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Sugarcane genome architecture decrypted with chromosome-specific oligo probes

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SUMMARY

Sugarcane (*Saccharum* spp.) is probably the crop with the most complex genome. Modern cultivars (2*n* = 100–120) are highly polyploids and aneuploids derived from interspecific hybridization between *Saccharum officinarum* (2*n* = 80) and *Saccharum spontaneum* (2*n* = 40–128). Chromosome-specific oligonucleotide probes were used in combination with genomic *in situ* hybridization to analyze the genome architecture of modern cultivars and representatives of their parental species. The results validated a basic chromosome number of *x* = 10 for *S. officinarum*. In *S. spontaneum*, 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. Each step involved three chromosomes that were rearranged into two. Further polyploidization led to the wide geographical extension of clones with *x* = 8. We showed that the *S. spontaneum* contribution to modern cultivars originated from cytotypes with *x* = 8 and varied in proportion between cultivars (13–20%). Modern cultivars had mainly 12 copies for each of the first four basic chromosomes, and a more variable number for those basic chromosomes whose structure differs between the two parental species. One–four of these copies corresponded to entire *S. spontaneum* chromosomes or interspecific recombinant chromosomes. In addition, a few inter-chromosome translocations were revealed. The new information and cytogenetic tools described in this study substantially improve our understanding of the extreme level of complexity of modern sugarcane cultivar genomes.

Keywords: *Saccharum*, chromosome-specific oligo FISH, GISH, polyploid, interspecific hybrid, chromosome rearrangements.

INTRODUCTION

Sugarcane is the number one crop by harvested tonnage, produces 80% of the world's sucrose, and has recently become a primary crop for biofuel production. Modern cultivars (*Saccharum* spp., 2*n* = 100–120) are highly polyploid, aneuploid and of interspecific origin with a genome complexity that exceeds that of all other crops.

Saccharum officinarum was cultivated until the end of the 19th century, together with *Saccharum barberi* and *Saccharum sinense*. *Saccharum officinarum* (2*n* = 80), the sugar-producing species (referred to as noble cane), is believed to have been domesticated from the wild species *Saccharum robustum* (2*n* = 60 or 80 and up to 200) in New Guinea possibly as far back as 8000 years ago. *Saccharum barberi* and *S. sinense* derived from natural interspecific hybridization most probably between *S. officinarum* and the wild species *Saccharum spontaneum* (D'Hont *et al.*, 2002). They were cultivated in India and China, and led to the emergence of sugar manufacturing (Daniels and Daniels, 1975).

Stimulated by disease outbreaks, the first man-made interspecific hybrids were produced in Java and India involving mainly *S. officinarum* and *S. spontaneum*. *Saccharum spontaneum* (2*n* = 40–128) is a wild species with a very large distribution from the Mediterranean to the Pacific. It has thin stalks, no or very low sugar, high vigor, and resistance to biotic and abiotic stresses. Restoration of a high sugar content was obtained through repeated backcrosses with *S. officinarum*. This procedure, called 'nobilization', was facilitated by 2*n* chromosome transmission to the F1 and BC1 Bremer (1961).

Interspecific hybridization provided a major breakthrough in sugarcane breeding, solving some of the disease problems, but also providing additional and

unexpectedly large benefits in increasing yield and improving ratooning ability and adaptability under various stress conditions (Roach, 1972). Modern sugarcane cultivars derived essentially from intercrossing of these first 'nobilized' hybrids that involved a small number of parental clones (Arceneaux, 1965; Price, 1965). They are propagated vegetatively. Given this genomic complexity, sugarcane genetics has lagged behind that of other major crops, and sugarcane breeding is still essentially based on conventional methods.

Past classical cytogeneticists studies have shown that all *Saccharum* species are polyploid, and that aneuploidy is frequent in this genus (for review, see Sreenivasan *et al*., 1987). The lowest ploidy level recorded is 4*x*, and is found only in *S. spontaneum*. Pairing behavior surprisingly showed mainly bivalents, but various types of meiotic irregularities, such as univalent and multivalent, were also found (Bremer, 1922; Price, 1963; Burner, 1991; Vieira *et al*., 2018). However, because all Saccharum species are highly polyploid and have small chromosomes of similar size both within and among species, information such as basic chromosome number and the precise behavior of wild chromosomes during introgression processes, including their exact contribution to present cultivars, could not be precisely established.

Molecular cytogenetics was developed in sugarcane in the 1990s, and has opened new avenues for genome structure analysis. The basic chromosome number in sugarcane and related germplasm had been actively debated for decades, and numbers of $x = 5$, 6, 8, 10 and 12 were proposed for the Saccharum species (for review, see Sreenivasan *et al*., 1987). Using fluorescent *in situ* hybridization (FISH) for physical mapping of two types of rRNA genes (5S and 45S), D'Hont *et al*. (1998) demonstrated a basic chromosome number of $x = 10$ in *S. officinarum* and *S. robustum*, and a basic chromosome number of $x = 8$ in *S. spontaneum*. Recently, Garsmeur *et al*. (2018) proposed, based on a dense single nucleotide polymorphism (SNP) genetic map of a cultivar, that this variation in basic chromosome number in *S. spontaneum* with $x = 8$, compared with *S. officinarum* with $x = 10$, resulted from two pairs of three chromosomes that were rearranged into two chromosomes.

The ability to differentiate by genomic *in situ* hybridization (GISH) chromosomes from the parental species in intergeneric and interspecific hybrids was then established (D'Hont *et al*., 1995) and used to characterize the interspecific genomic constitution of modern cultivars (D'Hont *et al*., 1996). GISH with total genomic DNA of *S. officinarum* and *S. spontaneum* performed on chromosome preparations of several modern cultivars uncovered 10–23% of entire *S. spontaneum* chromosomes and 8–13% of chromosomes resulting from exchanges between *S. officinarum* and *S. spontaneum* chromosomes (D'Hont *et al*., 1996; Piperidis and D'Hont, 2001; Cuadrado *et al*., 2004; Piperidis *et al*., 2010). Whether these exchanges occurred by translocations or by meiotic recombination was assessed by molecular genetic mapping of the cultivar R570 (Grivet *et al*., 1996; Hoarau *et al*., 2001). Several co-segregation groups included segments bearing *S. spontaneum*-specific markers, as well as segments bearing *S. officinarum*-specific markers showing that recombination occurred between the two parental genomes.

Several genetic maps have been developed in modern sugarcane cultivars (for review, see Zhang *et al*., 2014), and comparative genetic mapping with other Poaceae species revealed extensive genome-wide collinearity with Sorghum, which thus became a model for sugarcane (Dufour *et al*., 1997; Glaszmann *et al*., 1997; Guimarães *et al*., 1997). However, none of the current sugarcane maps is saturated, and thus to date it has been impossible to precisely outline the genome architecture of modern cultivars, in particular to determine the number of copies of each basic chromosome.

Molecular cytogenetics has been very important in the last 30 years in helping to understand the genome organization of plants (for review, see Jiang and Gill, 2006; Jiang, 2019), particularly those with complex genomes such as sugarcane (D'Hont, 2005; Piperidis *et al*., 2010). The availability of reference sequence genomes for many plants has allowed the design of synthetic oligonucleotide (oligo) probes for FISH applications a paradigm shift from the traditional FISH probes using cloned DNA sequences (Beliveau *et al*., 2012; Jiang, 2019). Oligo probes producing a unique barcode for each basic chromosome, as well as chromosome-specific oligo probes, have started to be used, in particular to establish karyotypes and to identify chromosome rearrangements (Braz *et al*., 2018; Albert *et al*., 2019; Song *et al*., 2020; Xi *et al*., 2020), and to study chromosome pairing at meiosis (Han *et al*., 2015; He *et al*., 2018; do Vale Martins *et al*., 2019).

Due to its genome complexity, sugarcane genome reference sequences have only recently become available. Garsmeur *et al*. (2018) assembled a monoploid genome reference sequence based on cultivar R570, and Zhang *et al*. (2018) assembled the sequence of the 32 chromosomes of a *S. spontaneum* haploid clone.

Here, we exploited the sequence assembly of Garsmeur *et al*. (2018) to design chromosome-specific oligo probes, and used them to analyze the genome architecture of some modern cultivars and their parental species.

RESULTS

Development of chromosome-specific oligo-based FISH probes in *Saccharum*

Based on the sugarcane reference genome sequence assembly of Garsmeur *et al*. (2018), that was assembled

into 10 basic chromosomes, we designed 10 oligo-based FISH probes, one for each assembled chromosome. Specific oligos were designed in regions regularly spaced along chromosomes arms, representing about 1.2 Mb for each chromosome.

These probes were tested on chromosome preparations from *S. officinarum*. Each of the 10 oligo probes painted both arms of eight (in one case nine) chromosome copies in *S. officinarum* with $2n = 8x = 80$ (or 81), and thus painted one basic chromosome set in this species (Figures 1a,e and S1a,b). These results validated the chromosome specificity of these probes in *S. officinarum* that will be further referred as probes P1–P10, corresponding to chromosomes 1–10 in *S. officinarum*.

The probes mainly painted the distal parts of the chromosomes as expected, as they were designed on chromosome arms. Some variation in the intensity of painting of individual chromosomes was sometimes observed, perhaps corresponding to technical artifact or to variation in the degree of homology between the various chromosome copies.

Genome architecture of *Saccharum officinarum*

In the clone Badila, we found 81 chromosomes instead of the $2n = 80$ previously reported for this clone (Bremer, 1924; Jagathesan *et al.*, 1970; Sreenivasan *et al.*, 1987), and each of the oligo probes painted both arms of eight chromosomes, with the exception of probe P9 that painted both arms of nine chromosomes (Figure 1a). Most *S. officinarum* clones have $2n = 80$ chromosomes, but some exceptions have been reported and were suggested to correspond to interspecific hybrids. We therefore performed GISH using *S. officinarum* and *S. spontaneum* total DNA as probes on chromosome preparations of Badila but we found no chromosomes intensively labeled by the *S. spontaneum* genomic probe, showing that this clone is not a hybrid between these two species (Figure S2a,b). This clone is thus aneuploid, with eight copies of each basic chromosome and one additional copy of chromosome 9 (Figure 2).

In the clone Black Innis, we found $2n = 80$ chromosomes as expected, and each of the oligo probes painted both arms of eight chromosomes (Figures 1e and S1a,b). This confirmed that this clone is octoploid with $2n = 8x = 80$. In addition, probe P5 painted a small chromosome segment on another chromosome, revealing a small translocation from chromosome 5 to one copy of chromosome 2 (Figures 2 and S2c,d).

Genome architecture of *Saccharum spontaneum*

The 10 oligo probes were used individually or in combination on chromosome preparations of five *S. spontaneum* accessions with different chromosome numbers.

Coimbatore is a *S. spontaneum* accession originating from India (Madras region) with $2n = 64$ chromosomes (Panje and Babu, 1960). In this accession, probes P1, P2, P3 and P4 each painted both arms of eight chromosomes (Figure S1c–f). Probes P5 and P6 each painted one arm and part of the second arm of eight chromosomes. Probe P9 painted the remaining distal parts of these 16 chromosomes (Figure 1b). Probes P7 and P8 each painted one arm and part of the second arm of eight chromosomes. Probe P10 painted the remaining distal parts of these 16 chromosomes (Figure 1f). We noted that two of the chromosome segments painted by probe P10 appear to be detached from the chromosomes labeled by probe P7 (Figures 1f and S3a). This can be explained by the fact that these *S. spontaneum* chromosomes carry an intercalary rDNA site that when de-condensed results in an apparent break of the chromosomes on some chromosome preparations (Figure S3b; D'Hont *et al.*, 1998). These results demonstrated a basic chromosome number of $x = 8$ in the *S. spontaneum* clone Coimbatore and the hexaploid level of this clone with $2n = 8x = 64$ (Figure 2).

Glagah is a *S. spontaneum* clone originating from Indonesia with a chromosome number of $2n = 112$ (Panje and Babu, 1960). In this accession, probes P1, P2, P3 and P4 each painted 14 chromosomes. Probes P5, P6, P7 and P8 each painted one arm and part of the second arm of 14 chromosomes (Figure 1d). Probes P9 or P10 painted the remaining parts of these chromosomes. One exception to this pattern was seen in one chromosome copy labeled exclusively with probe P5 (Figure 1d) that may result from a deletion or a translocation with an undetermined chromosome. These results demonstrated a basic chromosome number of $x = 8$ in the *S. spontaneum* Glagah, with the same basic chromosome organization as the one observed in Coimbatore but with a ploidy level of 14 with $2n = 14x = 112$ (Figure 2).

Mol5904 is a *S. spontaneum* clone originating from Molokai islands/New Guinea reported to have $2n = 80$ chromosomes (Panje and Babu, 1960). In this clone, we found 79 chromosomes. Probes P1, P2 and P4 each painted both arms of 10 chromosomes. Probe P3 painted both arms of nine chromosomes (Figure S1h). Probes P5, P6 and P8 each painted one arm and part of the second arm of 10 chromosomes. Probes P9 or P10 painted the remaining distal parts of these chromosomes (Figure 1h). P7 painted one arm and part of the second arm of eight chromosomes, P10 painted the remaining distal parts of these chromosomes. Another chromosome was painted by probe P3 and probe P7 resulting from a translocation between *S. spontaneum* chromosomes 3 and 7. Finally, another chromosome was painted by probes P3, P7 and P10, also resulting from a translocation between *S. spontaneum* chromosomes 3 and 7 (Figures 2 and S2e). These results demonstrated a basic chromosome number of $x = 8$ in the *S. spontaneum* Mol5904, with the same basic

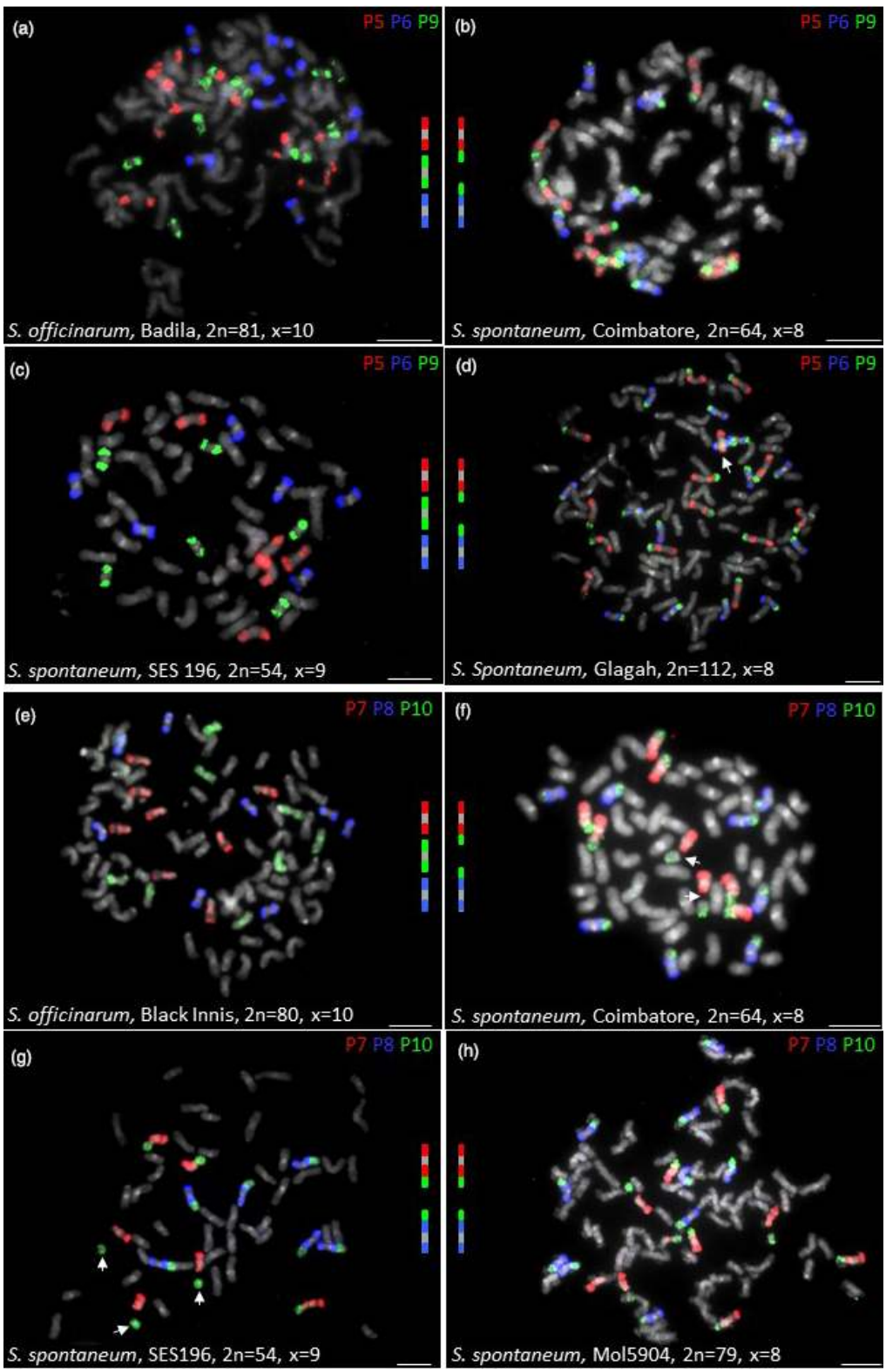

Figure 1. Fluorescent *in situ* hybridization (FISH) with chromosome-specific oligo probes on metaphase mitotic chromosomes.
Probe combinations and accession names are indicated on the pictures. Chromosomes were counterstained in 4′-6-diamidino-2-phenylindole (DAPI; displayed in gray). Schematic representations of the observed chromosome painting patterns are included. Arrow in (d) points to a peculiar copy of chromosome 5 (see text) P5. Arrows in (f and g) point to an apparent break in the chromosomes due to an intercalary rDNA site. Scale bar: 5 μm.

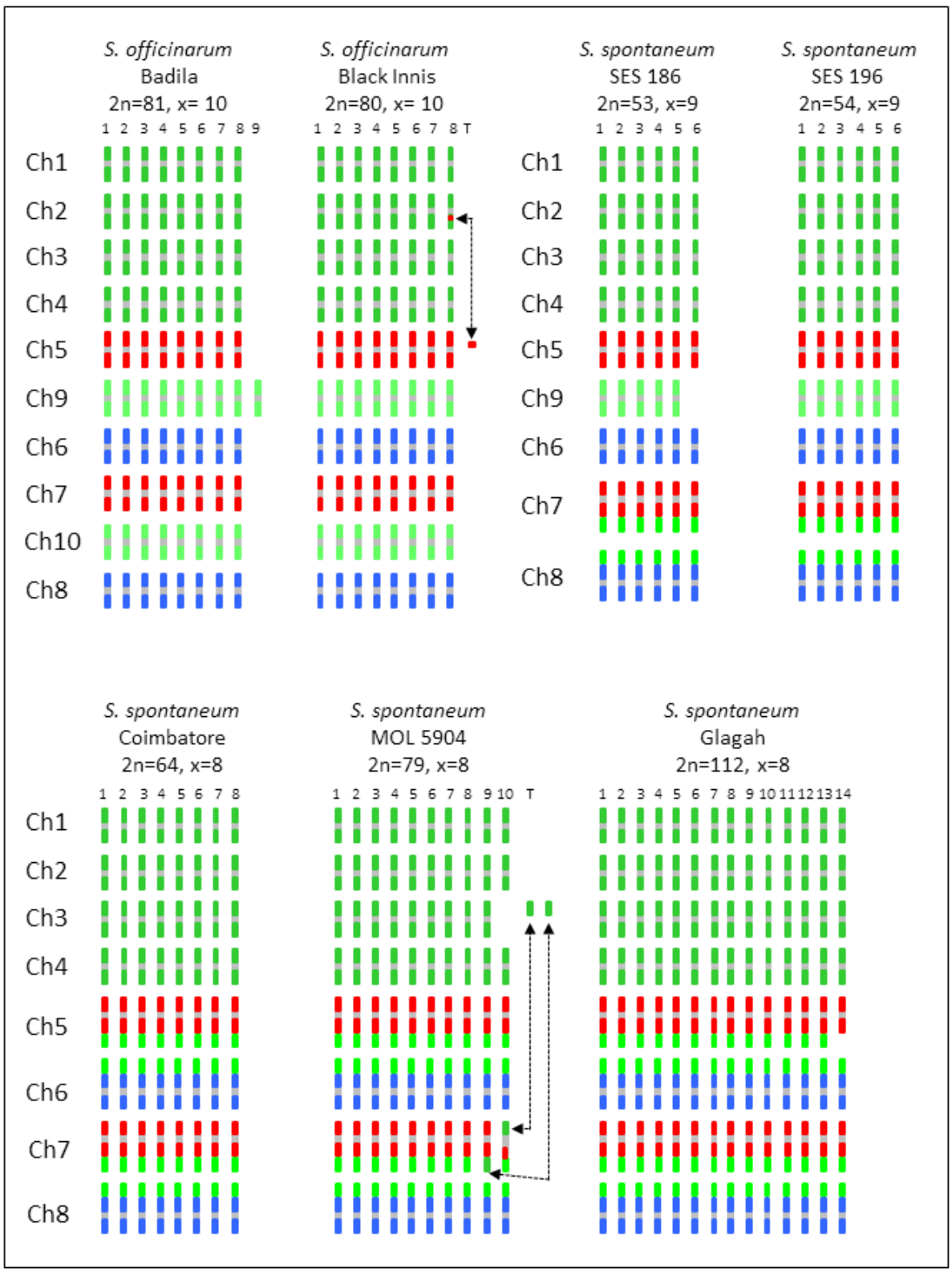

Figure 2. Karyotype schematic representations of the analyzed *Saccharum officinarum* and *Saccharum spontaneum* clones.
Each bar represents a chromosome, with colors corresponding to oligo probes used in Figure 1. Translocated chromosome segments are represented on the right, with black two-headed arrows linking the chromosome segments involved. The interspecific recombinant chromosome arms and translocated chromosome arms are positioned arbitrarily.

chromosome organization as the one observed in Coimbatore and Glagah but with a global ploidy level of 10. This clone has $2n = 10x = 79$ and thus is aneuploid. It also displayed two translocations with two undetermined chromosomes (Figure 2).

The results obtained on Coimbatore, Glagah and Mol5904 also validated that the variation in basic chromosome number in *S. spontaneum* with $x = 8$ compared with *S. officinarum* with $x = 10$ resulted from two pairs of three chromosomes that were rearranged in two chromosomes as proposed by Garsmeur *et al.* (2018).

SES 186 and SES 196 are *S. spontaneum* clones both originating from northern India (Orissa and West Bengal region, respectively) with reported chromosome numbers of 52 and 56, respectively (Panje and Babu, 1960).

In these clones, we observed 53 and 54 chromosomes, respectively. Probes P1, P2, P3, P4, P5, P6 and P9 each painted both arms of six chromosomes, except for probe P9 in accession SES 186, which painted both arms of five chromosomes (Figures 1c, 2, S1g and S2f). For both clones, probes P7 and P8 each painted one arm and part of the second arm of six chromosomes. Probe P10 painted the remaining distal part of these chromosomes (Figures 1g and 2).

These results revealed a basic chromosome number of $x = 9$ in these two *S. spontaneum* clones, with the same basic chromosome organization as the one observed in *S. officinarum* for chromosomes 1, 2, 3, 4, 5, 6, 9 and the same basic chromosome organization as in *S. spontaneum* with $x = 8$ for chromosomes 7 and 8. Therefore, SES 196 is a hexaploid with $2n = 6x = 54$. SES 186 is also globally hexaploid but lacks one copy of chromosome 9 and, thus, is an aneuploid with 53 chromosomes (Figure 2).

Genome architecture of modern cultivars

The 10 oligo probes were used individually or in combination on chromosome preparations of four modern sugarcane cultivars, R570, Q165, Q208 and Q209. The genome architecture of *S. officinarum* and *S. spontaneum* revealed by the oligo probes was used to help interpret the results, as modern cultivars combine chromosomes from both species. For cultivars R570, Q165 and Q208, GISH was performed as a second hybridization on the same slide after the FISH experiment with genomic DNA from *S. officinarum* and *S. spontaneum* to identify the specific origin of the chromosomes.

In cultivar R570, we counted 114 chromosomes from which GISH showed that 10 (9%) correspond to *S. spontaneum* chromosomes and 10 (9%) to interspecific recombinants chromosomes (Figures 3 and 4). We revealed 12 copies for chromosomes 1 and 4, with one–four copies corresponding to *S. spontaneum* or interspecific recombinant chromosomes. We revealed nine copies of *S. officinarum* chromosomes 5, 6 and 9, two copies of *S. spontaneum* chromosome 6, and three copies of *S. spontaneum* chromosome 5, of which two copies corresponded to interspecific recombinants. We revealed 10 copies of *S. officinarum* chromosomes 7, 8 and 10, and two copies of *S. spontaneum* chromosomes 7 and 8, one of them corresponding to an interspecific recombinant. In addition, we detected a translocation from a segment of *S. spontaneum* chromosome 5 to one copy of *S. officinarum* chromosome 8 (Figures S2g and 4).

In Q165, we counted 112 chromosomes, of which GISH showed that 17 (15%) correspond to *S. spontaneum* chromosomes and 10 (9%) to interspecific recombinant chromosomes (Figure 4). We revealed 12 copies for chromosomes 1 and 4, and 11 and 10 copies for chromosomes 2 and 3, respectively. Two–four of these chromosomes 1–4 corresponded to *S. spontaneum* or interspecific recombinant chromosomes. We revealed eight copies for *S. officinarum* chromosomes 6, 8, 9 and 10, and nine copies for *S. officinarum* chromosomes 5 and 7. We also identified three copies of *S. spontaneum* chromosomes 5, four copies of *S. spontaneum* chromosome 6, with one corresponding to an interspecific recombinant, and four copies of *S. spontaneum* chromosomes 7 and 9, with one corresponding to an interspecific recombinant. A small segment of chromosome 9 appeared translocated to one copy of chromosome 2. Finally, one segment of chromosome 2, two segments of chromosome 3 and one segment of chromosome 8 appeared translocated to other undetermined chromosomes. Because we counted 112 chromosomes for this cultivar, it is expected that the four translocated chromosome segments observed in this cultivar correspond each to part of two chromosomes.

In Q208, we counted 110 chromosomes from which GISH showed that 18 (16%) correspond to *S. spontaneum* chromosomes and seven (6%) to interspecific recombinant chromosomes (Figure 4). We revealed 11 copies for chromosome 1, and 12 copies for chromosomes 2, 3 and 4, of which three–four corresponded to *S. spontaneum* or interspecific recombinant chromosomes. One segment of *S. officinarum* chromosome 1 appeared translocated onto one copy of chromosome 6. We revealed 10, nine and eight copies for the *S. officinarum* chromosomes 5, 6 and 9, respectively, and two copies of *S. spontaneum* chromosomes 5 and 6. One of the copies of *S. spontaneum* chromosome 5 corresponded to an interspecific recombinant. We revealed 10, seven and eight copies for *S. officinarum* chromosomes 7, 8 and 10, respectively, and three and four copies of *S. spontaneum* chromosomes 7 and 8, respectively. One of the copies of *S. spontaneum* chromosome 8 corresponded to an interspecific recombinant. One copy of *S. spontaneum* chromosome 8 appeared

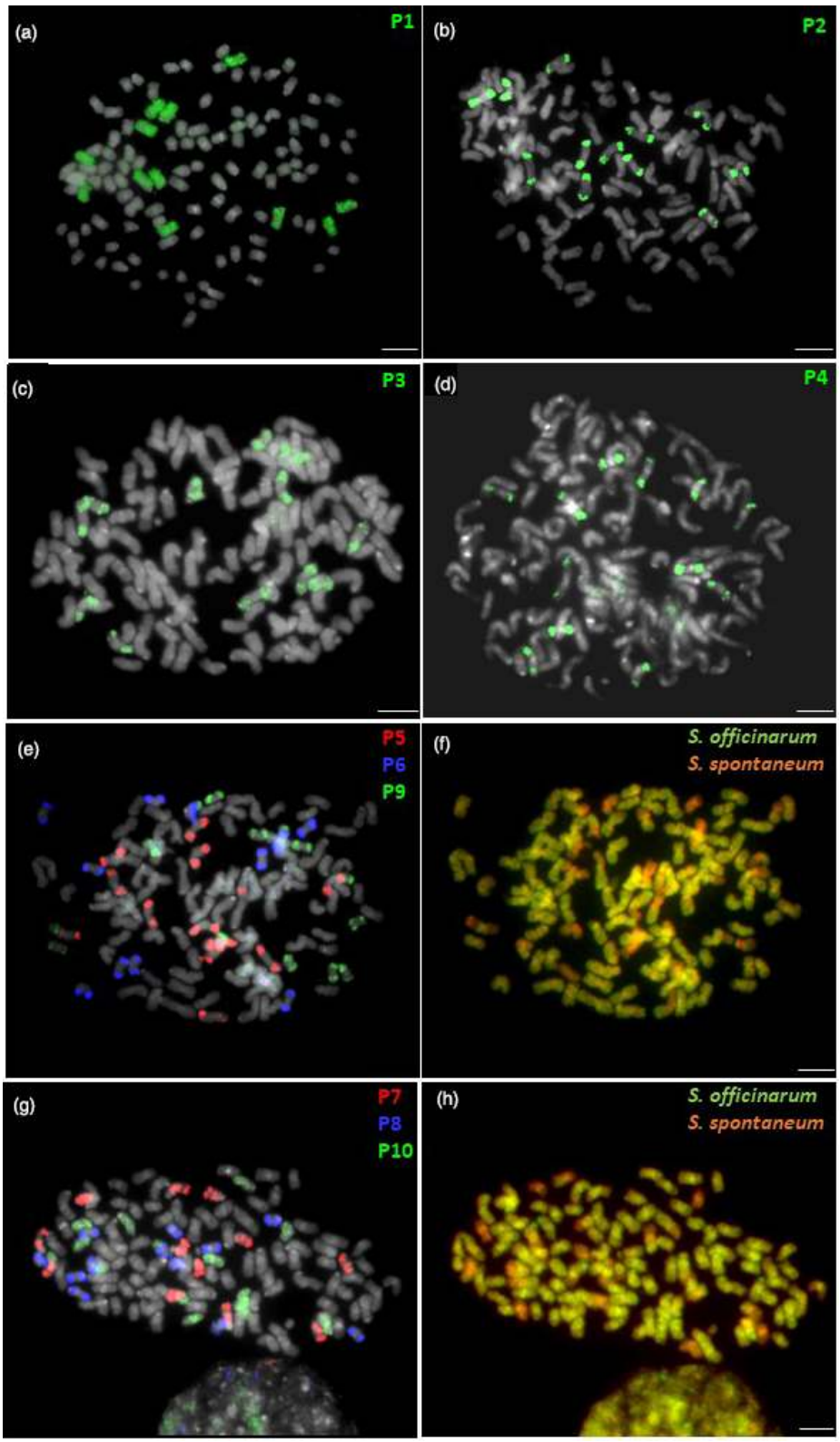

Figure 3. (a–e, g) Fluorescent *in situ* hybridization (FISH) on metaphase mitotic chromosomes of cultivar R570 with chromosome-specific oligo probes. Probe combinations are indicated on each picture. Chromosomes were counterstained in 4′-6-diamidino-2-phenylindole (DAPI; displayed in gray).
(f and h) Genomic *in situ* hybridization (GISH) with *Saccharum officinarum* and *Saccharum spontaneum* total DNA detected in green and orange, respectively, and performed as a second hybridization on the (e, g) metaphase cells. Scale bar: 5 μm.

Figure 4. Karyotype schematic representation of the analyzed cultivars.
Each bar represents a chromosome. On the left side, colors correspond to oligo probes used in Figure 4. On the right side, *Saccharum officinarum* chromosomes are represented in green and *Saccharum spontaneum* chromosomes in orange based on estimation from genomic *in situ* hybridization (GISH) results. No GISH was performed for Q209. Translocated chromosome segments are represented on the right, with black two-headed arrows linking the chromosome segments involved, when known. The interspecific recombinant chromosome arms and translocated chromosome arms are positioned arbitrarily.

truncated; this may have resulted from a translocation with an undetermined chromosome or from an interspecific recombination.

In Q209, we counted 107 chromosomes. We revealed 10–12 copies for chromosomes 1–4, and part of two copies of chromosome 2 that are translocated onto two undetermined chromosomes (Figure 4). We identified seven–nine copies of chromosomes 6–10, which we attributed to *S. officinarum* given their structures, and three copies that we attributed to *S. spontaneum* 5–8 given their structures. Finally, one segment of chromosome 5 appeared translocated to another undetermined chromosome. Because we counted 107 chromosomes for this cultivar, we consider that two of the translocated chromosome segments observed in this cultivar corresponded to part of one chromosome.

DISCUSSION

We analyzed the genome architecture of modern sugarcane cultivars and representatives of the two species from which they derived, *S. officinarum* and *S. spontaneum*, using chromosome-specific oligo probes. These probes were designed using the BAC-based sugarcane reference sequence of Garsmeur *et al.* (2018). The scaffolds (BAC sequences) of this reference sequence were assembled in 10 *S. officinarum* basic chromosomes based on the previously reported synteny conservation between sugarcane and sorghum (Dufour *et al.*, 1997; Guimarães *et al.*, 1997;

Ming *et al.*, 1998; Le Cunff *et al.*, 2008). One probe was designed for each of the 10 assembled chromosomes.

We showed that each of the 10 oligo probes painted both arms of eight chromosome copies in *S. officinarum* with $2n = 80$, and thus painted one basic chromosome set in this species. These results validated the chromosome specificity of these probes in *S. officinarum*, and confirmed at the genome scale the global synteny conservation between *S. officinarum* and sorghum. This synteny conservation has been previously assessed only based on genetic mapping, with genetic maps not saturated (Dufour *et al.*, 1997; Guimarães *et al.*, 1997; Ming *et al.*, 1998; Le Cunff *et al.*, 2008) due to the complexity of the genome.

The sugar-rich group of *S. officinarum* displays mainly $2n = 80$ chromosomes, and is thought to have been domesticated in New Guinea from the wild species *S. robustum* ($2n = 60$, 80 and up to 200; Brandes, 1956; for review, see Sreenivasan *et al.*, 1987). For *S. robustum* and *S. officinarum*, a basic chromosome number of $x = 10$ has been proposed based on it being the most common number in the Andropogoneae tribe (Bremer, 1961) and that the major cytotypes ($2n = 80$ or 60) more likely represent euploid forms (Bremer, 1961). This assumption was supported by physical mapping of two types of rRNA genes (5S and 45S) using FISH on a few *S. officinarum* and *S. robustum* clones (D'Hont *et al.*, 1998). The two *S. officinarum* clones we analyzed displayed eight (nine in one case) copies of 10 chromosomes confirming a basic number of $x = 10$. *Saccharum officinarum* has thus kept a basic chromosome number of $x = 10$ that is recognized as being ancestral in the Andropogoneae tribe.

Eight copies of each chromosome were found in the two *S. officinarum* clones analyzed, confirming a global octoploid level; an exception was one chromosome (Ch9) in clone Badila that had nine copies. Clones from the species *S. officinarum* were reported to mainly have $2n = 80$, with some exceptions in the range of (78–120) (for review, see Sreenivasan *et al.*, 1987). These exceptions were suggested to correspond to atypical aneuploids, for clones with minor deviation or, for larger deviations, to interspecific hybrids with *S. spontaneum* (Bremer, 1924; Price, 1960; Jagathesan *et al.*, 1970; Sreenivasan *et al.*, 1987), and this was demonstrated in a few cases using GISH (Piperidis *et al.*, 2010). We found no trace of interspecific hybridization in the Badila clone we analyzed; it thus appears to be a pure *S. officinarum* with an aneuploidy chromosome number of $2n = 81$.

The wild species *S. spontaneum* has a wide geographical distribution from the Mediterranean to the Pacific and shows great phenotypic variation. This species also has a wide range of chromosome numbers from $2n = 40$–128, the most frequent being in decreasing order 64, 112, 80, 128, 96, 120, 60 and 54 (Panje and Babu, 1960). Basic chromosome numbers of $x = 5$, 6, 8, 10 and 12 have been proposed by classical cytogeneticists based on chromosome counts and chromosome pairing observations, with $x = 8$ and $x = 10$ being the most plausible as multiples of eight and to a lesser extent multiples of 10 were the most frequent (for review, see Sreenivasan *et al.*, 1987). A basic chromosome number of $x = 8$ was demonstrated in six *S. spontaneum* clones with $2n = 64$, 80, 96 or 112 by physical mapping of two types of rRNA genes (5S and 45S) using FISH (D'Hont *et al.*, 1998; Ha *et al.*, 1999).

Garsmeur *et al.* (2018) proposed, based on a high-density SNP genetic map of a modern cultivar, that the rearrangements between the basic chromosome of $x = 10$ in *S. officinarum* and the basic chromosome of $x = 8$ in *S. spontaneum* consisted of two cases where three chromosomes were rearranged into two chromosomes. This scenario was then supported by the genome sequence assembly of a haploid *S. spontaneum* clone SES 208 that was assembled in 32 chromosomes corresponding to four copies of eight basic chromosomes (Zhang *et al.*, 2018).

For three of the *S. spontaneum* clones we analyzed, we revealed a basic chromosome number of $x = 8$ with architecture corresponding to that proposed by Garsmeur *et al.* (2018). These clones were Coimbatore, originating from southern India (Madras region), Glagah from Indonesia/Java, and Mol5904 from Molokai islands.

Recently, Meng *et al.* (2019) identified one *S. spontaneum* clone (Nepal-X) having a basic chromosome number of $x = 10$ with $2n = 4x = 40$ chromosomes using oligo-FISH-barcode probes. This clone showed a global chromosome architecture conserved with sorghum and by extension with *S. officinarum*, as synteny is globally conserved between *S. officinarum* and sorghum.

Here we identified, for the first time, *S. spontaneum* clones (SES 186 and SES 196) with a basic chromosome number of $x = 9$. In these clones, we showed that seven chromosomes had a global structure identical to seven *S. officinarum* chromosomes, and that two chromosomes had a distinct structure compared with *S. officinarum* but identical to the one observed in *S. spontaneum* with $x = 8$. Thus, *S. spontaneum* with $x = 9$ has one rearrangement in common with *S. spontaneum* with $x = 8$, and could represent an intermediate step between $x = 10$ and $x = 8$.

These results suggest that, after its divergence from the lineage of *S. officinarum* and its proposed wild ancestor *S. robustum,* rearrangements occurred in the *S. spontaneum* lineage involving cytotypes with $x = 10$ that through an intermediate step of $x = 9$ led to cytotypes with $x = 8$. Identifying the mechanisms involved will require further investigation, but could imply two successive reciprocal translocations, as reported in Brassicaceae and Poaceae (Mandkov *et al.*, 2010; Wang and Bennetzen, 2012).

This also suggests that polyploidization steps occurred independently in *S. officinarum/S. robustum* and *S. spontaneum* lineages after their divergence about 1.5–3.5 Mya; Jannoo *et al.*, 2007; Vilela *et al.*, 2017; Zhang *et al.*, 2018;

Garsmeur *et al.*, 2018), or if we consider that a change in basic chromosome number can occur at a tetraploid level, one of these steps of polyploidization from 2*x* to 4*x* may have occurred before the divergence of *S. officinarum*/*S. robustum* and *S. spontaneum* lineages.

Panje and Babu (1960) analyzed chromosome numbers in a very large sample of 442 *S. spontaneum* clones, and proposed a subdivision into three groups corresponding to three main geographical sectors. The first sector corresponded to a western sector (Africa, Mediterranean) predominantly with clones $2n = 112$, 120 and 128 that are all multiples of eight. The second group corresponds to an Eastern sector (South East Asia and Pacific) with mainly $2n = 80$, 96 and 112 clones. We analyzed two clones from this region in the present study, Glagah from Indonesia and Mol5904 from Molokai islands, and showed that both had a basic chromosome number of $x = 8$ with $2n = 14x = 112$ and $2n = 8x = 79$, respectively. D'Hont *et al.* (1998) analyzed clones from this sector with $2n = 80$, 96 and 112, and also found a basic chromosome number of $x = 8$. The third central sector corresponds to the Indian subcontinent (including Nepal, east and west Pakistan, and Sri Lanka) and contains the broader range of chromosome numbers from 40 to 80 with mainly $2n = 64$, the larger number of aneuploid forms and clones with the lowest chromosome numbers. The clone identified by Meng *et al.* (2019) with $x = 10$ and $2n = 4x = 40$, and the one we identified with $x = 9$ and $2n = 6x = 54$, 53, all came from the northern part of this central sector. Most of the clones with low chromosome numbers and $x = 10$ or $x = 9$ are found in this region, which also includes clones with high chromosome numbers multiples of $x = 8$ (Panje and Babu, 1960). This region, that covers the sub-Himalayan foothills and the alluvial plains of the Ganges, seems thus to correspond to a region of evolution of *S. spontaneum*. *Saccharum spontaneum* clones with low chromosome numbers are much less frequent than clones with high chromosome numbers, and are much restricted in their geographic distribution. High polyploidization seems to have been an important factor in the expansion of this species, which has been reported in some cases as being invasive (Westbrooks and Miller, 1993; Hammond, 1999; Grivet *et al.*, 2006). Polyploidization can provide increased adaptability to harsher conditions in novel environments (for review, see Comai, 2005; Van De Peer *et al.*, 2017), and may favor invasiveness (Te Beest *et al.*, 2012). Clones with $x = 10$ and $x = 9$ are also much less frequent than those with $x = 8$. The chromosome rearrangements that led to $x = 8$ may have been associated with selective advantages and/or the rearranged chromosomes may be preferentially transmitted to the progeny and thus may have colonized the species (Martin *et al.*, 2017), or they may have favored further polyploidization resulting in an increased adaptability leading to expansion of the species to the south, east and west. Therefore, it is proposed that from the northern part of India *S. spontaneum* cytotypes with $x = 8$ emerged, polyploidized, and then extended largely to other areas.

The four cultivars that we analyzed had between 107 and 114 chromosomes. In the three cases analyzed with GISH, we identified between 10 and 16% of entire *S. spontaneum* chromosomes and 6–10% chromosomes resulting from interspecific exchanges. This sits in the range previously reported (D'Hont *et al.*, 1996; Cuadrado *et al.*, 2004; Piperidis *et al.*, 2010). This proportion remains approximate as GISH experiments were performed as a second hybridization on the same slide resulting in some instances with low differentiation on some chromosomes. The number of interspecific recombinants is also an approximation as interspecific recombination involving small chromosome segments may not be detectable with the resolution of GISH. We showed that the vast majority of the chromosomes that result from interspecific exchanges were derived from recombination between homeologous chromosomes; only in one case we showed that it resulted from a translocation between distinct basic chromosomes. These results confirmed that interspecific recombination occurs between *S. officinarum* and *S. spontaneum* homoeologous chromosomes, as proposed based on genetic mapping studies (Grivet *et al.*, 1996; Hoarau *et al.*, 2001).

In the four modern cultivars we analyzed, the *S. spontaneum* chromosomes that could be distinguished between cytotypes with $x = 8$ and $x = 10$ or $x = 9$ all came from *S. spontaneum* cytotypes with $x = 8$. This is consistent with the facts that *S. spontaneum* with $x = 8$ are the most frequent, and that the two *S. spontaneum* clones reported to be involved in the first interspecific hybridizations made a century ago, and from which all modern cultivars are derived, involved two clones with $x = 8$. These clones are Glagah from Java with $2n = 14x = 112$ and Coimbatore from India with $2n = 8x = 64$ (Arceneaux, 1965; Sreenivasan *et al.*, 1987).

In the four cultivars and for the four basic chromosomes with a chromosome structure conserved between *S. officinarum* and *S. spontaneum* with $x = 8$ (chromosomes 1–4), we observed mainly 12 chromosome copies. For the chromosomes with a distinct structure between the two parental species, we observed a more variable number of chromosome copies (between 10 and 13). Large chromosome structural variations such as the one observed between *S. officinarum* and *S. spontaneum* are known to generate unbalanced gametes. Aneuploidy or segmental aneuploidy can result from normal segregation of bivalents between chromosomes with distinct chromosome structures or from resolution of multivalent pairing and univalent (Martin *et al.*, 2017; Baurens *et al.*, 2019). This could explain the more variable chromosome copy numbers for basic chromosomes with a distinct structure between the two parental species.

In addition, one–four inter-chromosome large translocations were revealed in the cultivars we analyzed, as well as

one in one *S. officinarum* clone and two in a *S. spontaneum* clone. Smaller inter-chromosome translocations may also exist, but may not be detected due to the resolution limits of FISH experiments. In addition, inversions also cannot be detected using whole-chromosome probes, although they probably exist as shown in the SES208 *S. spontaneum* sequence assembly (Zhang *et al.*, 2018). Variation of ploidy between chromosomes or chromosome segments, i.e. aneuploidy or segmental aneuploidy due to the coexistence of distinct basic chromosome structure from the parental species or due to inter-chromosome translocations are probably tolerated in sugarcane cultivars and their parental species thanks to the high polyploid context. This buffering capacity of polyploidy was shown in banana where aneuploidy or segmental aneuploidy is tolerated at the triploid level but not at the diploid level, where it can generate lethal gametes due to the absence of a chromosome or chromosome segment (Martin *et al.*, 2017; Baurens *et al.*, 2019).

Surprisingly, despite this very high level of genome complexity, meiosis of sugarcane cultivars involved mainly bivalent pairing with only a few irregularities found at anaphase I, such as chromosome bridges and laggards (Burner, 1991; Burner and Legendre, 1993; Thumjamras *et al.*, 2016; Vieira *et al.*, 2018).

Genetic mapping studies suggested that in cultivars pairing is mainly polysomic with some preferential pairing occasionally leading to disomy (Grivet *et al.*, 1996; Hoarau *et al.*, 2001; Jannoo *et al.*, 2004). We can imagine that the cases of disomy or preferential pairing may involve chromosomes from the same species when chromosome structure differs between the two ancestral species.

Our study provides important and new information regarding the architecture and evolution of the genomes of the two species, *S. officinarum* and *S. spontaneum*, at the origin of modern cultivars. Our findings highlight the extreme level of complexity of modern sugarcane cultivar genomes that we showed included: (i) chromosomes from two species with distinct basic chromosome architectures; (ii) various chromosome copy numbers depending on chromosomes or chromosome segment; (iii) various chromosome copy numbers originating from the two parental species depending on chromosomes; (iv) interspecific recombinant chromosomes; and (v) inter-chromosome translocations.

This genome complexity will undoubtedly have to be taken into account when developing genomics-assisted breeding approaches that have started to be experimented for the improvement of this challenging crop.

EXPERIMENTAL PROCEDURES

Plant material

The accessions analyzed in this study included two *S. officinarum* (Badila and Black Innis), five *S. spontaneum* (Coimbatore, Glagah, Mol 5904, SES 186 and SES 196) all from the Sugar Research Australia (SRA) collection based in Meringa, Australia, and four cultivars (R570, Q165, Q208 and Q209) that were sourced from the SRA stations in Meringa or Mackay (Australia). Sugarcane is propagated vegetatively, so accessions consisted of clones.

Chromosome preparation

Root tips were harvested from sugarcane plants grown in a glasshouse in 20-L pots containing a mixture of 50% vermiculite/perlite or in an aeroponic system with liquid fertilizer (Ionic Grow, Growth Technology), in Mackay, Australia. Actively growing root tips were pre-treated in a solution of 0.05% 8-hydroxyquinoline for 4 h, oxygenated every hour with a pipette, transferred into an ethanol:acetic acid (3:1) fixative solution for 72 h, and stored in 70% ethanol until use. Fixed root tips were sequentially washed twice for 10 min in H_2O, in a 0.25 N HCl solution, in H_2O, and finally in a digestion citrate buffer. Meristem tips were then incubated at 37°C in a mixture of pectinase (1% final concentration) and cellulase (5% final concentration) in digestion citrate buffer for 2–4 h depending on their size, before being spread on a slide with tweezers in a 3:1 fixative solution.

Chromosome oligo probe design

We used the sugarcane reference sequence assembly of Garsmeur *et al.* (2018) that was assembled in 10 chromosomes to design one oligo probe per chromosome (probes 1–10). Probes were designed by Arbor Biosciences (Ann Arbor, MI, USA) using their proprietary software. The design was based on targeting interspaced regions (islands) spread over the chromosome. Target sequences were cut into overlapping probe candidate sequences with a size ranging from 43 to 47 bases to accommodate for a narrow Tm distribution. Probe candidate sequences were compared with the rest of the genome sequence. A combination of sequence similarity and thermodynamic parameters was used to reject any candidates with significant potential cross-hybridization. Overlapping qualified probes were discarded to obtain a final set of unique non-overlapping target-specific probes. The number and size of targeted islands varied according to chromosome assembly size, but the final probe represents about 27 000 oligos of about 45 nucleotides, and thus about 1.2 Mb for each chromosome. Probes were labeled depending on chromosome and experiments with ATTO 488 (green fluorescence), ATTO 550 (red fluorescence), ATTO 594 and ATTO 633 (far-red fluorescence).

FISH/GISH preparation

The FISH with chromosome-specific oligo probes was performed as described previously (Piperidis *et al.*, 2013; Piperidis, 2014), except that no blocking DNA was used. The hybridization mixture consisted of 50% formamide, 10% dextran sulfate, 1 × of 20 × standard sodium citrate (SSC), 10% sodium dodecyl sulfate, 1 μg salmon sperm DNA, and 50–100 ng of probes, and H_2O to 50 μl.

Chromosome-specific oligo probes were used separately or in combination depending on the experiment. Not all combinations of probes were used, so in the few cases where we observed inter-chromosome translocation, it was often not possible to identify one of the two chromosomes involved.

For GISH, genomic DNA was labeled by random priming according to the manufacturer's directions. We used the BIOPRIME DNA labeling system with rhodamine 5-d-UTP to label genomic DNA from *S. officinarum* (clone Badila or BNS 3066), and

with fluorescein 12-d-UTP to label genomic DNA from *S. spontaneum* (clone Coimbatore or Mandalay).

The GISH experiments were performed on the same slides used for FISH after removing the cover slips in 4 × SSC/Tween20 at 42°C for 10 min, followed by a 2 × SSC at 42°C for another 10 min. Slides were then denatured with a formamide solution following the same protocol used for the chromosome-specific oligo probes.

Slides were then counterstained with 4′-6-diamidino-2-phenylindole (DAPI; Vectashield Mounting Media with DAPI). Images were digitally captured with a CCD camera attached to a BX53 Olympus microscope, and final contrast of images was processed with the Olympus Cellsens software. Chromosomes for each clone were analyzed from at least four cells and at least two root tips.

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AUTHOR CONTRIBUTIONS

NP performed the experiments; NP and AD designed the study, analyzed the data and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are included in the manuscript or are available in supplementary materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. (a–h) FISH with chromosome-specific oligo probes on metaphase mitotic chromosomes. Probes and accession names are indicated on the pictures. Chromosomes were counterstained in DAPI (displayed in gray). Two white arrows on (h) point to two chromosomes with only one arm painted by P3. Scale bar: 5 μm.

Figure S2. (a, c–g) FISH with chromosome-specific oligo probes on metaphase mitotic chromosomes. Probe combinations and accession names are indicated on the pictures. Chromosomes were counterstained in DAPI (displayed in gray). (b) GISH with *S. officinarum* and *S. spontaneum* total DNA detected in green and red, respectively, and performed as a second hybridization on the (a) metaphase cell (showing no sign for the presence of *S. spontaneum* chromosomes). (c,d) White arrow points to the translocation of a small segment of chromosome 5 in one copy of chromosome 2. (e) Schematic representations of the observed chromosome painting patterns are included for the two translocated chromosomes. (f) Six copies of chromosomes 5 and 6 and five copies of chromosome 9. (g) The white arrow points to a translocated segment from chromosome 5 onto one copy of chromosome 8. Scale bar: 5 μm.

Figure S3. FISH on metaphase mitotic chromosomes of *S. spontaneum* Coimbatore with (a) chromosome-specific oligo probes 7, 8 and 10, and (b) a 45S rDNA probe. Chromosomes were counterstained in DAPI. Arrows in (a) point to an apparent break in the chromosomes due to the intercalary rDNA sites. Arrows in (b) point to rDNA sites, crosses indicate signals interpreted as background. Scale bar: 5 μm.

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