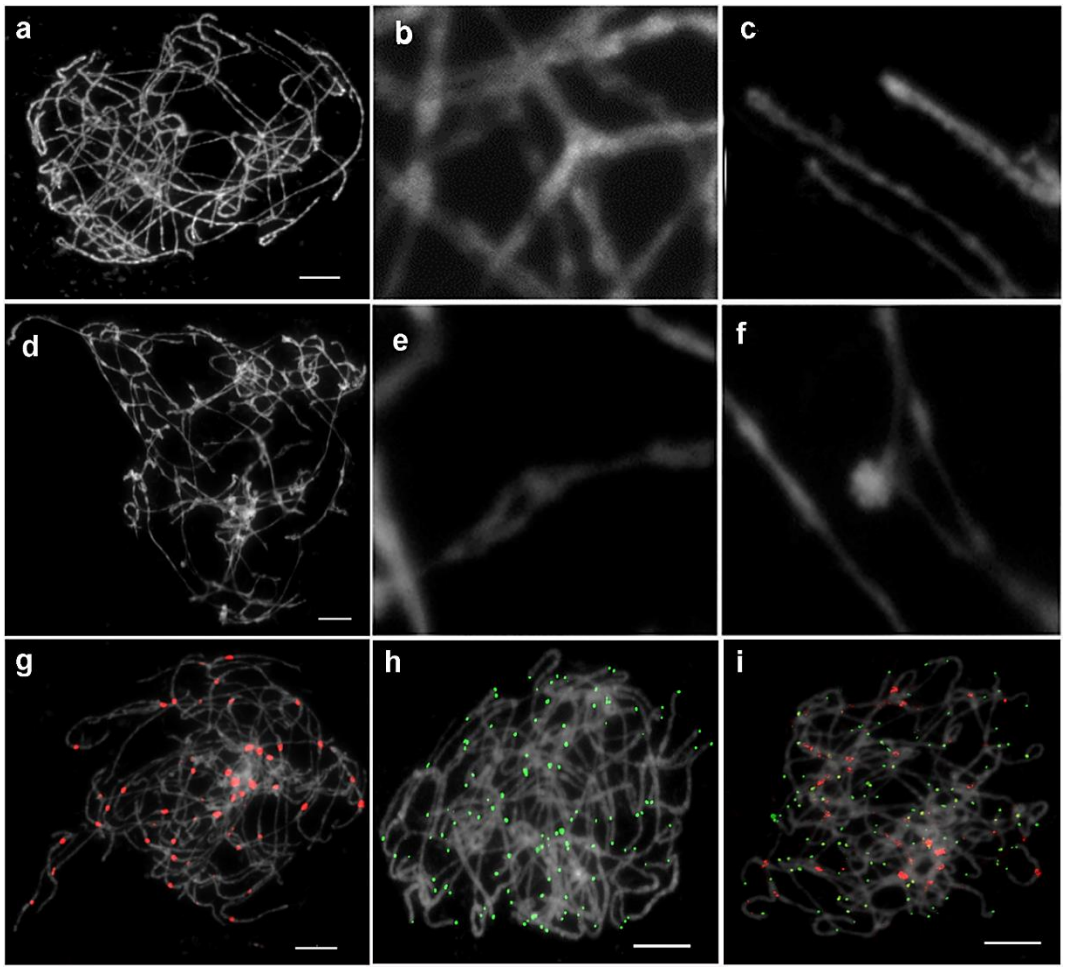


**Fig. 5** SP80-3280 typical pachytene cells (a, d, g-i). Possible sites of pairing partner switching (b, e-f) Unpaired chromosomal regions (c). Fluorescent *in situ* hybridization with centromeric probes detected with anti-DIG-rhodamine (red), indicating 56 centromere signals (g). Fluorescent *in situ* hybridizations with telomeric probes (green) (h). Centromere and telomere signals (i). Bar, 10 µm.

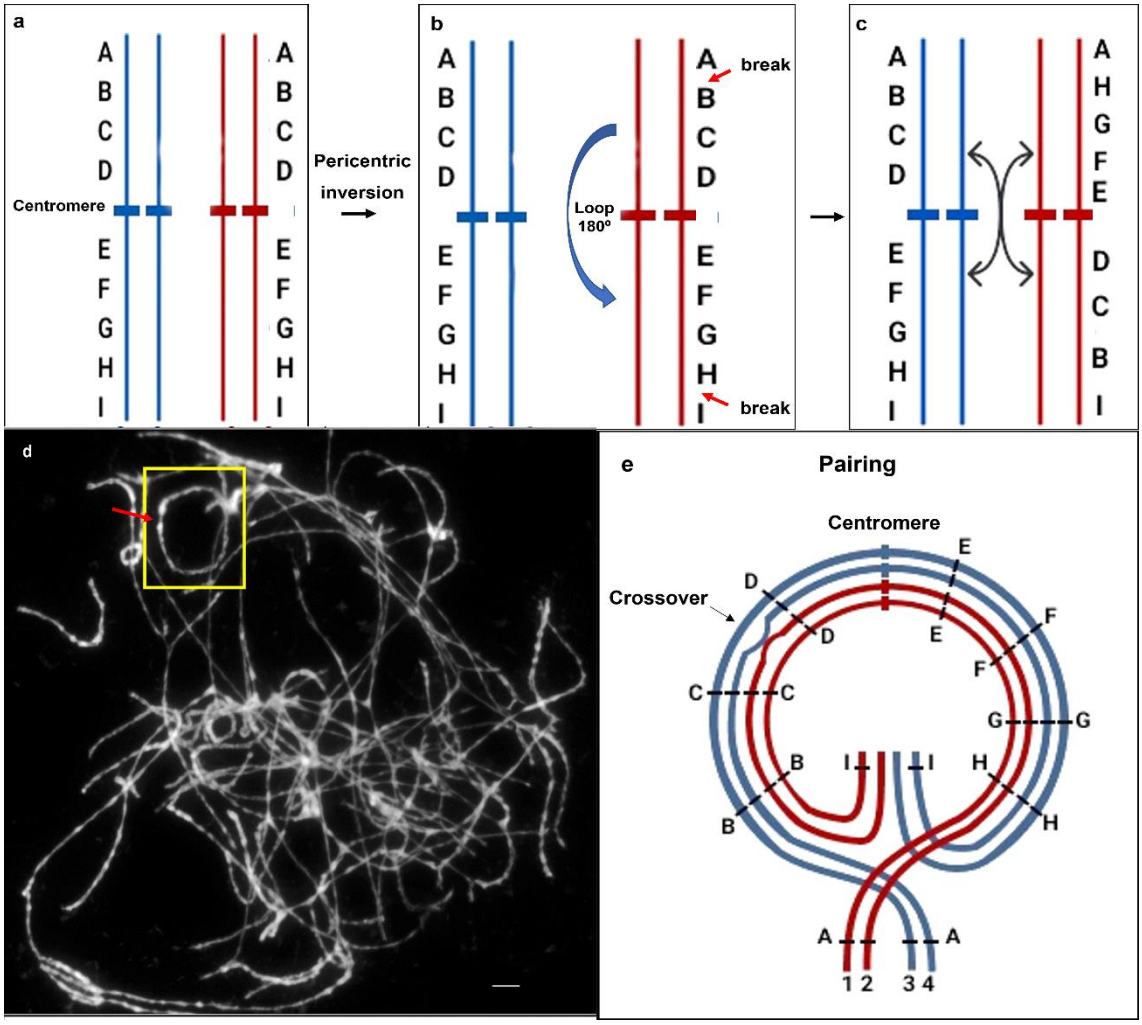


**Fig. 6** Schematic representation of a hypothetical pair of homologs (a): Two breaks occur on opposite sides of the centromere, and the region between the breaks is inverted, and the ends rejoined to the rest of the chromosome (b-c); An inversion loop in a SP80-3280 pachytene cell (yellow box); the red arrow indicates the centromere (d) (Bar 10 µm); Pairing during meiosis and loop formation so that homologs (or homeologs) can line up along their lengths (e).

## Chromosome composition of SP80-3280

Before performing GISH, we examined 23 SP80-3280 mitotic cells to determine their chromosome numbers. The modal value was $2n = 112$ chromosomes (Supplementary Fig. 5). The combination of cycloheximide and 8-hydroxyquinoline resulted in good chromosome spreads and accumulation of prometaphase and metaphase cells, confirming the effectiveness of this method for sugarcane chromosome counting and allowing us to proceed with GISH.

Herein, we describe Caiana Fita and SES205 genomic probe hybridization on SP80-3280 chromosomes. These *Saccharum* representatives were selected based on our current knowledge of the pedigrees of some commercial sugarcane varieties. For instance, Caiana Fita, a noble cane, was one of the first accessions of *S. officinarum* introduced into and cultivated in Brazil ($x = 10$,

$2n = 8x = 80$) (Figueiredo 2008). However, SES205 is a very divergent accession introduced from India and a representative of the wild species, *S. spontaneum* ($x = 8$, $2n = 8x = 64$) (see Medeiros et al. 2020).

We examined 10 cells, all with $2n = 112$ chromosomes. Eighty-nine (80%) chromosomes were entirely labeled in green and corresponded to *S. officinarum*; 13 (11%) were entirely labeled in orange corresponding to *S. spontaneum*; and 10 (9%) were labeled in green/orange, revealing their interspecific origins due to chromosome exchanges or recombination (Fig. 7). There were no non-hybridized chromosome regions.

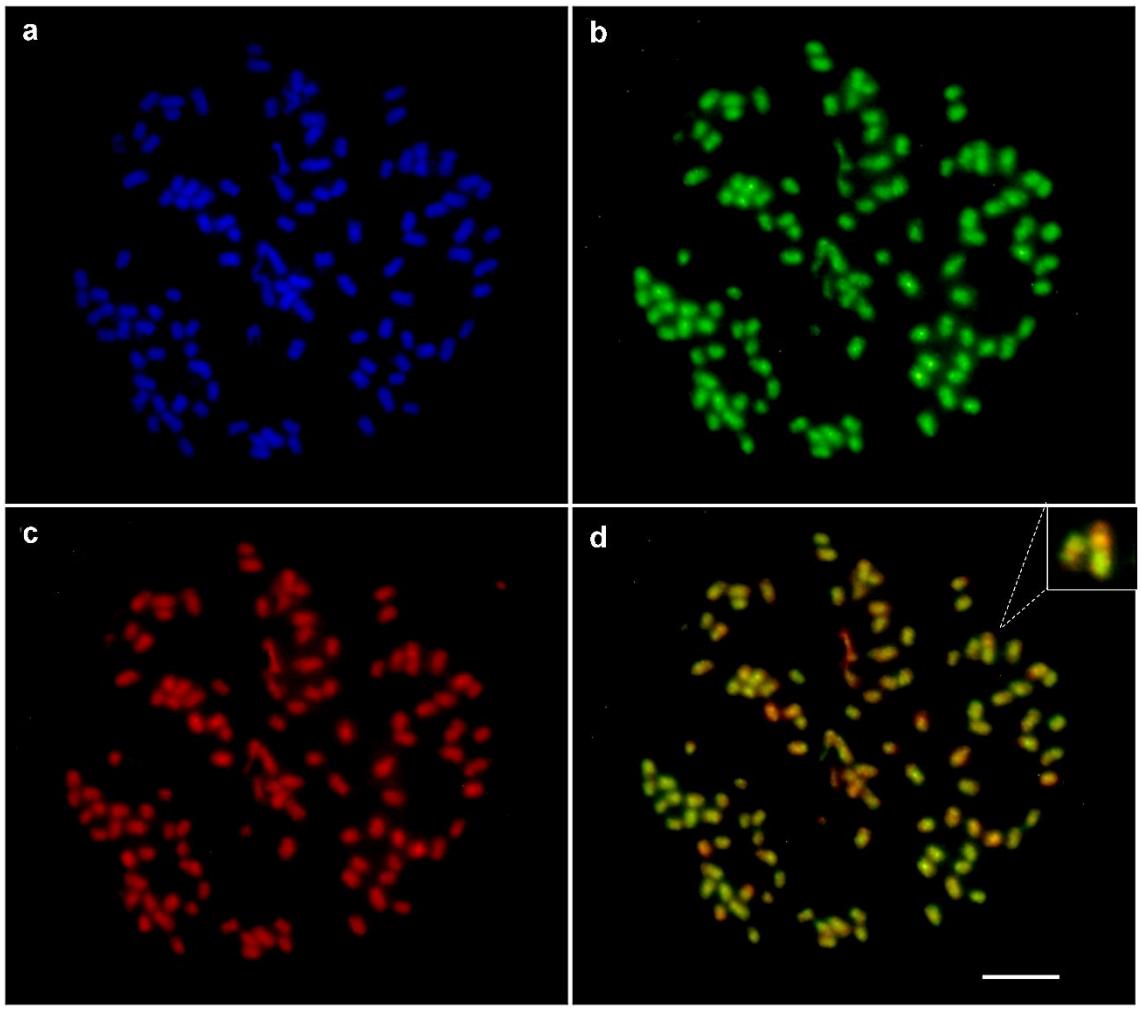


**Fig. 7** Genomic *in situ* hybridization of chromosome preparations of the SP80-3280 variety using labeled genomic DNA of Cana Fita (*S. officinarum*) and SES205 (*S. spontaneum*): Mitotic metaphase counterstained with DAPI (a); Yellow-green fluorescence (FITC) indicating hybridization with *S. officinarum* DNA (b); Red fluorescence (TRITC) indicating hybridization with *S. spontaneum* DNA (c); Merged images (b/c). The inset shows a typical recombinant chromosome (d). Bar, 10 µm.

## Discussion

The genus *Saccharum* is well-known for its exclusively higher-order polyploid species, such as *S. officinarum*, *S. robustum* and *S. spontaneum*, the latter two with variable chromosome numbers or cytotypes. In this study, we confirmed that *S. officinarum* exhibits a relatively low number of meiotic abnormalities (~6%), i.e., regular meiosis, similar to that of other polyploids in the grass family (Leofanti et al. 2017; Aissat et al. 2019; Risso-Pascotto et al. 2003). In contrast to our findings, these studies report abnormalities higher than 30%.

Approximately 50% of the cells of SES205 (representing *S. spontaneum*) exhibit abnormalities, including lagging chromosomes from metaphase I to subsequent phases. Moreover, we visualized some asynchronous cells, confirming the classic findings (Sreenivasan and Jagathesan 1975). The evolutionary history of the autopolyploid *S. spontaneum* may explain the meiotic chromosomal instability observed herein.

To clarify, *S. spontaneum* is a mixoploid species (see Zhang et al. 2018), with chromosome numbers in multiples of 8 ($2n$ = 40 to 128). According to the classic study by Panje and Babu (1960), the cytotypes should have a typical geographic distribution: in West Asia the numbers range from 2n = 112 to 128, in the East, $2n$ = 80 to 112 and in the Center, $2n$ = 40 to 80. Chromosome numbers of $2n$ = 64 are common in India, the origin of SES205.

Subsequently, a novel tetraploid accession (Np-X) that belongs to the ancient Pan-Malaysia group was found to have $2n$ = 40 and an unusual $x$ =10 (Meng et al. 2020). According to the authors, this finding suggests a parallel evolution pathway of genomes and polyploid series with different basic chromosome numbers. However, at the time, it was also proposed that rearrangements occurred from a basic chromosome of $x$ = 10 (probably in the Northern part of India) in two steps, leading to $x$ = 9 and then $x$ = 8 (descending disploidy). Each step involved three chromosomes that were rearranged to form only two. Further polyploidization led to wide geographical distribution of clones with $x$ = 8 (Piperidis and D´Hont 2020). Insertional dysploidy has been recorded in three grass subfamilies and appears to be the dominant mechanism of basic chromosome number reduction in grasses (Luo et al. 2009).

Despite the interspecific origin of modern varieties, bivalent pairing prevails, and this type of chromosome association has been documented in classic studies. Subsequently, using FISH on meiotic chromosomes of the IACSP93-3046 variety ($2n$ = 112), Vieira et al. (2018) confirmed the predominance of the bivalent configuration. Chromosomal abnormalities were visualized in approximately 70% of IACSP93-3046 meiotic cells, e.g., chromosomes not aligned to the equatorial plate, laggards and chromosomes not incorporated into telophase I nuclei, resulting in micronuclei at the end of division and explaining, at least in part, the origin of univalents.

Similarly, it was possible to identify a significant number of cells with irregularities (~77%) in the SP80-3280 variety, including asynchronous cells which were also present in SES205 but not in the representative of *S. officinarum*. Remarkably, the strategy of using centromeric probes proved to be critical for confirming the predominance of bivalents at diakinesis, including laggards at anaphase. It is not always possible to recognize lagging bivalents using conventional protocols.

In conclusion, our findings lend weight to the idea that stable chromosome segregation occurs in modern sugarcane varieties and this has implications, for instance, on linkage analysis. One may assume that sugarcane behaves as a diploid during meiosis. Our results suggest that a synapse regulatory mechanism exists in *Saccharum*, in which probable multivalent associations are resolved into bivalents towards the end of prophase I. This mechanism has been extensively researched and proven to exist in wheat and *Brassica* (Jenczewski et al. 2003; Nicolas et al. 2009; Riley and Chapman 1958; Griffiths et al. 2006; Rey et al. 2017; Rey et al. 2021). *B. napus* is an established allopolyploid species with good meiotic control (see Quezada-Martinez 2022). There are gene clusters responsible for regulating the progression of meiosis and the most promising candidate gene to play this role is thought to be *ZIP4* (reviewed in Soares et al. 2021). It has been suggested that *ZIP4* acts as a scaffold protein containing tetratricopeptide repeats (TPRs), facilitating the assembly of protein complexes and promoting homologous crossovers (Chelysheva et al. 2007; Shen et al. 2012).

With this in mind, we decided to observe the pachytene cells in SP80-3280. The pachytene is one of the most informative meiotic phases from a chromosomal standpoint. We were able to visualize several possible sites of pairing partner switching, in which a chromosome can synapse with more than one partner simultaneously. It is a common phenomenon in polyploids due to the occurrence of complex interactions and the pairing of three or more chromosomes starting simultaneously at different points along their length (see Choudhary et al. 2020). Our findings seem to show that this is corrected by the end of pachytene to produce bivalents.

In addition, inversion loops were observed as a result of pericentric inversions leading to unbalanced gametes, confirming a previous report. The Thai sugarcane KPS 01-01-25 cultivar exhibited pairing partner switches and a few small loops that point to inversions (though not typified), duplications or deletions (Thumjamras et al. 2016). No dicentric chromosomes derive from this type of inversion.

Some chromosomal bridges were visualized in anaphase I and telophase I cells of SP80-3280 and SES205 (Supplementary Fig. 6), but not in the representative of *S. officinarum*. Finally, we also detected bridges and other irregularities in the final stages of division. These bridges

originate due to the formation of dicentric chromosomes. Homolog pairing during meiosis in a paracentric inversion heterozygote is also maximized by the formation of an inversion loop. If a crossover happens within this loop, dicentric and acentric chromosomes are formed. The acentric fragment is lost during meiosis as it cannot be pulled to either pole due to the absence of the centromere. However, the chromosome bridge will be mechanically broken in a random place, some would lead to 100 % of unbalanced products. Bridges and fragments in the second meiotic division are not as common as they are in the first, but bridges without fragments occur more frequently at this stage (see Huang 2020). Finally, as first described by McClintock (1939), inverted duplications may also form a bridge configuration in anaphase I or II, depending on the position of the crossover.

Recent pioneering studies on mitotic chromosomes have established that the constitution of modern varieties consists of approximately 75 to 80% *S. officinarum*, 10 to 25% *S. spontaneum* and 10 to 15% recombinant chromosomes (D'Hont et al. 1996; Piperidis et al. 2010; Piperidis and D'Hont, 2020). However, using *S. spontaneum*-specific chromosome probes, Wang et al. (2022) reported unexpected proportions of interspecific recombinants (11.9 to 40.9%) in some cultivars. The above figures are corroborated herein, i.e., a respective 80, 11 and 9% of *S. officinarum*, *S. spontaneum* and recombinant chromosomes. Importantly, as recently reported in *Brassica* allohexaploids (Quezada-Martinez et al 2022), *Saccharum* hybrids and sugarcane modern varieties were able to tolerate multiple chromosome rearrangements over generations, despite the putative impact of these on meiosis.

It would be interesting, in the near future, to focus on immunolocalization of the axis and synaptonemal complex proteins in the parental contributors of sugarcane to characterize their role in stabilizing sugarcane meiosis. Future work could also focus on the pairing partner switches observed at pachytene that could be confirmed and visualized in greater detail, as was the case in *Arabidopsis arenosa* (Morgan et al. 2020). Finally, for the first time ever, we found that both types of inversions occur in sugarcane, and progress on sugarcane genome architecture decrypting may elucidate their chromosome localization.

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**Conflict of interest**

549 The authors declare no conflicts of interest.
550
551 **Data Availability Statement**
552 The authors affirm that all data necessary for confirming the conclusions of the article are present
553 within the article, figures, tables, and supplementary material.
554
555 **Authors' contribution**
556 GKO, NRS and ZPC carried out the experiments, including the preparation of the slides, DNA
557 extraction and preparation of genomic probes. GKO prepared the figures and tables and drafted
558 the manuscript. CBA has designed and made available the CENT probes for chromosome FISH.
559 RMM and ATM collaborate with the FISH experiments under the supervision of ERFM. MSC
560 provided the experimental material and information about. MM collaborated with the
561 interpretation of the data. MLCV conceptualized the study and has written the final version of the
562 manuscript.
563
564 **Ethics approval**
565 Not applicable.
566
567 **Consent to participate**
568 Not applicable.
569
570 **Consent for publication**
571 Not applicable.
572
573 **References**
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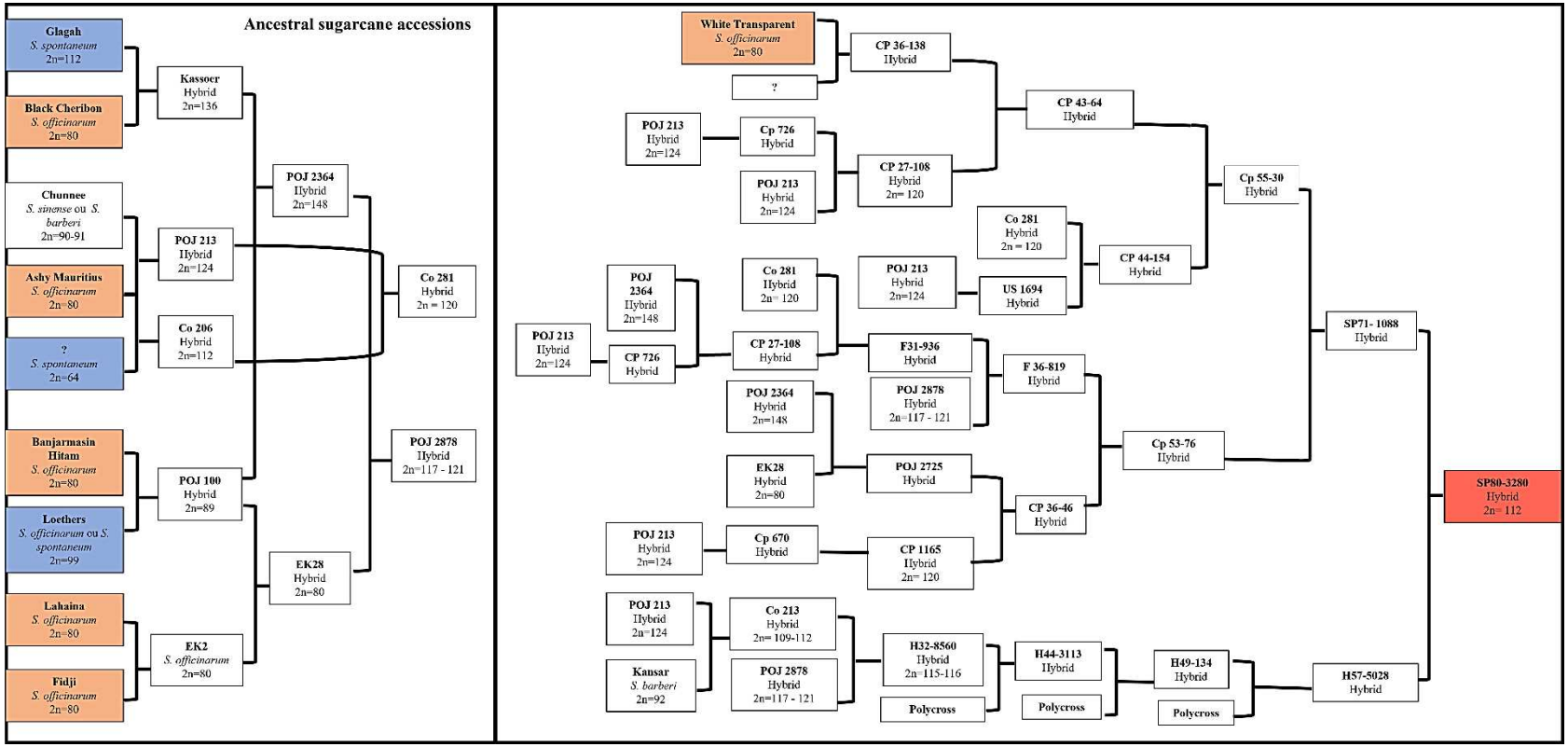
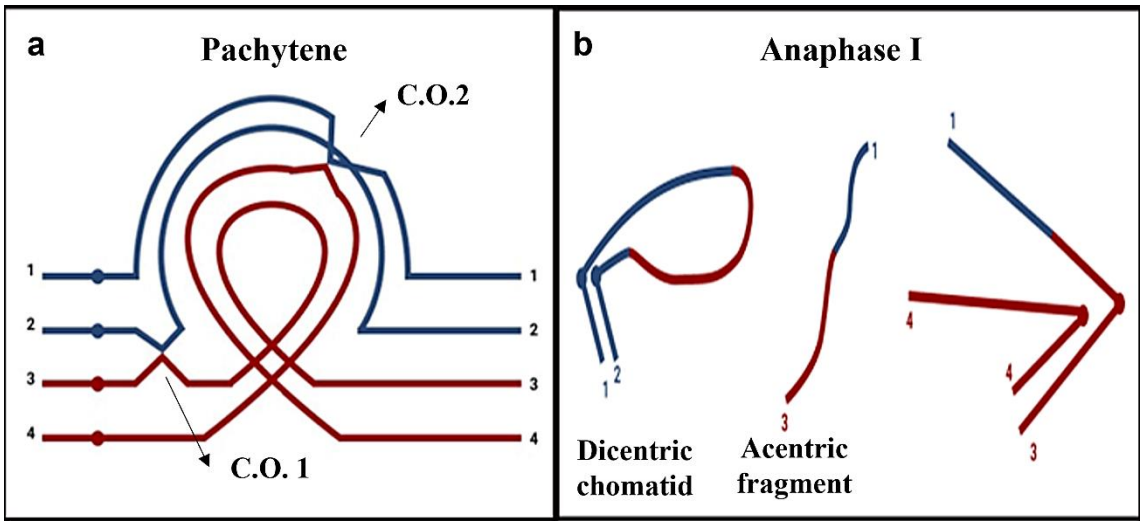
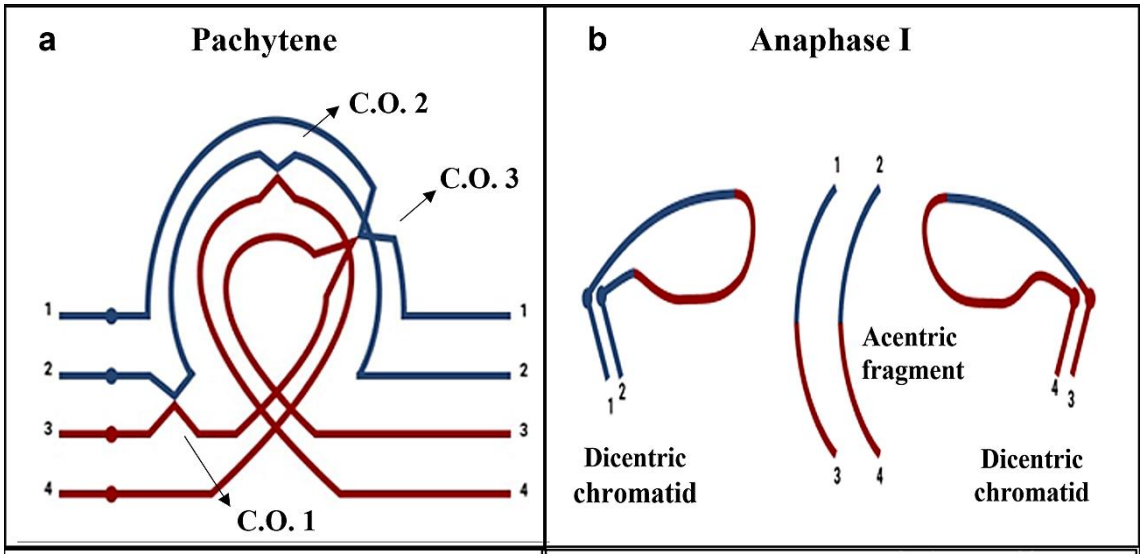
## Supplementary material



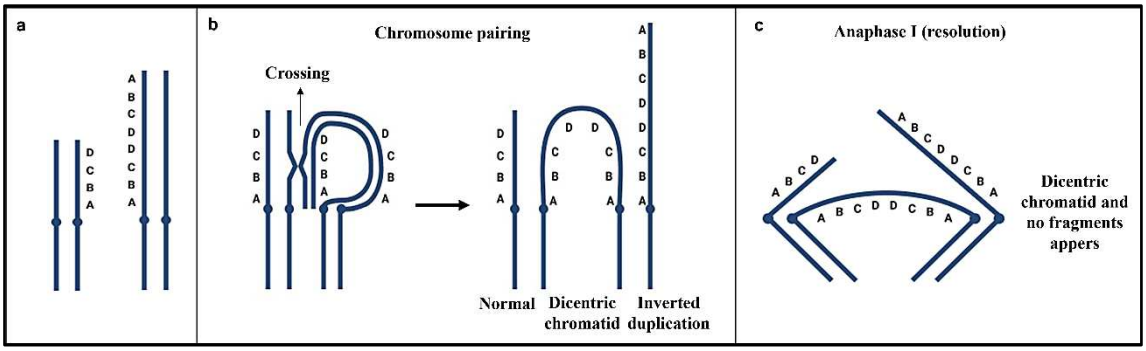

**Supplementary Fig. 1** The SP80-3280 pedigree. Ancestral sugarcane accessions Glagah, Black Cheribon, Chunee, Bendjermasin Hitam, Loethers, Fidji (cultivated in Java, Indonesia), White Transparent and Ashy Mauritius (cultivated in India). Representative clones of *S. officinarum* are highlighted in brown and those of *S. spontaneum* in blue. Kassoer is a spontaneous hybrid of Javanese *S. officinarum* (*2n* = 80) and *S. spontaneum* (*2n* = 112, the wild Glagah). EK2 and EK28, *S. officinarum* are cultivated by the indigenous people of Java. D74 is a *Saccharum* hybrid. POJ100, *S. officinarum* hybrid is a noble cane. POJ *Saccharum* hybrids are grown in Java (robust canes, immune to sereh disease). Co are early cultivars improved in Coimbatore, India. POJ2878 is found in the pedigrees of almost all the varieties grown worldwide. Many countries with breeding programs of their own continue to rely on Co clones. SP and IAC are Brazilian varieties (Modified from Sforça 2019).



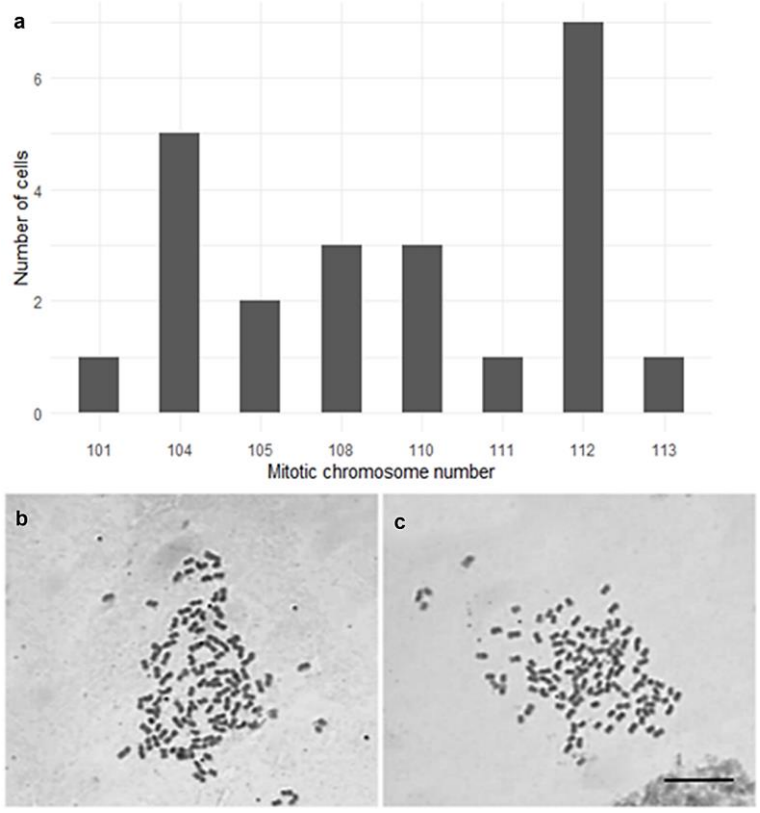

**Supplementary Fig. 2.** Schematic representation of a paracentric inversion in the pachytene and the formation of a dicentric chromatid in anaphase I. C.O. 1 and C.O. 2 are crossovers between the chromatids 2 and 3 (outside the inversion loop), and 1 and 4 (inside the loop), respectively (a); The resulting three chromatids, in late anaphase I, including one which produces a bridge, and an acentric fragment (b).



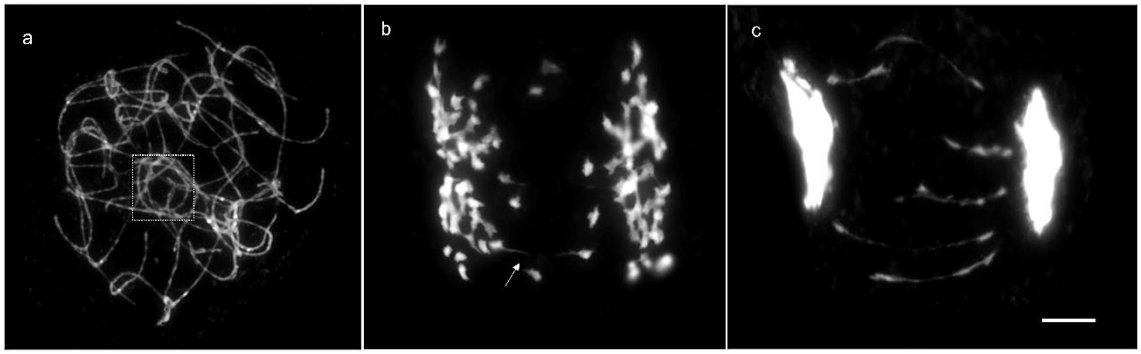

**Supplementary Fig 3.** Schematic representation of a paracentric inversion in the pachytene and the formation of two dicentric chromatids in anaphase I; C.O. 1 is a crossover between the chromatids 2 and 4, (outside the inversion loop); C.O. 2 and C.O. 3 are crossovers between the chromatids 2 and 4, and 1 and 3, both inside the inversion loop, respectively (a); The resulting dicentric chromatids and two acentric fragments in late anaphase (b). Both dyads will show bridges in anaphase II.




**Supplementary Fig. 4.** Alternative model proposed for the origin of a chromatid bridge. The occurrence of inverted duplications cannot be discarded in sugarcane. In this case, a recombined chromosome containing the inverted duplication may pair with the normal chromosome (a). Supposing that a crossover have occurred, a bridge will be formed in anaphase I, together with no fragments (b,c).




**Supplementary Fig. 5** The graph shows the distribution of chromosome counts in 23 mitotic cells of the SP80-3280, pretreated with 8-hydroxyquinoline (300 ppm) combined with cycloheximide (25 ppm) (a); Metaphases showing $2n = 112$ chromosomes (b-c). Bar, 10 µm.




**Supplementary Fig. 6** Meiotic cells of the *S. spontaneum* representative (SES205) stained with DAPI: A possible inversion loop in a pachytene cell (white box) (a); Anaphase I chromosome bridges (arrowed) (b-c). Bar, 10 µm.