Unexpected Inheritance Pattern of *Erianthus arundinaceus* Chromosomes in the Intergeneric Progeny between *Saccharum* spp. and *Erianthus arundinaceus*

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**Abstract**

*Erianthus arundinaceus* is a valuable source of agronomic traits for sugarcane improvement such as ratoonability, biomass, vigor, tolerance to drought and water logging, as well as resistance to pests and disease. To investigate the introgression of the *E. arundinaceus* genome into sugarcane, five intergeneric F1 hybrids between *S. officinarum* and *E. arundinaceus* and 13 of their BC1 progeny were studied using the genomic in situ hybridization (GISH) technique. In doing so, we assessed the chromosome composition and chromosome transmission in these plants. All F1 hybrids were aneuploidy, containing either 28 or 29 *E. arundinaceus* chromosomes. The number of *E. arundinaceus* chromosomes in nine of the BC1 progeny was less than or equal to 29. Unexpectedly, the number of *E. arundinaceus* chromosomes in the other four BC1 progeny was above 29, which was more than in their F1 female parents. This is the first cytogenetic evidence for an unexpected inheritance pattern of *E. arundinaceus* chromosomes in sugarcane. We pointed to several mechanisms that may be involved in generating more than 2n gametes in the BC1 progeny. Furthermore, the implication of these results for sugarcane breeding programs was discussed.

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**Introduction**

Sugarcane (*Saccharum* spp.) plays a pivotal role in world agriculture as a primary sugar-producing crop and has significant potential as a renewable bioenergy crop [1]. The genus *Saccharum* is comprised of six species: The two wild species are *S. spontaneum* and *S. robustum*, and the four cultivated species are *S. officinarum*, *S. barberi*, *S. sinense* and *S. edule*. *S. officinarum* (2n = 80) is known as the noble cane due to its high sugar content and thick and juicy culms. The wild species *S. spontaneum* (2n = 40–128; chromosome number varies) has very low sugar content but exhibits high vigor, profuse tillers and strong ratooning ability, as well as resistance to diseases and pests. Modern sugarcane cultivars are highly complex polyploid aneuploids and typically have 100–130 chromosomes derived from a combination of these two species [2].

During the early 20th century, interspecific hybridization was used to introgress desirable traits from wild species into sugarcane cultivars, and this practice led to substantial improvements in sugarcane agriculture [3]. In particular, interspecific hybridization between *S. officinarum* as the female parent and *S. spontaneum* as the male parent, followed by successive backcrosses of the hybrids to different clones of *S. officinarum* as the recurrent parent, significantly increased cane yields and resistance to biotic and abiotic stresses. In this hybridization strategy, F1 hybrids and plants in the first backcross generation (BC1) receive 2n gametes from female parent and n gametes from male parent, and plants in the second backcross generation (BC2) receive n gametes from both the female and male parents [4]. The purpose of the process termed mobilization, which refers to the crossing and backcrossing of intergeneric hybrids to noble cane, was to retain high-sugar producing clones and to eliminate the negative effects of wild germplasm [5]. Over the past decades, much insight has been gained into the mechanisms underlying 2n gamete formation through the use of molecular genetic and cytological techniques. Using simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP) and diversity arrays technology (DarT), Hermann et al. [6] provided molecular marker data that suggested the mechanism was second division restitution (SDR) or megaspore tetrad cell fusion (MTCF). Bielig et al. [7] provided cytological evidence that 2n male gamete formation was probably...
attributable to SDR. Nevertheless, the mechanisms underlying 2n gamete formation in sugarcane are still not fully understood.

Indeed, the occurrence of 2n gametes is not rare in the plant kingdom and it has been reported in many genera, including *Brassica*, *Paspalum*, *Brachiaria*, *Citrus*, *Fragaria*, *Malus*, *Manihot*, *Medicago*, *Saccharum*, and *Trifolium* [8–11]. Several explanations for 2n gamete formation have been proposed, including pre-meiotic and post-meiotic genome doubling, and meiotic restitution. Among these possibilities, the majority of reports have identified a restitution of the meiotic cell cycle in several species [12–14], suggesting that it is the predominant mechanism of 2n gamete formation in plants. However, a small number of reports have documented pre-meiotic and post-meiotic genome duplications, indicating that these mechanisms of 2n gamete formation are quite rare. Three cytological processes can lead to 2n gamete formation during abnormal meiosis: first division restitution (FDR), second division restitution (SDR) and indeterminate meiotic restitution (IMR). In FDR, homologous chromosomes remain together when the nucleus fails to divide after telophase I, and after a normal second division, sister chromatids derived from each chromosome move to opposite poles. In SDR, normal separation of the homologous chromosomes at first division is followed by the absence of the second meiotic division and sister chromatids fail to migrate to opposite poles at second division. IMR has been best described in lily meiocytes, and simultaneously shows characteristics similar to both SDR and FDR within a single meiocyte [15–18].

Modern sugarcane cultivars have limited genetic diversity, due to the small number of progenitors used in the initial interspecific hybridizations during the process of nobilization [19–21]. This genetic bottleneck has impeded further sugarcane improvement for certain traits such as tolerance to biotic and abiotic stresses. Therefore, it is urgent to broaden the genetic base of sugarcane by introgressing favorable genes from closely related *Erianthus*, *Miscanthus*, *Narenga* and *Sclerostachya* genera [22].

*Erianthus* is one of the most closely related genera to *Saccharum* and has attracted the interest of sugarcane breeders worldwide. *Erianthus arundinaceus* (*E. arundinaceus*, 2n = 20, 40, 60) is one of eight species in the genus *Erianthus* [23], and it possesses valuable agronomic traits for sugarcane improvement such as high biomass, vigor, rootatenability, tolerance to drought and water logging, and resistance to pests and disease [23–28]. Favorable alleles can be introduced into modern sugarcane cultivars for yield and stability improvement, although the hybrid progeny is often sterile [26]. However, significant progress has been made to produce genuine F1 hybrids and to backcross the progeny successfully. Molecular markers and genomic in situ hybridization (GISH) techniques have been used to identify true intergeneric hybrids between *Saccharum* spp. and *Erianthus* spp. [23,24,26,29]. According to histological staining character of root tips, Fukuhara et al. [30] concluded that F1 hybrids were successfully obtained from the intergeneric hybridization between *Saccharum* spp. hybrid and *E. arundinaceus*.

Using GISH, N. Piperidis et al. [29] reported that chromosome transmission was n+n in both F1 (*S. officinarum* × *E. arundinaceus*) and BC2 (BC1 × sugarcane cultivar) generations, but was 2n+n in the BC1 (F1 × sugarcane cultivar) cross. In similar crosses six F1 hybrids had fewer than 70 chromosomes and one had more than 70, indicating that all F1 crosses were aneuploid [26]. In this report, we studied chromosome transmission in (*E. arundinaceus* × *S. officinarum*) hybrids by using GISH to determine the chromosome composition of two generations including five intergeneric F1 hybrids and 13 BC1 progeny.

**Materials and Methods**

**Plant materials**

The plant materials used in this study consisted of 18 clones derived from two generations of intergeneric hybrids (Table 1). The male parent of the F1 generation was either *E. arundinaceus*...
HN 92-77 (2n = 60) or HN 92–105 (2n = 60) from Hainan, China. *S. officinarum* Badila (2n = 80) was used as the female parent for the F1 generation. Female parents of the BC1 generation were YCE 95-41, YCE 96-40 and YCE 96-66, which were derived from crosses between Badila and HN 92-77 or HN 92–105. The male parent of the BC1 generation was CP 84–1198 (2n = 120), which is a commercial cultivar containing germplasm from *S. officinarum*, *S. spontaneum*, *S. barberi* and *S. robustum* without contribution from *E. arundinaceus*. F1 and BC1 plants were generated at the Hainan Sugarcane Breeding Station of Guangzhou Sugarcane Industry Research Institute. All clones were planted in the greenhouse at Fujian Agriculture and Forestry University.

**Genomic in situ hybridization procedure**

Chromosome preparation, chromosome spreading and GISH experiments were performed as described in D’hont et al. [24]. Genomic DNA from *E. arundinaceus* HN 92-77 and HN 92–105 was labeled with digoxigenin-11-dUTP (Roche) and genomic DNA from *E. arundinaceus* HN 92-77 and HN 92–105 was labeled with digoxigenin-11-dUTP (Roche) and genomic

**Figure 1. GISH analysis of the F1 hybrids and BC1 progeny.** *Saccharum* spp. chromosomes were visualized in red and *E. arundinaceus* chromosomes in green. (A) YCE 96-66 (F1): 29 chromosomes from *E. arundinaceus* and 40 chromosomes from *Saccharum* spp.; (B) YCE 01–102 (BC1): 22 chromosomes from *E. arundinaceus* and 96 chromosomes from *Saccharum* spp.; (C) YCE 01–36 (BC1): 36 chromosomes from *E. arundinaceus*, 96 chromosomes from *Saccharum* spp. and one terminally translocated chromosome; (D) YCE 01–61 (BC1): 31 chromosomes from *E. arundinaceus* and 85 chromosomes from *Saccharum* spp.; (E) YCE 01–69 (BC1): 31 chromosomes from *E. arundinaceus* and 88 chromosomes from *Saccharum* spp.; (F) YCE 01–92 (BC1): 35 chromosomes from *E. arundinaceus*, 95 chromosomes from *Saccharum* spp. and one terminally translocated chromosome. The arrowhead in Figure 1C and Figure 1F shows the translocated chromosome. Scale bars: 5 μm.

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### Table 2. Chromosome composition of F1 hybrids and BC1 progeny.

| Generation | Clones   | Chromosome Composition | No. of cells observed |
|------------|----------|------------------------|-----------------------|
|            |          | **Modal Number**       | **Range**             | **Modal Number**       | **Range**             | **Recombinants** |
|            |          | **Zn cell**             | **Saccharum spp.**    | **E. arundinaceus**    |                      |                |
| F1         | YCE 95-41| 68                     | 68–70                 | 40                     | 28                    | 28–30            | 0               | 4               |
| F1         | YCE 96-40| 69                     | 68–70                 | 40                     | 40                    | 29                | 28–30            | 0               | 21              |
| F1         | YCE 96-43| 69                     | 68–70                 | 40                     | 40                    | 29                | 28–30            | 0               | 4               |
| F1         | YCE 96-45| 69                     | 67–70                 | 40                     | 40                    | 29                | 27–30            | 0               | 12              |
| F1         | YCE 96-66| 69                     | 67–70                 | 40                     | 40                    | 29                | 27–30            | 0               | 22              |
| BC1        | YCE 01-33| 120                    | 118–121               | 93                     | 91–93                 | 27                | 26–28            | 0               | 5               |
| BC1        | YCE 01-46| 125                    | 122–127               | 96                     | 95–97                 | 29                | 28–29            | 0               | 6               |
| BC1        | YCE 01-48| 120                    | 117–121               | 93                     | 93–94                 | 27                | 26–29            | 0               | 15              |
| BC1        | YCE 01-63| 125                    | 123–126               | 97                     | 95–98                 | 28                | 26–28            | 0               | 8               |
| BC1        | YCE 01-99| 118                    | 117–120               | 95                     | 95–97                 | 23                | 21–23            | 0               | 4               |
| BC1        | YCE 01-102| 118                   | 115–119              | 96                     | 94–97                 | 22                | 19–24            | 0               | 23              |
| BC1        | YCE 01-105| 117                  | 115–119              | 94                     | 93–96                 | 23                | 22–23            | 0               | 12              |
| BC1        | YCE 01-116| 122                  | 119–124              | 94                     | 92–95                 | 28                | 26–29            | 0               | 10              |
| BC1        | YCE 01-134| 121                 | 120–122              | 93                     | 93–95                 | 28                | 26–29            | 0               | 8               |
| BC1        | YCE 01-36| 134                    | 130–136               | 96                     | 95–97                 | 36                | 35–36            | 1               | 10              |
| BC1        | YCE 01-92| 130                    | 129–132              | 95                     | 94–96                 | 35                | 34–36            | 1               | 8               |
| BC1        | YCE 01-61| 116                    | 114–118              | 85                     | 84–85                 | 31                | 30–31            | 0               | 3               |
| BC1        | YCE 01-69| 119                    | 115–120              | 88                     | 87–90                 | 31                | 29–32            | 0               | 8               |

**Note:** The modal number of chromosomes is presented for the sugarcane clones analysed, since small variation of chromosome counts can occur due to the loss or the overlapping of a few chromosomes from the preparation. doi:10.1371/journal.pone.0110390.t002
DNA from Badila and CP 84–1198 was labeled with biotin-16-dUTP (Roche) using the Nick Translation Kit (Roche). To detect signal from biotin-labeled probes, Avidin D, Rhodamine 600 (XRITC) and biotinylated anti-avidin antibody (Vector Laboratories, Burlingame, CA) were used. To detect signal from digoxigenin-labeled probes, sheep-anti-digoxin-FITC (Roche, Lewes, UK) and rabbit-anti-sheep-FITC (Roche, Lewes, UK) were used. Chromosomes were then counter stained using DAPI in Vectashield anti-fade solution Vectashield (Vector Laboratories, Burlingame, CA). The hybridization signals were observed on an AxioScope A1 Imager fluorescent microscope (Carl Zeiss, Gottingen, Germany). Images were captured digitally with an AxioCam MRc5 and AxioVision v.4.7 imaging software (Carl Zeiss, Gottingen, Germany).

Results and Discussion

Aneuploidy in F1 hybrids

Five F1 hybrids analyzed by GISH were characterized by the presence of 68–69 chromosomes, consisting of 40 Saccharum-derived chromosomes and 28–29 E. arundinaceus-derived chromosomes (Figure 1A, Figure S1–S4 in File S1). Therefore, the five F1 hybrids were the products of n+n chromosome transmission (Table 2), and all hybrids were also aneuploid. These results are consistent with G. Piperidis’s studies [26]. Notably, the high rate of aneuploidy in F1 hybrids contributes to the production of unbalanced gametes, which might be associated with the high degree of sterility in F1 hybrids [31,32]. Chromosomes inherited from divergent parents are often unable to pair with each other in meiosis [33–36], producing very few or no viable pollen grains [26,37]. In order to obtain backcross generations, an adjustment in sugarcane breeding was implemented, involving F1 hybrids as female parents and CP 84–1198 as the male parent, although this does not conform perfectly to the fundamental principles of backcross breeding. Even though F1 hybrids were used as female parents, their fertility was still very low. Among the five F1 hybrids, only YCE 96-40, YCE 96-66 and YCE 95-41 as female parents generated several BC1 progeny by backcrossing with CP 84–1198. Attempts to backcross YCE 96-43 and YCE 96-45 to CP 84–1198 were not successful.

Unexpected inheritance pattern in BC1 progeny

GISH analysis of nine BC1 progeny revealed plants with a total chromosome complement ranging from 117 to 125 (Table 2), of which 93 to 97 chromosomes were derived from Saccharum and...
22 to 29 chromosomes were derived from *E. arundinaceus* (Figure 1B, Figure S5–S12 in File S1). These results indicated that the nine BC₁ progeny were products of 2n+n transmission. G. Piperidis et al. [26] and N. Piperidis et al. [29] reported similar results. GISH analysis of another four BC₁ progeny revealed plants with a total chromosome complement ranging from 116 to 132, and evidence of an unusual mode of chromosome transmission (Table 2, Figure 1C–1F). In YCE 01–36, YCE 01–61, YCE 01–69 and YCE 01–92, 85 to 96 chromosomes were derived from *Saccharum* and 36, 31, 31 and 35 chromosomes were derived from *E. arundinaceus*, respectively. These results indicated that, in these four BC₁ progeny, more than 29 *E. arundinaceus*-derived chromosomes (Table 2, Figure 1) were transmitted, which is a greater number than was detected in the F₁ generation. To our knowledge, ours is the first report to document that the *E. arundinaceus*-derived chromosome number was above 29 in BC₁ progeny. Within the plant kingdom, this unusual phenomenon has rarely been reported. It is especially noteworthy that four BC₁ progeny out of 13 exhibited greater than 2n female-inherited chromosomes, suggesting that this newly discovered phenomenon can occur at relatively high frequency in sugarcane (above 30%).

**Possible mechanisms**

The unexpected inheritance pattern that we observed in BC₁ progeny is not in accordance with prevailing theories of chromosome transmission in hybrids. If meiosis occurs normally, four gametes are generated with different numbers of *E. arundinaceus*-derived chromosomes, due to the 29 *E. arundinaceus* chromosomes in F₁ hybrids. As a result, two gametes are produced containing 14 *E. arundinaceus* chromosomes and the other two gametes contain 15 *E. arundinaceus* chromosomes. (Figure 2A). In FDR, two gametes are produced with 29 *E. arundinaceus* chromosomes (Figure 2B). In SDR, one gamete is produced with 28 *E. arundinaceus* chromosomes and a second contains 30 *E. arundinaceus* chromosomes (Figure 2C). SDR has been extensively reported in sugarcane [7,38,39]. In addition,
Narayanaswami [40] discovered that 2n gametes originated from the fusion of the two innermost cells of the megaspore tetrad (megaspore tetrad cell fusion, MTCF). Post-meiotic restitution (PMR), in which chromosome doubling occurs after the second meiotic division, was observed by Bremer [38].

The normal separation of chromosomes in the first meiotic division or sister chromatids in the second meiotic division is called disjunction. Nondisjunction can occur in the first meiotic division (nondisjunction of homologous chromosomes; NHC) or second meiotic division (nondisjunction of sister chromatids; NSC). These distinct processes of nondisjunction create gametes with different numbers of chromosomes (Figure 2D, Figure 2E). In this study, we propose two possible mechanisms responsible for the formation of gametes with chromosome number greater than 2n. The first possibility (Model I) involves both NHC and SDR, which would generate two gametes with different even numbers of \( E. \) arundinaceus chromosomes after meiosis (Figure 2F; NHC + SDR). The second possibility (Model II) involves both FDR and NSC, which would generate two gametes with different odd or even numbers of \( E. \) arundinaceus chromosomes after meiosis (Figure 2G; FDR + NSC).

According to the results obtained from plants in the F₁ generation, their meiocytes contained 29 \( E. \) arundinaceus chromosomes. During S phase of pre-meiotic interphase, all chromosomes are duplicated and each chromosome is comprised of two sister chromatids. Consequently, after meiosis, the total number of \( E. \) arundinaceus chromosomes in the F₁ gametes should be 58. According to Model I, if there are \( i \) pair(s) of NHC in the first meiotic division, this yields a difference of \( 2(2i+1) \) \( E. \) arundinaceus chromosomes between the two gametes. According to Model II, if there are \( j \) chromosomes with NSC in the second meiotic division, this yields a difference of \( 2j \) \( E. \) arundinaceus chromosomes between the two gametes. Thus, the following two simultaneous linear equations are obtained:

**Model I**

\[
\begin{align*}
    g &= g' + 2(2i + 1) = g' + 4i + 2 \\
    g &= 58 - g'
\end{align*}
\]

**Model II**

\[
\begin{align*}
    g &= g' + 2j \\
    g &= 58 - g'
\end{align*}
\]
In these equations, \( g \) and \( g' \) are the total number of \( E. \) \textit{arundinaceus} chromosomes in each gamete after meiosis, and \( i \) and \( j \) are the number of NHC and NSC, respectively. From our experimental observations of 29 \( E. \) \textit{arundinaceus} chromosomes in a meioocyte and 50 \( E. \) \textit{arundinaceus} chromosomes in two gametes, we required that 0 \( \leq g \leq 50 \); 0 \( \leq g' \leq 50 \); 0 \( \leq i \leq 14 \); and 0 \( \leq j \leq 29 \). The four variables \( g \), \( g' \), \( i \), and \( j \) were integral. After solving these simultaneous linear equations, we obtained two formulas:

\[
\text{Model I: } g = 2i + 30 \\
\text{Model II: } g = 2j + 29
\]

Based on these formulas, graphs of these linear equations are shown in Figure 3 and Figure 4.

In this study, eight different \( E. \) \textit{arundinaceus} chromosome numbers were observed (22, 23, 27, 28, 29, 31, 35 and 36) in BC1 progeny. Due to the fact that we detected odd numbers of \( E. \) \textit{arundinaceus} chromosomes in BC1 progeny, we speculate that Model II may be a more likely mechanism than Model I, but Model I cannot be ruled out as a mechanism occurring in plants that inherited even numbers of \( E. \) \textit{arundinaceus} chromosomes. It is possible that both models are valid, suggesting that both mechanisms can occur.

Modern sugarcane cultivars are characterized by a high degree of inbreeding depression, so any increase in the heterozygosity of gametes may be beneficial to breeding efforts [6]. Depending on the specific mode of chromosome segregation, gametes can exhibit different degree of heterozygosity. During normal meiosis, crossing-over occurs between two non-sister chromatids. In FDR, 2n gametes always possess two non-sister chromatids and consequently maintain equivalent levels of parental heterozygosity and epistatic interactions. In SDR, sister chromatids do not separate and these gametes exhibit high levels of homozygosity. As a result, most parental heterozygosity and epistatic interactions are lost [41,42]. The highest degree of heterogeneity is found in gametes originated through IMR, since these gametes result from a mixture of FDR and SDR [15]. In addition, when chromatids migrate to the same pole as in NHC and NSC, chromosomes are doubled. In NHC+SDR or FDR+NSC, some \( E. \) \textit{arundinaceus} chromosomes would be doubled twice. This process likely creates a larger number of new multilocus allelic combinations and provides the opportunity to select the resulting germplasm for new, desirable traits.

Future directions

In order to understand the underlying mechanisms involved in generating the number of \( E. \) \textit{arundinaceus} chromosomes in BC1 progeny, detailed cytological observations of female gametes and chromosomal dynamics in the embryonic sac of \( F_1 \) hybrids are needed. Although difficult to access, a more thorough understanding of the megagametophyte may result in possible applications for improving sugarcane through 2n gamete transmission.

Supporting Information

File S1 Supporting Information Figures. Figure S1. YCE 95-41(F1); 28 chromosomes from \( E. \) \textit{arundinaceus} and 40 chromosomes from \( Saccharum \) spp. Figure S2. YCE 96-40(F1); 29 chromosomes from \( E. \) \textit{arundinaceus} and 40 chromosomes from \( Saccharum \) spp. Figure S3. YCE 96-43(F1); 29 chromosomes from \( E. \) \textit{arundinaceus} and 40 chromosomes from \( Saccharum \) spp. Figure S4. YCE 96-45(F1); 29 chromosomes from \( E. \) \textit{arundinaceus} and 40 chromosomes from \( Saccharum \) spp. Figure S5. YCE 01–33 (BC1); 27 chromosomes from \( E. \) \textit{arundinaceus} and 93 chromosomes from \( Saccharum \) spp. Figure S6. YCE 01–46 (BC1); 29 chromosomes from \( E. \) \textit{arundinaceus} and 96 chromosomes from \( Saccharum \) spp. Figure S7. YCE 01–48 (BC1); 27 chromosomes from \( E. \) \textit{arundinaceus} and 93 chromosomes from \( Saccharum \) spp. Figure S8. YCE 01–63 (BC1); 28 chromosomes from \( E. \) \textit{arundinaceus} and 97 chromosomes from \( Saccharum \) spp. Figure S9. YCE 01–99 (BC1); 23 chromosomes from \( E. \) \textit{arundinaceus} and 95 chromosomes from \( Saccharum \) spp. Figure S10. YCE 01–105 (BC1); 23 chromosomes from \( E. \) \textit{arundinaceus} and 94 chromosomes from \( Saccharum \) spp. Figure S11. YCE 01–116 (BC1); 28 chromosomes from \( E. \) \textit{arundinaceus} and 94 chromosomes from \( Saccharum \) spp. Figure S12. YCE 01–134 (BC1); 28 chromosomes from \( E. \) \textit{arundinaceus} and 93 chromosomes from \( Saccharum \) spp.

(DOC)

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Author Contributions

Conceived and designed the experiments: JYW YJH ZHD. Performed the experiments: JYW YJH ZHD. Analyzed the data: JYW YJH. Contributed reagents/materials/analysis tools: YQL CF SML QWL ZXH RKC. Designed the picture: YJH.

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