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DR YONGJI HUANG (Orcid ID : 0000-0003-2513-5983)
DR JIMING JIANG (Orcid ID : 0000-0002-6435-6140)

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Chromosome-specific painting unveils chromosomal fusions and distinct allopolyploid species in the *Saccharum* complex

Fan Yu[1,2,§], Xinwang Zhao[1,§], Jin Chai[1], Xueer Ding[1], Xueting Li[1], Yongji Huang[3], Xianhong Wang[4], Jiayun Wu[1,5], Muqing Zhang[2], Qinghui Yang[4], Zuhu Deng[1,2,6,*], Jiming Jiang[7]

[1] National Engineering Research Center for Sugarcane, Fujian Agriculture and Forestry University, Fuzhou 350002, Fujian, China

[2] State Key Laboratory for Protection and Utilization of Subtropical Agro-bioresources Guangxi University, Nanning, 530004, China

[3] Marine and Agricultural Biotechnology Laboratory, Fuzhou Institute of Oceanography, Minjiang University, Fuzhou, 350108, China

[4] College of agriculture, Yunnan Agricultural University, Kunming, 650201, Yunnan, China

[5] Institute of Nanfan＆Seed Industry, Guangdong Academy of Sciences, Guangzhou, 510316, China

[6] Key Lab of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture, Fujian Agriculture and Forestry University, Fuzhou 350002, Fujian, China

[7] Department of Plant Biology, Department of Horticulture, MSU AgBioResearch, Michigan State University, East Lansing, MI 48824, USA

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Dr. Fan Yu (ORCID ID: 0000-0003-4550-1936)
Dr. Jiming Jiang (ORCID ID: 0000-0002-6435-6140)

§ These authors contributed equally to this work
* Address correspondence to: dengzuhu@163.com

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Summary

- Karyotypes provide key cytogenetic information on the phylogenetic relationships and evolutionary origins in related eukaryotic species. Despite our knowledge of the chromosome numbers of sugarcane and its wild relatives, the chromosome composition and evolution among the species in the *Saccharum* complex have been elusive due to the complex polyploidy and the large numbers of chromosomes of these species.
- Oligonucleotide (oligo)-based chromosome painting has become a powerful tool of cytogenetic studies especially for plant species with large numbers of chromosomes. We developed oligo-based chromosome painting probes for all 10 chromosomes in *Saccharum officinarum* (2n = 8*x* = 80). The ten painting probes generated robust fluorescence *in situ* hybridization (FISH) signals in all plant species within the *Saccharum* complex, including species in genera *Saccharum*, *Miscanthus*, *Narenga*, and *Erianthus.*
- We conducted comparative chromosome analysis using the same set of probes among species from four different genera within the *Saccharum* complex. Excitingly, we discovered several novel cytotypes and chromosome rearrangements in these species.
- We discovered that fusion from two different chromosomes is a common type of chromosome rearrangement associated with the species in the *Saccharum* complex. Such fusion events changed the basic chromosome number and resulted in distinct allopolyploids in the *Saccharum* complex.

Keywords: Sugarcane, *Saccharum* complex, chromosome painting, oligo-FISH, chromosome fusion, allopolyploids

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Introduction

The karyotype is the basic genetic makeup of a eukaryotic species and can be used to understand chromosomal relationships among genetically related plant species. Development of a karyotype in a plant species relies on accurate identification of individual chromosomes. Unfortunately, chromosome identification is a challenge in most non-model species, especially those with large numbers of chromosomes or with chromosomes of a similar size. Sugarcane has one of the most complex genomes in crop plants due to its complexity associated with not only polyploidy but also aneuploidy. Sugarcane belongs to the genus *Saccharum*, the core member of the *Saccharum* complex that also includes four other genera, namely *Miscanthus*, *Erianthus*, *Narenga* and *Sclerostachya* (Amalraj & Balasundaram, 2006). Modern sugarcane cultivars are mostly derived from a narrow genetic base, including a few clones of *Saccharum officinarum* and *Saccharum spontaneum* (Arceneaux, 1967; Roach, 1989). Species from the four other genera have been increasingly recognized as important germplasm for future sugarcane breeding (Chang *et al.*, 2020). Nevertheless, the highly variable chromosome numbers (20 - ~128) and diversity in the basic chromosome number (x = 5-19) have hindered cytogenetic research of plant species within the *Saccharum* complex. To date, the genetic and cytogenetic information of the *Saccharum* complex remains enigmatic.

Fluorescence *in situ* hybridization (FISH) has been the most important tool in plant chromosome identification (Jiang, 2019). For decades, various types of DNA probes, i.e., bacterial artificial chromosome (BAC) clones and repetitive DNA sequences etc., were widely used to identify chromosomes in different plant species (Mukai *et al.*, 1993; Kim *et al.*, 2002; Kato *et al.*, 2004; Iovene *et al.*, 2008; Dong *et al.*, 2018). Unfortunately, these probes are time-consuming to develop and are often not reliable to identify the homologous/homoeologous chromosomes among related species or among different genotypes (Jiang, 2019). The recent development and application of oligonucleotide (oligo)-based probes have dramatically improved the efficiency and accuracy of FISH-based methodology in chromosome identification. Oligo probes based on conserved DNA sequences were successfully used in chromosome identification among plant species diverged more than 15 million years (MYs) (Braz *et al.*, 2018; He *et al.*, 2020; Xin *et al.*, 2020). Oligos specific to a chromosome region or to an entire chromosome can be developed to track chromosomes or chromosomal rearrangements in both mitosis (Han *et al.*, 2015; Braz *et al.*, 2018; Braz *et al.*, 2020a)

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and meiosis (Han *et al.*, 2015; He *et al.*, 2018; Braz *et al.*, 2021). Oligo-based chromosome barcode or painting probes have been developed in an increasing number of plant species (Han *et al.*, 2015; Qu *et al.*, 2017; Braz *et al.*, 2018; Albert *et al.*, 2019; Martins *et al.*, 2019; Simonikova *et al.*, 2019; Agrawal *et al.*, 2020; He *et al.*, 2020; Meng *et al.*, 2020; de Oliveira Bustamante *et al.*, 2021; Hoang *et al.*, 2021).

The phylogenetic relationship of plant species in the *Saccharum* complex has been well studied using molecular markers associated with chloroplast DNA, mitochondrial DNA, as well as nuclear genes (D'Hont *et al.*, 1993; Selvi *et al.*, 2005; Singh *et al.*, 2010; Viola *et al.*, 2011; Raj *et al.*, 2016). However, the karyotypes and chromosomal evolution of these species remain largely unknown. Although genome sequencing and assembly has become increasingly less expensive, it is still costly and technically challenging for complex plant species such as sugarcane, which is confounded not only by high levels of polyploidy, but also by aneuploidy. FISH using oligo-based probes has already been demonstrated to be an effective approach for sugarcane chromosome identification (Meng *et al.*, 2018; Meng *et al.*, 2020; Piperidis & D'Hont, 2020).

We developed oligo-based chromosome painting probes for all 10 chromosomes of *S. officinarum* (LA Purple) (2n = 8x = 80). We were able to identify every chromosome in *S. officinarum* by multiple rounds of sequential FISH using the chromosome painting probes. We conducted comparative chromosome painting using these probes in four species that are closely related to sugarcane, including *Miscanthus sinensis, Narenga pophyrocoma*, *Erianthus rockii* and *Erianthus fulvus*. We discovered several novel cytotypes [varieties or races of a species whose chromosome complement differs quantitively (in chromosome number) or qualitatively (in chromosome structure) from the standard karyotype of the species], which were not reported in the literature. We discovered that chromosome fusions derived from two chromosomes were a common rearrangement associated with species in the *Saccharum* complex, which resulted in distinct allopolyploids.

Materials and Methods

Plant materials and chromosome preparation

Saccharum complex species, including *S. officinarum* LA purple, *S. spontaneum* Np2013-6 and Gx-spon, *S. robustum* NG77-004, 51NG63 and 51NG3-1, *M. sinensis* Jiangxi91-8, *N.*

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pophyrocoma Guangdong64, *E. rockii* Yunnan83-224 and *E. fulvus* Yunnan2009-3, were used for FISH mapping. To prepare mitotic metaphase chromosomes, root tips were harvested from stalks or plants, pre-treated with saturated solution of para-dichlorobenzene and α-bromonaphthalene at room temperature (25°C) for 4 h, fixed in Carnoy's fixative for 12 h, and then stored in 70% ethanol at -20°C until use. An enzyme mixture (1% pectolyase Y23, 2% pectinase, 2% RS and 4% cellulase Onozuka R-10) was used to digest the root tips for 4 h at 37°C. Finally, the suspension cells were dropped onto glass slides and 10 μl acetic acid was used to spread the cells.

Development and synthesis of oligo libraries

We used RepeatMasker software (http://www.repeatmasker.org/) to filter the repetitive sequences in the *S. officinarum* (LA Purple) haplotype reference genome (chromosome set A, unpublished data). The remaining sequences were used to design and select the oligos using the Chorus2 software (Zhang *et al.*, 2021). *S. officinarum* genomic shotgun sequences were used for further repeat filtering using the ChorusNGSfilter.py and ChorusNGSselect.py scripts (-q 0.1; -p 0.9; -d 25, 45 or 50) that are included in the Chorus2 software. Finally, the ten oligo libraries (**Supplemental Dataset S1**) were synthesized by CustomArray (Genscript, Nanjing, China). Each oligo contained 45-50 nt (**Table S1**), a 23-nt forward (T7 RNA polymerase promoter sequence), and a 20-nt reverse primers (**Table S2**).

Oligo-FISH and karyotyping

The oligo libraries were labeled with FAM-green (direct label) or Cy3-red (direct label) following published protocols (Braz *et al.*, 2020b). The 5S rDNA and 35S rDNA probes were labeled with FITC-dUTP and Cy3-dUTP by nick translation (DIG-Nick Translation Mix, Roche). Mapping of 5S and 35S rDNAs was performed by additional rounds of FISH after chromosome painting. FISH was performed according to published protocols (Han *et al.*, 2015) with minor adjustments. Approximately 10 μl hybridization solution containing 1.5 μl FAM-green and 1.5 μl Cy3-red probes was placed onto each dried slide. Subsequently, the slides were heated for 3 min at 55°C, followed by overnight incubation at 37°C. Coverslips were gently removed and the slides were washed 3 min in 2×SSC, 10 min in 2×SSC and 3 min in 1×PBS, respectively. The slides were dried and counterstained with 4',6-diamidino-2-phenylindole (DAPI).

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For multiple rounds of sequential FISH, probes were removed by washing the slides in 4×SSC (included Tween-20) for three times (5 min each) and in 2×SSC three times (3 min each) after removing the coverslips. The slides were dehydrated in 70% and 100% ethanol (3 min each step). The slides were then denatured again in 70% formamide at 70°C for 1 min for the following round of FISH. Chromosome images were captured using a Zeiss fluorescence microscope with CoolSNAP DYNO CCD camera and processed using Adobe Photoshop CS software. DRAWID (Kirov *et al.*, 2017) was used to measure the length of individual chromosomes for karyotyping. Measurement was performed on every chromosome in complete 10 metaphase cells from each species.

Results

Development of chromosome-specific painting probes in *S. officinarum*

We developed a complete set of 10 chromosome-specific painting probes in sugarcane. The painting probes were developed based on the sequenced genome of *S. officinarum* (2n = 8x = 80) (unpublished data) using the Chorus2 software (Zhang *et al.*, 2021). The lengths of the 10 *S. officinarum* pseudomolecules range from 66 megabase (Mb) of chromosome 8 to 150 Mb of chromosome 1 (**Figure 1a**). We intended to retain all single-copy oligos (45-50 nt), which would allow to evenly cover the entire chromosomes and to generate uniform chromosome painting signals. Nevertheless, some chromosomal arms or regions, such as the short arm of chromosome 2, appeared to contain a relatively low density of single-copy oligos (**Figure 1a**), possibly due to high percentages of repetitive DNA sequences in these arms and regions. Lack of oligos was observed in the putative centromeric region in most chromosomes (**Figure 1a**). The subtelomeric regions of chromosome 1 showed a low density oligos on the short arm (**Figure 1a**). Finally, we selected 41,990 ~ 103,312 oligos from each of the ten pseudomolecules (**Table S1**). The average oligo density of the 10 painting probes range from 0.49 to 0.84 oligos per kilobase (kb) (**Table S1**).

Chromosome painting in *S. officinarum*

The 10 chromosome painting probes were labeled by either Cy3- or FAM-, which produce red and green signals, respectively. The 10 painting probes were referred to Chr1-Chr10, which correspond to pseudomolecule 1-10, respectively. A group of two probes were hybridized to the metaphase chromosomes prepared from *S. officinarum*. For example, painting probes of chromosomes

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1 and 8 were hybridized to a metaphase cell (**Figure S1a**). The slide was then washed and re-probed by painting probes of chromosomes 2 and 6 (**Figure S1b**). All ten chromosomes were individually identified after five rounds of sequential FISH experiments (**Figure S1**). Each of the ten probes generated bright and specific signals on eight homologous chromosomes. These results validated the chromosome specificity of the painting probes. The FISH signals on the short arm of chromosome 2 were weaker than those on the long arm (**Figure S1b**), which is consistent with the distribution of oligo density on this chromosome (**Figure 1a**). The nucleolar organizing region (NOR) is located on the short arm of chromosome 6 (Meng *et al.*, 2018). Very weak FISH signals were detected on this chromosomal arm (**Figure S1b**), likely due to its highly repetitive sequence content, which is similar with painting pattern of chromosome 6 in maize (Albert *et al.*, 2019). In addition, no signals or very weak signals were observed in the centromeric region of most *S. officinarum* chromosomes (**Figure 1b**).

New cytotypes of *S. robustum* revealed by chromosome painting

We next evaluated the quality of the painting probes in hybridization to chromosomes from *S. robustum,* which is considered as the ancestor of *S. officinarum*. Three different accessions of *S. robustum* (51NG63, NG77-004 and 51NG3-1) were used in the analysis. Previous studies indicated that *S. robustum* has different cytotypes with chromosome numbers ranging from 60 to 200 (Brandes, 1965). However, most researchers believed that the true chromosome numbers of *S. robustum* should be either 60 or 80, and other "cytotypes" with different chromosome numbers are likely hybrids between *S. robustum* and other *Saccharum* species (Brandes, 1965).

All 10 painting probes produced bright and chromosome-specific signals with minimal hybridization background in all three accessions (**Figure 2a, 2b**; **Figure S2**). However, different chromosomal compositions were detected in the three *S. robustum* accessions. Each painting probe hybridized to 8 copies of a chromosome in 51NG63 ($2n = 8x = 80$, **Figure S2a**), but 9 copies in NG77-004 ($2n = 9x = 90$, **Figure 2a**), and amazingly, 11 copies in 51NG3-1 ($2n = 11x = 110$, **Figure 2b**). These results unveiled that *S. robustum* is associated with various levels of ploidy in nature, expanding our knowledge of the previously confirmed hexaploidy and octoploidy in this species (Brandes, 1965).

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We were intrigued by the possibility that accessions NG77-004 and 51NG3-1 are hybrids derived from *S. robustum* and *S. spontaneum*. A *S. spontaneum*-specific repetitive DNA probe, which is a cocktail probe containing repeats SsRetro1-SsRetro4 (Huang *et al.*, 2020), was used to examine the potential presence of *S. spontaneum* chromosomes in these accessions. We did not detect any hybridization signals in both accessions (**Figure S2, b5 and Figure S2, c5**). Thus, we conclude that accessions NG77-004 and 51NG3-1 are new cytotypes of *S. robustum*, which were not recorded previously.

Chromosome composition of *S. spontaneum* revealed by chromosome painting

Classical cytogenetic studies based on rDNA probes established the basic chromosome number of *S. spontaneum* as $x = 8$ (D'Hont *et al.*, 1998; Garsmeur *et al.*, 2018). This conclusion has been validated by genome sequencing of *S. spontaneum* AP85-441 (Zhang *et al.*, 2018). Interestingly, *S. spontaneum* cytotypes with basic chromosome numbers of $x = 10$ and $x = 9$ have recently been reported (Meng *et al.*, 2020; Piperidis & D'Hont, 2020). Hence, we intended to use our chromosome painting probes for further study of the chromosome compositions in different cytotypes of *S. spontaneum.*

Two accessions of *S. spontaneum*, Np2013-6 (2n = 40) and Gx-spon (2n = 80), were used in the analysis (**Figure 3**). Each of the ten painting probes hybridized to four homologous chromosomes in accession Np2013-6 (**Figure 3, a1-a5**). Thus, Np2013-6 represents an euploidy tetraploid clone. Each Np2013-6 chromosome is homologous to one of the 10 chromosomes in *S. officinarum.*

Accession Gx-spon was confirmed to be a decaploid with a basic chromosome $x = 8$. Each of the four chromosomes of Gx-spon (Ss1, Ss3, Ss4, and Ss8) hybridized only to a single painting probe (**Figure 3**, **Figure S3b**). The remaining four chromosomes (Ss2, Ss5, Ss6, and Ss7) hybridized to multiple painting probes. For example, Ss5 hybridized to painting probes Chr5 and Chr6. Thus, Ss5 of Gx-spon is an equivalent of fusion of chromosomes 5 and 6 from *S. spontaneum* ($x = 10$) (**Figure 4**). Similarly, Ss2, Ss6 and Ss7 were derived from similar chromosome fusion events (**Figure 4**). Both chromosomes 5 and 8 in *S. spontaneum* ($x = 10$) fused with two different chromosomes, resulting in a cytotype with $x = 8$ (**Figure 4**). In addition, we observed a reciprocal translocation between one copy

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of Ss5 and one copy of Ss8, and an inversion in one copy of Ss7 (indicated by the box in **Figure 3b5**; **Figure S3a**).

Distinct chromosome fusion and allotetraploids revealed by chromosome painting

We were intrigued if the 10 painting probes can be used to analyze chromosomes of wild species that are more distantly related to sugarcane. *Miscanthus sinensis* (Jiangxi91-8), *Narenga pophyrocoma* (Guangdong64), *Erianthus rockii* (Yunnan83-224) and *Erianthus fulvus* (Yunnan2009-3) were selected for chromosome painting analyses. Excitingly, the painting probes generated robust signals on chromosomes in all four species (**Figure 5**, **Figure 6**, **Figure S4**, **Figure 7**).

In *M. sinensis* Jiangxi91-8 (2n = 4*x* = 38), each of the 10 painting probes generated distinct signals (**Figure 5**). Most probes hybridized to four copies of a single chromosomes in Jiangxi91-8 (**Figure 5**). However, two chromosomes of Jiangxi91-8 were resulted from fusion of a *S. officinarum* 7-like chromosome with a *S. officinarum* 4-like chromosome (**Figure 5c**, **f**), which caused the reduction of chromosome number from 40 to 38. This is consistent with the published *M. sinensis* genome in which a sorghum 4-like and a sorghum 7-like chromosomes fused to a new chromosome (Mitros *et al.*, 2020).

In *N. pophyrocoma* Guangdong64 (2n = 30), each of the 10 painting probes hybridized to one complete pair of chromosomes (**Figure 6, a, b**). Each of the five remaining pairs of Guangdong64 chromosomes hybridized to two painting probes. For example, chromosome 11 hybridized to painting probes 1 and 8 (**Figure 6b**). Thus, each of these five chromosomes was derived from fusion of two *S. officinarum*-like chromosomes. Interestingly, chromosomes 11 and 15 showed a similar fusion pattern: one *S. officinarum*-like chromosome inserted in the middle of another *S. officinarum*-like chromosome (**Figure 6c**). Similarly, chromosomes 12 and 14 also showed a similar fusion pattern from two *S. officinarum*-like chromosomes (**Figure 6c**). These results show that *N. pophyrocoma* Guangdong64 is a distinct allotetraploid (2n = 4*x* = 30). One subgenome of this allotetraploid is the same as the basic *Saccharum* genome with n = 10 chromosomes. The second subgenome contains only n = 5 chromosomes that originated from fusions of chromosomes from the basic *Saccharum* genome.

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Surprisingly, we found that the karyotype of *E. rockii* Yunnan83-224 (2n = 4x = 30) is highly similar to that of *N. pophyrocoma* (**Figure 6c**, **Figure S4**). Thus, the chromosomes of *E. rockii* were named as chromosomes 1-15 similarly as those in *N. pophyrocoma* (**Figure 6c**).

E. fulvus Yunnan2009-3 (2n = 2x = 20) contained 10 pairs of chromosomes and each chromosome hybridized to only one of the 10 painting probes (**Figure 7**). Thus, *E. fulvus* is a diploid with the basic chromosome number x= 10, representing an ancestral chromosomal composition of all species in the *Saccharum* complex.

Comparative karyotyping of *Saccharum* complex species based on individually identified chromosomes

The 10 chromosome painting probes allow us to essentially identify every chromosome in all *Saccharum* complex species. Thus, a true karyotype, in which each chromosome is individually identified, can now be established in the *Saccharum* species based on sequential FISH experiments using 2-3 probes in each round of FISH. A similar karyotyping strategy has recently been demonstrated in two woody plant species (He *et al.*, 2020; Xin *et al.*, 2020). We developed the karyotype in four species, including *S. officinarum*, *S. robustum*, *N. pophyrocoma, E. rockii* and *E. fulvus* (**Table 1**).

We observed a highly similar karyotype between *S. officinarum* and *S. robustum*. Chromosome 1 and 8 represent the longest and the shortest chromosomes in both species, which is consistent with the *S. officinarum* genome sequencing data (unpublished data). Most chromosomes are metacentric or submetacentric in both species (**Table 1**). In *N. pophyrocoma*, three fused chromosomes (11, 12, and 13) are longer than chromosome 1 (**Table 1**). All five fused chromosomes (11-15) are longer than chromosome 1 in *E. rockii*. In addition, chromosome 10, rather than chromosome 8, was found to be the shortest chromosome in both species (**Table 1**).

Comparative FISH mapping of the 5S and 35S rDNAs

The 5S and 35S rDNAs have been widely used as cytogenetic markers for chromosome identification in plants. However, variability of chromosomal locations of rDNAs were reported in many plant species (Kolano *et al.*, 2012; Wang *et al.*, 2019). It is unknown if the chromosomal

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locations of rDNAs are conserved in the *Saccharum* species. Thus, we intended to map the 5S and 35S rDNAs to specific chromosomes in the *Saccharum* complex species using the sequential FISH procedure. Interestingly, the 5S rDNA was mapped to *S. officinarum*-9 like chromosome in all *Saccharum* complex species (**Figure 8**). However, the 35S rDNA was mapped to *S. officinarum*-6 like chromosome in most species, except for *M. sinensis* and *S. spontaneum* (x = 8) (**Figure 8**).

In four species with the basic chromosome number of x = 10, including *S. officinarum*, *S. robustum*, *S. spontaneum* (x = 10) and *E. fulvus*, the 5S rDNA and 35S rDNA were located to chromosomes 9 and 6, respectively (**Figure 8**). Thus, the rDNA loci have been conserved among these species. In *S. spontaneum* with x = 8 (2n = 10x = 80), nine copies of chromosome Ss7 carried the 5S rDNA with a similar centromeric location as chromosome 9 in *S. officinarum* (**Figure 8**). However, the 5S rDNA was located in a sub-telomeric region in the last copy of Ss7 (**Figure 8**), suggesting a paracentric inversion associated with this chromosome (indicated by the arrow in **Figure S5a**; **Figure S5c**), which is consistent with the chromosome painting result (**Figure S3a**). Unexpectedly, we detected only six 35S rDNA loci associated with chromosome Ss5 (**Figure 8**). Three additional 35S rDNA loci were translocated to Ss8 (indicated by the arrow in **Figure S5b**, **Figure S5d**). Of these three 35S rDNA loci, one copy of Ss8 also included the Chr5 probe signal that is consistent with the chromosome translocation between Ss5 and Ss8 (**Figure S3a**).

In *N. pophyrocoma* Guangdong 64 and *E. rockii* Yunnan83-224, 5S rDNA was mapped to chromosomes 9 and 15 (**Figure 8**). However, 35S rDNA was only detected on chromosome 6. Thus, another 35S rDNA locus, which is presumably associated with chromosome 12, was either lost during evolution or was not detectable in these species (**Figure 8**).

Unexpectedly, the 35S rDNA was mapped to close to the centromeric region of chromosome 1 in *M. sinensis* Jiangxi91-8 (**Figure 8**), rather than *S. officinarum* 6-like in other species. Thus, the 35S rDNA moved to the chromosome 1 in *M. sinensis* during evolution. In contrast to the variable number and position of 35S rDNA, 5S rDNA showed a highly conserved location on *S. officinarum* 9-like chromosome in all species in the *Saccharum* complex (**Figure 8**).

Discussion

Identification of individual chromosomes has long been an impossible task for plant species like sugarcane. Traditional cytogenetic markers, such as repetitive sequences and BAC clones,

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allowed only identification of a few chromosomes in sugarcane (D'Hont *et al.*, 1998; Garsmeur *et al.*, 2011; Dong *et al.*, 2018). In addition, probes based on repetitive DNA sequences often generate variable number of FISH foci in different cells (Thumjamras *et al.*, 2016) and many BAC probes produce strong background signals in sugarcane (Dong *et al.*, 2018). Oligo-based FISH probes have recently been developed for sugarcane chromosome research. These oligo probes were developed based on the genome sequences of sorghum (Meng *et al.*, 2018), *S. spontaneum* AP85-441 (2n = 4x = 32) (Meng *et al.*, 2020), or a monoploid genome of sugarcane cultivar R570 (2n = 114, x = 10+8) (Piperidis & D'Hont, 2020). These oligo-based probes produced robust FISH signals in sugarcane, especially in *S. officinarum* and *S. spontaneum*. However, these probes are specific to only a restricted region (0.6 Mb ~ 9.8 Mb) on each chromosome. Thus, the FISH signals do not cover entire chromosomes, which limit the power to reveal the details of rearranged or fused chromosomes. We demonstrate that a complete set of whole chromosome painting probes are highly powerful to reveal rearrangements and evolution of chromosomes in the *Saccharum* complex species.

S. officinarum is well known to be an euploid autopolyploid with 2n = 80 chromosomes, although accessions with an odd chromosome number of 2n = 81 were reported in literature (Irvine, 1999; Piperidis & D'Hont, 2020). *S. officinarum* was hypothesized to have been domesticated in New Guinea from the wild species *S. robustum* (2n = 60, 80 and up to 200) (Brandes, 1965). Most recently, Pompidor *et al.* (2021) confirmed the common view that *S. officinarum* was domesticated from *S. robustum* and proposed that it was derived from interspecific hybridization between two unknown ancestors (A and B genomes) (Pompidor *et al.*, 2021). However, sequence-based analysis suggested that *S. officinarum* and *S. robustum* diverged about 385,000 years ago (Zhang *et al.*, 2018). Our comparative chromosome painting analysis showed that these two species share a nearly identical karyotype. *S. robustum* accessions with x = 10 (2n = 60 or 80) were considered to be the major cytotypes and represent euploid forms (Bremer, 1961). This popular view was supported by mapping of 5S and 35S rDNAs using FISH in few *S. robustum* clones (D'Hont *et al.*, 1998). Our analysis of multiple *S. robustum* accessions showed that *S. robustum* (51NG63) is a classical octoploid. Surprisingly, *S. robustum* (NG77-004, 2n = 9x = 90) is a nonaploid and *S. robustum* (51NG3-1, 2n = 11x = 110) is a hendecaploid. We predict that additional cytotypes may exist in nature. It will be interesting to investigate meiosis in these different cytotypes and reveal possible mechanisms

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responsible for the formation of gametes with different numbers of the basic set (x = 10) of chromosomes.

S. spontaneum is the most intensively studied species in genus *Saccharum* (Ming *et al.*, 2006). Recently, oligo-based FISH probes designed based on the genome of sorghum (2n = 2x = 20) or R570 sugarcane (2n = 114, x = 10+8) have been used to analyze the chromosomes of *S. spontaneum* (2n = 53-112). These FISH mapping efforts revealed the chromosome rearrangements that caused the reduction of x = 10 to x = 9, 8 (Meng *et al.*, 2018; Piperidis & D'Hont, 2020). Similarly, oligo-based probes developed based on *S. spontaneum* genome (x = 8) were used to analyze the chromosomes of *S. officinarum* (2n = 8x = 80), which also revealed the chromosome rearrangements that caused the reduction of x = 10 to x = 8 (Meng *et al.*, 2020). However, these analyses using the regional specific oligo probes did not reveal the details of the chromosome fusion events occurred during chromosome number reduction. Using whole chromosome painting probes we revealed that both chromosomes 5 and 8 in *S. spontaneum* (x = 10) broke and fused with two different chromosomes, resulting in *S. spontaneum* with x = 8 (**Figure 3**). We also detected unexpected chromosome translocation between nonhomologous chromosomes Ss5 and Ss8 (**Figure 3**) and an inversion in one copy of Ss7. Thus, whole chromosome painting probes provide a powerful tool to reveal the details of chromosomal rearrangements occurred during the evolution of species in the *Saccharum* complex.

Miscanthus species were traditionally considered to have a basic chromosome number n = x =19 (Swaminathan *et al.*, 2012). However, genome sequencing studies revealed that *M. sinensis* and *M. lutarioriparius*, both 2n = 38, are allotetraploids containing the A and B subgenomes (Mitros *et al.*, 2020; Miao *et al.*, 2021). These two subgenomes include 9 and 10 chromosomes, respectively. Two chromosomes in the A subgenomes, which are homologous to sorghum chromosomes 4 and 7, fused into a single chromosome in subgenome B (Mitros *et al.*, 2020). Our chromosome painting results confirmed the chromosome fusion between a *S. officinarum* 4-like chromosome and a *S. officinarum* 7-like chromosome (**Figure 5**). Most painting probes hybridized to four *M. sinensis* chromosomes that show a similar morphology and FISH signal pattern (**Figure 5**). Thus, most homoeologous chromosomes in the two subgenomes appear to maintain similar structures.

N. porphyrocoma (2n = 30) was considered as the closest diploid relative of sugarcane (Al-Janabi *et al.*, 1994) and diverged from sugarcane 2.5 Mya (Garsmeur *et al.*, 2011). Excitingly,

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chromosome painting analysis allowed us to unveil, for the first time, that *N. porphyrocoma* is a distinct allotetraploid. We propose that one subgenome of *N. porphyrocoma* contains 10 complete *S. officinarum*-like chromosomes, and the second subgenome contains five fused chromosomes (**Figure 6**). However, we cannot exclude the possibility that both subgenomes contain fused chromosome(s). Interestingly, the genetic composition and fused pattern of chromosome 14 in *N. porphyrocoma* (**Figure 6**) is nearly identical to the single fused chromosome in *M. sinensis* (**Figure 5**), suggesting that these chromosomes may share the common evolutionary path. Thus, we cannot exclude the possibility that *N. porphyrocoma* was derived from additional chromosome fusions of a *M. sinensis*-like species. In addition, *E. rockii* have a similar karyotype and chromosome fusion patterns with *N. pophyrocoma*, although these two species belong to different genera. A similar chromosome composition (x = 10 + 5) was found in polyploidy *Brachypodium* species, and it was proposed that the x = 10 + 5 species was the hybrids between x = 10 and x = 5 species (Hasterok *et al.*, 2020). It is possible that both *N. porphyrocoma* and *E. rockii* were derived from two diploid species with 2n = 2x = 20 and 2n = 2x = 10, respectively. While *E. fulvus* represents an ancestral diploid species with x = 10, the ancestral diploid species with x = 5 may no longer exist in the nature.

Chromosome fusion events are the main factor leading to the dysploidy in plants. Insertional or nested chromosome fusion, where both ends of one ancestral chromosome is flanked the chromosome arms of a second ancestral chromosome, have been found in many grass species (Luo *et al.*, 2009; Salse *et al.*, 2009; International Brachypodium Initiative, 2010; Murat *et al.*, 2010), but less frequently in *Arabidopsis thaliana* and related Brassicaceae species (Lysak *et al.*, 2006). In the present study, we observed similar chromosome fusion patterns in *N. porphyrocoma* and *E. rockii*. Symmetric nested chromosome fusion was found in chromosomes 11 and 15 (**Figure 6b**). Nested fusion by asymmetric with donor arm re-positioning (Luo *et al.*, 2009) was detected in chromosomes 12 and 14 (**Figure 6b**). However, chromosome 13 showed an unusual complex fusion that may have undergone an asymmetric nested fusion together with a small inversion (**Figure 6b**). Altogether, these results confirmed that the insertional chromosome fusions play the key role in evolution of the species in *Saccharum* complex, which resulted in distinct cytotypes and allopolyploids.

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Author contributions

Z.D. designed the research; F.Y., X.Z., J.C., X.D., and X.L. performed experiments; X.W., J.W., X.Z., M.Z., and Q.Y. provided resources; F.Y., Y.H., Z.D. and J.J. analyzed the results and wrote the manuscript. All authors read and approved the final manuscript. F.Y. and X.Z. contributed equally to this work.

Data availability

Data available in the supporting information. The sequences of all oligos for the 10 chromosome-specific libraries are included in Dataset S1.

Supporting Information

Figure S1. Chromosome painting on metaphase chromosomes of *S. officinarum* LA Purple.
Figure S2. Chromosome painting on metaphase chromosomes of *S. robustum*.
Figure S3. Structure and composition of chromosomes from *S. spontaneum*.
Figure S4. Chromosome painting on metaphase chromosomes of *E. rockii* Yunnan83-224.
Figure S5. Locations of the 5S rDNA and 35S rDNA in *S. spontaneum* Gx-spon (2n = 10x = 80).
Table S1. Information of the 10 synthetic oligo libraries.
Table S2. Primers tagged to each selected oligo.
Dataset S1. All oligos included in the 10 chromosome-specific oligo libraries.

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Table 1. Arm ratios and relative lengths of individual chromosomes of five species from the *Saccharum* complex

Chr.	*Saccharum officinarum* (2n = 8*x* = 80)		*S. robustum* (2n = 8*x* = 80)		*Narenga pophyrocoma* (2n = 4*x* = 30)		*Erianthus rockii* (2n = 4*x* = 30)		*E. fulvus* (2n = 2*x* = 20)	
	Arm ratio[a]	Relative length[b] (%)	Arm ratio[a]	Relative length[b] (%)	Arm ratio[a]	Relative length[b] (%)	Arm ratio[a]	Relative length[b] (%)	Arm ratio[a]	Relative length[b] (%)
1	1.47+0.26[a]	12.75+0.82[b]	1.28+0.23	12.91+0.97	1.24+0.03	7.99+0.35	1.09+0.06	6.71+0.07	1.56+0.08	13.34+1.09
2	1.65+0.15	10.67+0.81	1.41+0.20	11.57+0.49	1.05+0.04	6.45+0.22	1.13+0.02	6.05+0.17	1.39+0.05	10.99+0.30
3	1.49+0.20	12.21+1.33	1.48+0.26	11.21+0.76	1.36+0.07	6.49+0.08	1.36+0.20	6.06+0.43	1.42+0.11	13.09+0.38
4	1.38+0.15	11.03+0.93	1.34+0.20	10.59+0.78	1.24+0.14	5.21+0.44	1.28+0.09	5.12+0.16	1.33+0.24	10.43+0.44
5	1.19+0.12	9.60+0.66	1.25+0.18	9.04+0.86	1.13+0.04	7.48+0.25	1.09+0.02	7.08+0.07	1.19+0.06	10.83+1.34
6*	1.67+0.21	10.11+1.08	1.69+0.21	8.86+0.94	1.52+0.12	5.96+0.26	1.43+0.05	4.90+0.17	1.42+0.07	8.67+0.36
7	1.13+0.09	8.26+0.97	1.10+0.08	8.73+0.97	1.24+0.21	4.88+0.39	1.16+0.16	4.72+0.35	1.16+0.07	7.84+0.25
8	1.23+0.08	7.17+0.45	1.16+0.06	8.47+0.86	1.19+0.12	5.29+0.05	1.25+0.06	4.78+0.26	1.69+0.15	8.85+1.17
9	1.17+0.17	9.42+0.55	1.11+0.07	8.73+0.96	1.18+0.09	5.07+0.24	1.15+0.01	4.42+0.26	1.34+0.18	8.43+0.48
10	1.29+0.16	9.62+1.10	1.20+0.17	9.47+0.71	1.14+0.09	4.46+0.04	1.20+0.12	4.40+0.20	1.09+0.02	7.50+0.80
11					1.22+0.15	9.39+0.84	1.14+0.03	9.43+0.08		
12					1.54+0.13	8.47+0.14	1.64+0.05	9.42+0.27		
13					1.13+0.02	8.40+0.18	1.41+0.02	9.29+1.29		
14					1.41+0.12	7.66+0.27	1.40+0.10	7.17+0.31		
15					1.25+0.05	6.80+0.32	1.10+0.03	7.11+0.78		

[a]Arm ratio, length of the long arm/length of the short arm. Data is presented as the average (in micrometers) with standard deviation.

[b]Relative length = 100 × length of the chromosome length/length of all chromosomes. Data is presented as the average (in micrometers) with standard deviation.

*The 35S rDNA on the short arm of chromosome 6 was excluded in the measurement.

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Figure legends

Figure 1. Development of chromosome-specific painting probes based on the *Saccharum officinarum* genome. (**a**) The ten pseudomolecules of *S. officinarum* were divided into 500-kb windows and the number of oligos was calculated for each window. The distribution of the number of oligos is shown in the line plot and heatmap. The *x*-axis is the position of the chromosome. The *y*-axis is the number of oligos in each 500 kb window. (**b**) FISH-labeled chromosomes were digitally excised from the same image of Figure S1. Chromosomes were prepared from *S. officinarum* LA Purple. The asterisk indicates that one copy of chromosome 8 was lost in this cell during slide preparation.

Figure 2. Chromosome painting on metaphase chromosomes of *Saccharum robustum*. (**a**) Painting of Chr7 (red) and Chr8 (green) on a metaphase cell prepared from *S. robustum* NG77-004. (**b**) Painting of Chr7 (red) and Chr8 (green) on a metaphase cell prepared from *S. robustum* 51NG3-1. Bars = 10 μm.

Figure 3. Chromosome painting on metaphase chromosomes of *Saccharum spontaneum*. (**a1-a5**) Five rounds of sequential chromosome painting on a metaphase cell prepared from *S. spontaneum* Np2013-6. (**b1-b5**) Five rounds of sequential chromosome painting on a metaphase cell prepared from *S. spontaneum* Gx-spon. The dotted boxes in (b5) indicate the inversion associated with chromosome Ss7 and a translocation between chromosomes Ss8 and Ss5. Bars = 10 μm.

Figure 4. Ideograms illustrating chromosome evolution between the $x = 10$ and $x = 8$ cytotypes of *Saccharum spontaneum*. (**a**) Chromosomal ideogram of $x = 10$ and $x = 8$ cytotypes of *S. spontaneum.* (**b**) An ideogram illustrating rearrangements associated with chromosomes 5 and 8.

Figure 5. Chromosome painting on metaphase chromosomes of *Miscanthus sinensis* Jiangxi91-8. (**a**) Chr1 (red) and Chr2 (green). The dotted lines link the over-stretched chromosome 1 that is likely associated with a tertiary constriction. (**b**) Chr3 (red) and Chr8 (green). (**c**) Chr4 (red) and Chr7 (green). (**d**) Chr5 (green) and Chr6 (red). (**e**) Chr9 (red) and Chr10 (green). (**f**) An ideogram

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illustrating the chromosome fused from a *Saccharum officinarum* 4-like chromosome and *S. officinarum* 7-like chromosome. Bars = 10 μm.

Figure 6. Chromosome painting on metaphase chromosomes of *Narenga pophyrocoma* and *Erianthus rockii*. (**a1-a5**) Five rounds of sequential chromosome painting on a metaphase cell prepared from *N. pophyrocoma* Guangdong64. Bars = 10 μm. (**b**) FISH-labeled chromosomes were digitally excised from the same images of Figure 6a and Supporting Information Figure S4. (**c**) An ideogram illustrating the chromosomes 1-15 in *N. pophyrocoma* and *E. rockii.*

Figure 7. Chromosome painting on metaphase chromosomes of *Erianthus fulvus* Yunnan2009-3. (**a-e**) Five rounds of sequential chromosome painting on the same metaphase cell. Bars = 10 μm. (**f**) FISH-labeled chromosomes were digitally excised from the same metaphase cell.

Figure 8. Chromosomal locations of the 5S rDNA and 35S rDNA in *Saccharum* complex species.

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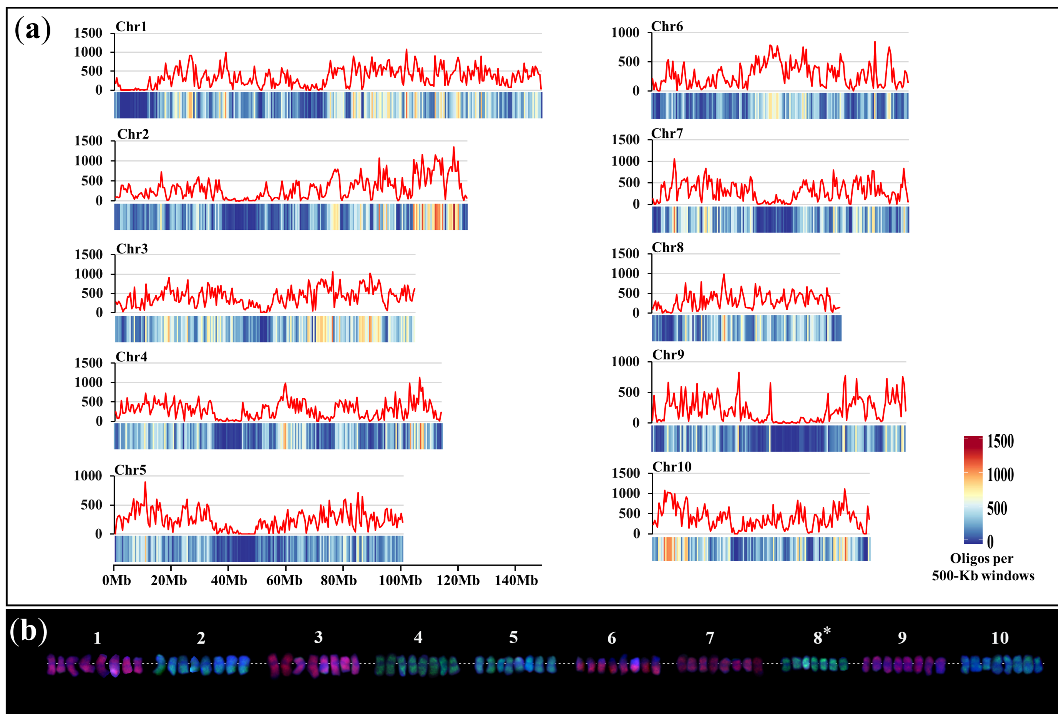

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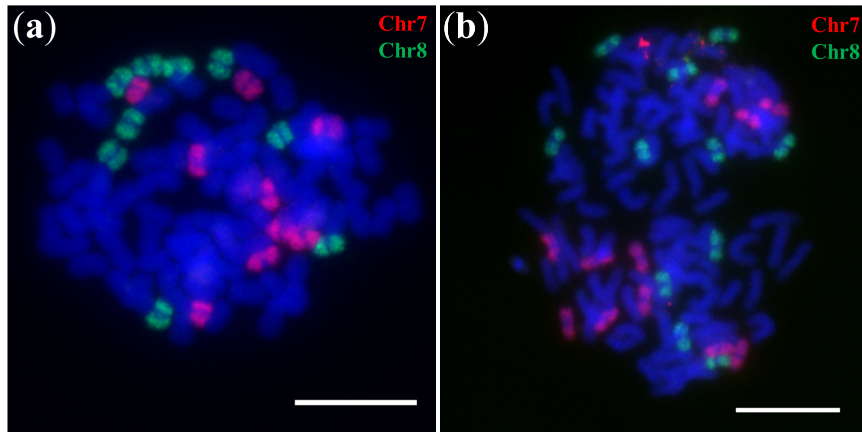

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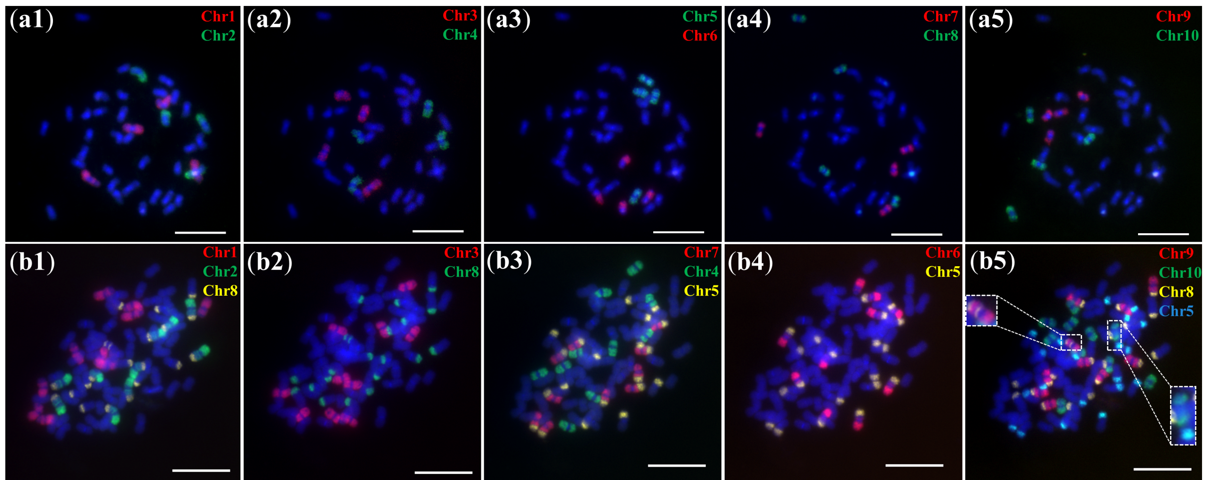

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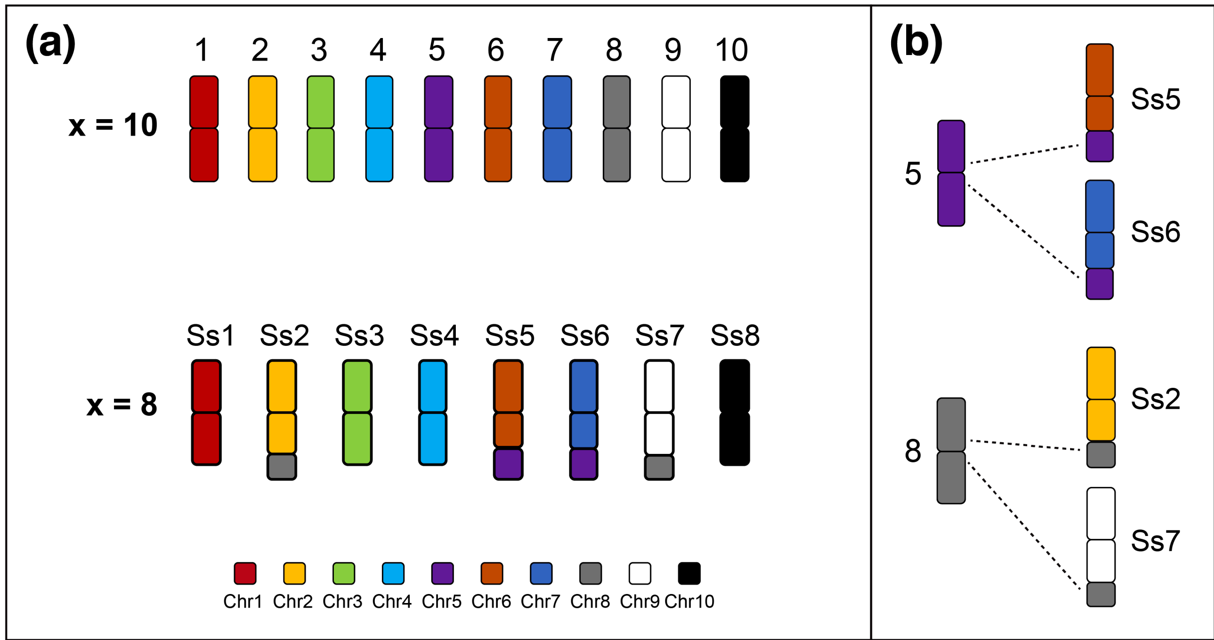

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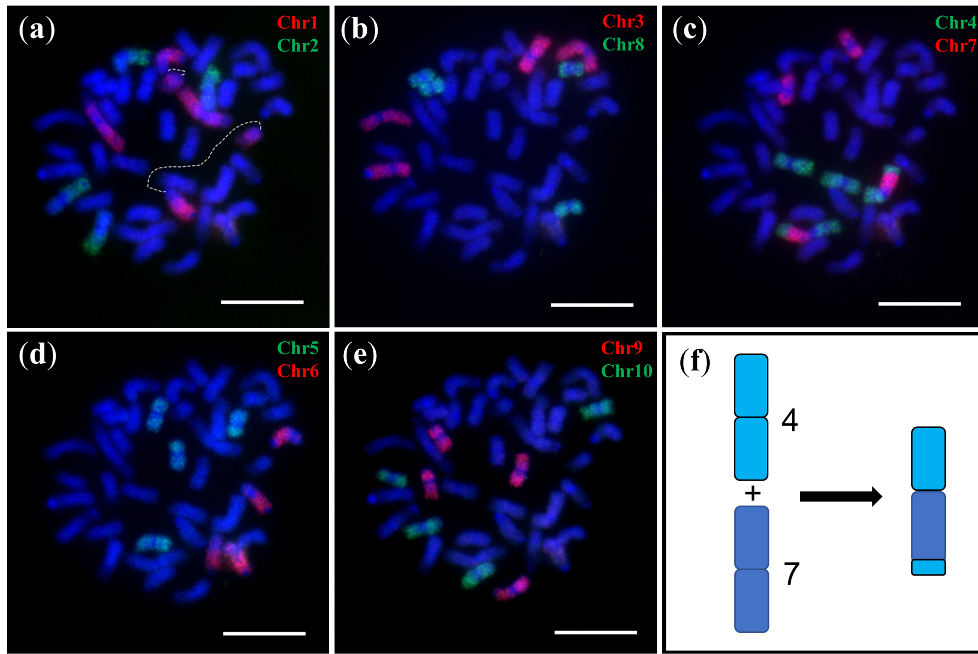

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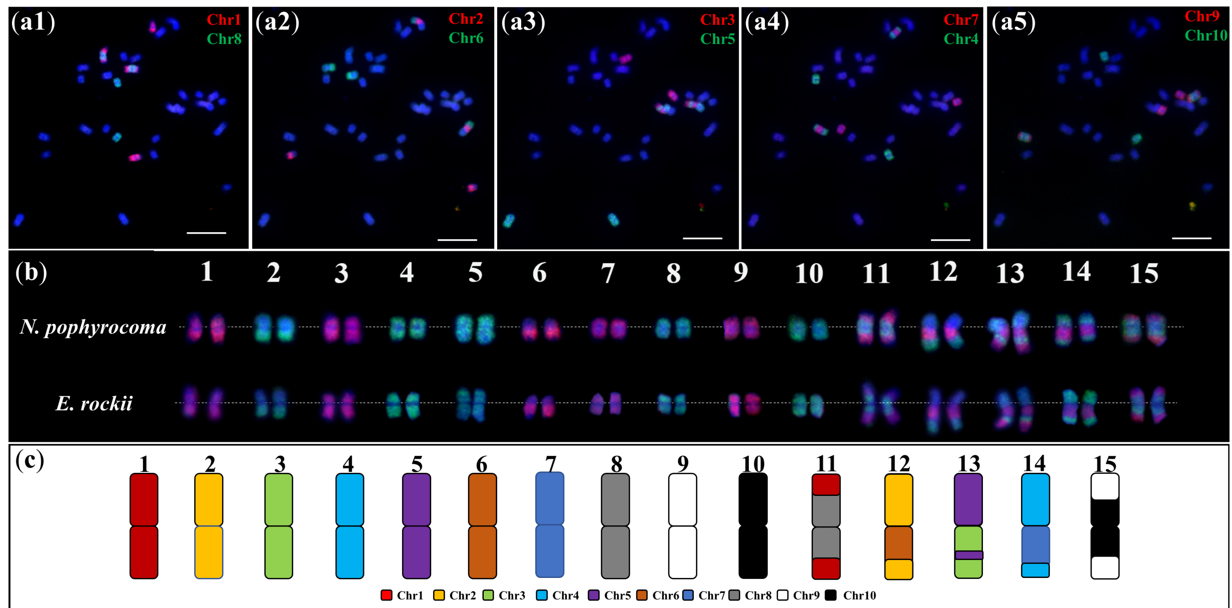

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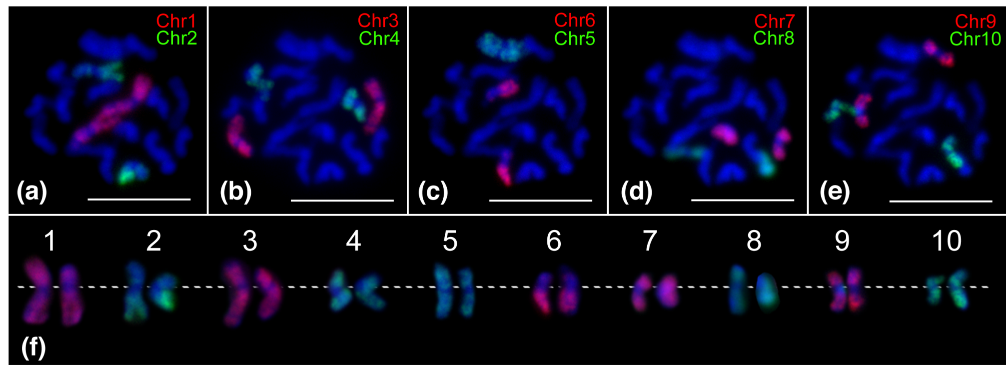

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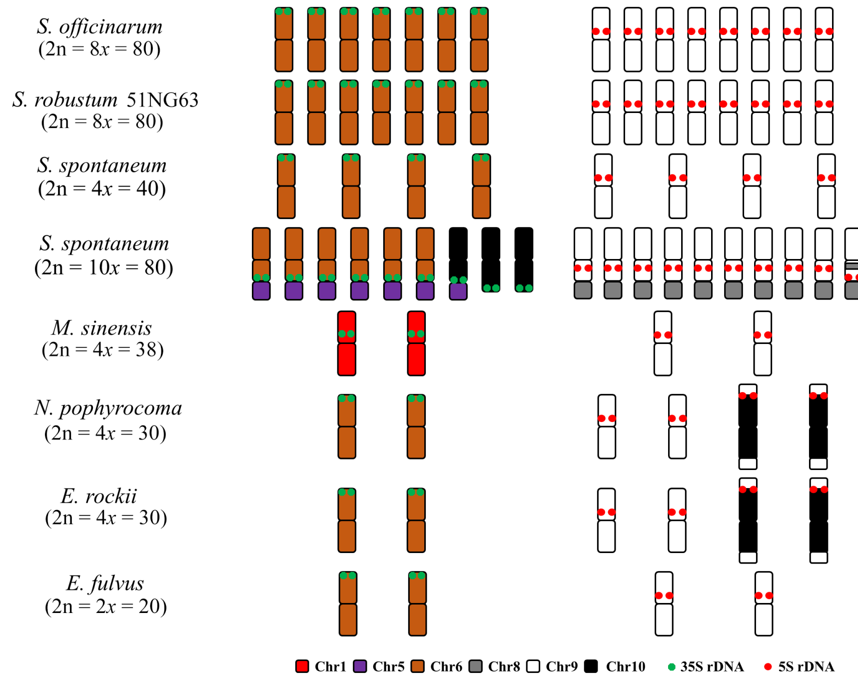

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