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Ploidy Identification and Analysis of *Saccharum* and Its Related Genera

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Abstract In order to provide basis for the genetic improvement efficiency of sugarcane, and to find out the ploidy of sugarcane and its related genera and cultivated species, the ploidy of sugarcane and its related genera was systematically analyzed and identified by flow cytometry for the first time in this study. The results showed that the ploidy of the tropical species of *Saccharum* was 8 and 10, in which the ploidy of typical tropical species Badila was 8 and that of atypical tropical species Kala Wa was 10. The ploidy of *S. barberry* was 8 and 16.8, and the ploidy of Mungo was 8 and that of Pansahi was 16. The ploidy of *S. sinense* Yuba and reed cane was 9.8 and 10, respectively. The ploidy of *S. spontaneum* was 6.3~9.2 and most of them were about 8, while the ploidy of *S. robustum* was 14.4. The ploidy of *Erianthus*, the related genera of *Saccharum*, was 3.3~7.9, and the ploidy of *S. arundinaceum* and Erianthus rockii was 7.1~7.9 and 3.3~3.8, respectively. There were two kinds of ploidy in *Miscanthus* (1.4 and 4.8) and the ploidy of *Narenga* was 3.6~5.2. The ploidy of hybrid cultivars of *Saccharum* was relatively higher, which was 8.2~12.3, and ploidy of 9~10 was the most common. The ploidy of *Saccharum* and its related genera and cultivated species was systematically proved for the first time in this research, and the lowest ploidy was in *Miscanthus* (1.4) and the highest ploidy was in Pansahi (16.8), indicating that *Saccharum* was an aneuploid plant with complex genetic background. The results of this study could be used to guide the configuration of sugarcane hybrid combinations.

Keywords *Saccharum*, Related genera, Ploidy identification, Flow cytometry

Background

Sugarcane is not only an important sugar crop in China, but also an important energy crop. With the continuous improvement of national living standards, people's demand for sugar consumption is also increasing. Sugarcane industry will play a more important role in the development of Chinese economy and society in the future. China's sugarcane producing areas are mainly concentrated in Guangxi, Yunnan, Guangdong, Hainan and Fujian (Li, 2010, China Agriculture Press, pp.102-156; Chen et al., 2015; Li et al., 2015; Xu et al., 2015; Li et al., 2016). Among them, Guangxi has the largest planting area, accounting for more than 65% of the national area, followed by Yunnan and Guangdong (Liu et al., 2017).

Saccharum belongs to Gramineae, Andropogoneae, Saccharinae, which is a C_4 high photosynthetic capacity plant. It contains six protospecies, including *S. officinarum*, *S. barberi*, *S. sinense*, *S. spontaneum*, *S. robustum* and *S. edule*. Among them, *S. edule* does little to the breeding of *Saccharum*. The related genera of *Saccharum* mainly contain *Erianthus* Michx., *Sclerostachya* (Hach.) A. Camus, *Narenga* Bor, *Miscanthus* Anderss et al., which are the main sources of *Saccharum* resistance. These related genera are collectively referred to as "*Saccharum* complex" together with *Saccharum* (Grivet and Arruda, 2001; Cai et al., 2002; Li, 2010, China Agriculture Press, pp.102-156; Liu et al., 2012a; 2012b).

Ploidy identification is an important link in ploidy breeding and application. Understanding plant ploidy can

effectively reduce blindness in breeding and accelerate the breeding process (Li et al. 2010, China Agriculture Press, pp.102-156). The methods of plant ploidy identification mainly include morphological identification, physiological and biochemical identification, fertility status identification, cytological identification, molecular level identification and so on. Sugarcane is an aneuploid heteroploidy plant with very complex heredity, many chromosomes and huge genome. The number of chromosomes varies greatly among different species or different types within the same species. 2n=100~130. It mainly contains three kindreds, which are *S. spontaneum*, *S. officinarum* and *S. barberi* (Grivet and Arruda, 2001; Li, 2010, China Agriculture Press, pp.102-156; Wang et al., 2015a; Hu et al., 2016). The genetic behaviors of sugarcane chromosomes are n+n, n+2n, 2n+n and 2n+2n, with complex transmission mode. Also, n often changes, which is not the original number of ploidy and is accompanied by increase or decrease. That is to say, "Unbalanced Inheritance" phenomenon occurs (Bremer, 1924; Huang et al. 2015). Therefore, compared with other homozygous crops, it is difficult to accurately identify sugarcane ploidy by conventional phenotypic analysis and cytological identification in the practical work of sugarcane breeding. At present, the studies on ploidy identification of sugarcane at home and abroad are only found in the typical tropical species Badila, which is an octoploid and 2n=10x=80 (Whalen, 1991; Irvine, 1999; Wang et al. 2008; Wang et al. 2015b). Ploidy identification of other sugarcane species and related genera has not been reported.

Flow cytometry (FCM) is a technique developed in 1970s. It is a method of molecular level identification. It mainly analyzes a large number of cells in tissues and determines the DNA content of isolated nuclei, as well as the DNA content of crops increases with the increase of ploidy, so as to judge the ploidy level. Flow cytometry is not limited by the sampling site and the cell stage, which is simple, sensitive, accurate and reproducible. It can rapidly detect the ploidy of plant (Jin et al., 2016). Up to now, flow cytometry has been widely used in ploidy analysis of rice (Tan et al., 2014), *Magnolia liliflora* (Zhao et al., 2016), mulberry (Yanget al., 2017) and other crops. In this study, the ploidy of sugarcane and its related genera was systematically identified and analyzed by flow cytometry for the first time in order to provide theoretical basis and scientific basis for conventional sugarcane breeding and distant cross utilization of germplasm resources, especially wild germplasm resources.

1 Results and Analysis

1.1 Ploidy identification of *Saccharum*

In this study, the typical tropical species Badila (2n=80, octaploid) was set as the control to identify the ploidy of *Saccharum* materials. Among them, the ploidy of the atypical tropical species Kala Wa, *S. barberry* Mungo and Pansahi was 10, 8 and 16.8, respectively; The ploidy of *S. sinense* Uba and Luzhe was 9.8 and 10; The ploidy of GSM56, GSM84-24, GSM85-2 and GSM79-9 in *S. spontaneum* was 7.7, 8.6, 9.2 and 6.3, respectively. The ploidy of both Yacheng *S. spontaneum* 12 and Fujian *S. spontaneum* 2 was 8; The ploidy of *S. robustum* was 14.4 (Figure 1; Table 1).

1.2 Ploidy identification of the related genera of sugarcane

From the data of ploidy detection (Table 1), we can see that among the related genera of sugarcane, the ploidy of

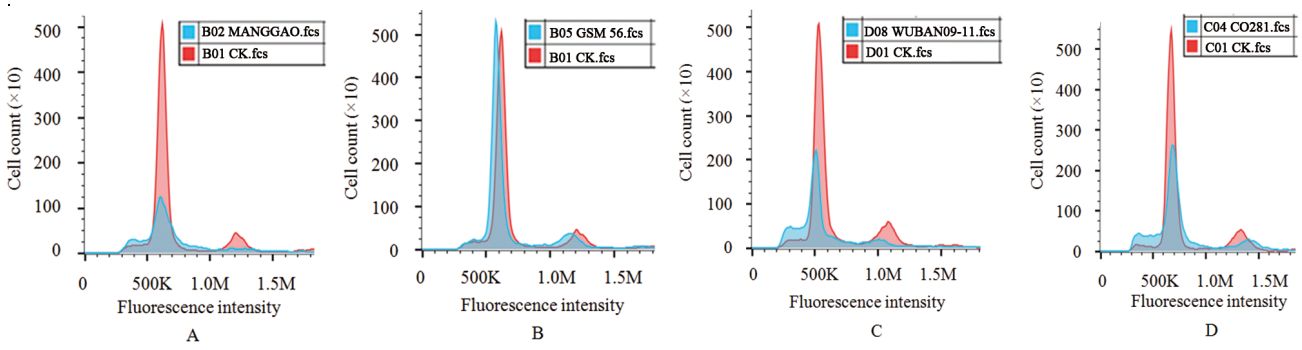

Figure 1 Sugarcane ploidy detection

Note: A: Mungo belonging to *S. barberi*; B: GSM56 belonging to *S. spontaneum*; C: WMBM09-11 belonging to *Erianthus* Michx.; D: Sugarcane hybrid cultivar Co281; Red peak: The fluorescence intensity of CK; Blue peak: The fluorescence intensity of the sample

Table 1 The data of ploidy detection

Genus	Species	Clones	Ploidy	Genus	Species	Clones	Ploidy
Saccharum L.	*S. officinarum*	Badila	8.0	Related genera	*Miscanthus* Anderss	M1	1.4
		Kala Wa	10.0			M84-7	4.8
	S. barberi	Mungo	8.0		*Narenga* Bor	GXN1	3.8
		Pansahi	16.8			GXN2	5.2
	S. sinense	Uba	9.8			GXN5	4.7
		Luzhe	10.0			HJCN	3.6
	S. spontaneum	GSM56	7.7	Hybrid cultivar		POJ2878	10.1
		GSM84-24	8.6			Co281	8.5
		GSM85-2	9.2			F134	8.6
		GSM79-9	6.3			CP49-50	9.4
		YCGSM12	8.0			ROC22	10.1
		FJGSM2	8.0			GT11	9.8
						GT21	9.7
	S. robustum	*S. robustum*	14.4			GT28	10.7
Related	*Erianthus*	GXBM32	7.6			GT29	9.1
genera	Michx.	XJZSBM3	7.6			GT42	9.4
		PXBM6	7.6			GT43	9.2
		WMBM09-11	7.6			GT45	11.4
		BM85-48	7.1			GT46	8.2
		BM52	7.9			LC05-136	12.1
		YFBM11	7.1			YT93-159	12.3
		SHBM4	7.8			GF1	9.9
		DZM1	3.3			GF2	9.6
		DZM2	3.8				

Miscanthus has two types (1.4, 4.8). In *S. arundinaceum* of *Erianthus*, there are four ploidy types (7.1, 7.6, 7.8, 7.9), while in *E. rockii* of *Erianthus*, there are two ploidy types (3.3, 3.8). The ploidy of *Narenga* is between 3.6 and 5.2. The ploidy of the related genera of sugarcane ranges from 1.4 to 7.9, with the lowest ploidy in *Miscanthus* (1.4) and the highest in *S. arundinaceum* (7.9). The genetic background of the related genera with higher ploidy is relatively complex. The ploidy results demonstrate again that sugarcane is an aneuploid plant, especially the ploidy of these wild sugarcane germplasms also exists as aneuploidy.

1.3 Ploidy identification of sugarcane hybrids

From the data of ploidy detection (Table 1), we can see that compared with the related genera of sugarcane, the ploidy of sugarcane hybrids has more types and larger value which is between 8.2~12.3. For example, the ploidy of POJ2878 and ROC22 is 10.1, Co281 is 8.5, F134 is 8.6, CP49-50 and GT42 is 9.4, GF1 is 9.9 and YT93-159 is 12.3. Sugarcane hybrids are the materials selected and bred from the conventional sugarcane breeding. They have more chromosomes and more complex genetic background. The ploidy of sugarcane hybrids should be larger than that of wild sugarcane varieties. The ploidy of sugarcane hybrids tested in this study was greater than that of related genera, among which nonuploid and decaploid were more common.

2 Discussion

2.1 Ploidy analysis of sugarcane and its related genera

In the complex of *Saccharum*, there are only reports about the ploidy of the typical tropical species Badila which is 8 at present (Whalen, 1991; Irvine, 1999; Wang et al., 2008; Wang et al., 2015b). This study is the first systematic study on ploidy of sugarcane and its related genera. In previous reports (Li, 2010, China Agriculture Press, pp.102-156), the chromosome numbers of the typical tropical species Badila, the atypical tropical species Kala Wa, *S. barberi*, *S. sinense*, *S. spontaneum*, and *S. robustum* were 2n=80, 2n=124~126, 2n=82~124, 2n=116~118, 2n=32~128 and 2n=60 or 80, respectively. The results of this study indicated that except the ploidy

of the typical tropical species Badila which was 8, the ploidy of the atypical tropical species Kala Wa was 10; the ploidy of *S. barberi* was 8 or 16.8; the ploidy of *S. sinense* was 10 or close to 10; the ploidy of *S. spontaneum* mainly was close to 8; and the ploidy of *S. robustum* was 14.4. It was thus clear that the ploidy of the tested tropical species, *S. barberi*, *S. sinense*, *S. spontaneum*, and *S. robustum* was between 6.3 and 16.8, and the agronomic traits among these species were also quite different. Whether the results of this study with large ploidy differences in *Saccharum* are related to their agronomic traits remains to be further studied. Among the tested materials, GSM79-9 of *S. spontaneum* has the lowest ploidy (6.3), while Pansahi of *S. barberi* has the highest ploidy (16.8). Most of the ploidy of *S. spontaneum* is about 8, indicating that most of the *S. spontaneum* species belong to octoploid. The chromosome number of *S. robustum* in *Saccharum* is relatively small, and other species have larger chromosome number, but *S. robustum* maintains a high ploidy level. Whether this indicates that *S. robustum* has more relatively complex genetic background information needs further investigation.

The results of this study showed that in the related genera of *Saccharum*, *Miscanthus* had the lowest ploidy (1.4). The genetic background of this material was relatively simple, but *Miscanthus* also contained species which had the ploidy of 4.8. Previous studies had shown that the chromosome number of *Miscanthus* was 2n=38~114 (Cai et al., 2002; Li et al., 2010, China Agriculture Press, pp.102-156). Chromosome numbers ranged from low to high, which might be the main reason for the ploidy types of 1.4 and 4.8 in *Miscanthus*. *Narenga* is a wild species in sugarcane complex, which has small chromosome number (2n=30) (Cai et al., 2002; Li, 2010, China Agriculture Press, pp.102-156). The ploidy of *Narenga* mainly concentrates between 3.8 and 5.2, with little difference, which may be related to its relatively stable chromosome number. Most *S. arundinaceum* of *Erianthus* (2n=60 mainly) (Cai et al., 2002) has the ploidy of 7~8, inferring that *S. arundinaceum* should belong to the species with stable ploidy.

In the hybrids of sugarcane, there are many ploidy types and the ploidy is relatively high, with the variation between 8.3~12.3. The reason may be that these hybrids are the varieties (lines) produced in the process of sugarcane "Nobilization" breeding. After hybridization, the number of chromosomes is more than 100. They contain *S. officinarum*, *S. barberi* and *S. sinense* (Li, 2010, China Agriculture Press, pp.102-156), and have relatively more heterologous genetic materials, and the genetic background is more complex, which leads to the increase of ploidy size and ploidy types. For instance, POJ2878 is the hybrid progeny of *S. spontaneum* and *S. officinarum*, which aggregates the genetic background of the two species. The ploidy of POJ2878 is 10.1, which is higher than the ploidy of the tested *S. spontaneum* species. However, there is also a special case, that is, the ploidy of the original species is higher than that of the hybrid varieties. For example, the ploidy of Pansahi of *S. barberi* is 16.8, which is higher than that of all the cultivated species tested. Whether this indicates that sugarcane protospecies Pansahi also has complex genetic background needs further study.

The results of this study indicated that the ploidy of most sugarcane materials tested was aneuploidy, which again illustrated that sugarcane might be an aneuploid plant with complex genetic background. Generally speaking, the higher the ploidy, the more the genome and the more complex the genetic background. Therefore, in the routine sugarcane breeding, it is suggested that the ploidy of the materials used should be clearly understood before the intraspecific and interspecific distant hybridization. The efficiency of genetic improvement of sugarcane varieties could be improved more effectively by selecting materials with high or low ploidy according to breeding objectives.

2.2 Feasibility of flow cytometry in ploidy identification of sugarcane

Flow cytometry has been successfully applied to sugarcane. Previous studies have used this technique to analyze genome size of different sugarcane varieties (lines) (Oliveira et al., 2015; Mondal et al., 2017), but no report has been reported to identify ploidy of sugarcane. Sugarcane is an allopolyploid plant. Except for some protospecies, such as Badila (2n=80), *Narenga* (2n=30), *Saccharum arundinaceum* (2n=60), other genera and species of sugarcane have many chromosomes, and there is no complete unified number. The chromosome number of a

sugarcane material could only be represented by the mode number (Cai et al., 2002; Li, 2010, China Agriculture Press, pp.102-156). The determination of the ploidy of sugarcane by morphological identification, physiological and biochemical index identification, fertility status identification and cytological identification is cumbersome and difficult.

Flow cytometry is based on the analysis of nuclear DNA content detected to determine ploidy. The more cells and the higher DNA content were analyzed, the more accurate the results were. In this research, the detected cell number of Badila, GXN1, Pansahi, Mungo, Luzhe and POJ2878 was 14 891, 15 824, 8 862, 5 193, 6 522 and 8 291, respectively. The detected cell number in the tested materials was all over 5 000, which was consistent with previous studies on other crops (Wu et al., 2014; Yang et al., 2017). Therefore, the results obtained in this study could be more accurate. Flow cytometry provides a reliable and rapid method for ploidy identification of heteroploid plants such as sugarcane.

3 Materials and Methods

3.1 Experimental materials

In this study, 46 sugarcane materials and its related genera were used (Table 2). Among them, there were 13 *S. officinarum*, *S. barberi*, *S. sinense*, *S. spontaneum* and *S. robustum*, 16 *Erianthus*, *Miscanthus* and *Narenga* of related genera, and 17 hybrid varieties. The materials were conserved in Hainan Sugarcane Hybrid Breeding Base of Sugarcane Research Center of Chinese Academy of Agricultural Sciences and Sugarcane Germplasm Resources Nursery of Sugarcane Research Institute of Guangxi Academy of Agricultural Sciences. The stems of the materials were collected on 14th July, 2017 and placed in ice boxes. After cleaning in the laboratory, the materials were put in a refrigerator at 4℃ for use. The reagents used were as follows: solution A: 10 mmol/L $MgSO_4 \cdot 7H_2O$ 0.246 g, 50 mmol/L KCl 0.370 g and 5 mmol/L Hepes 120 g. The volume of solution A was set to

Table 2 Names of tested sugarcane clones

Genus	Species	Clones	Origins	Genus	Species	Clones	Origins
Saccharum L.	*S. officinarum*	Badila	Unknon	Related	*Miscanthus*	M1	Guangxi
		Kala Wa	Unknon	genera	Anderss.	M84-7	Yunnan
	S. barberi	Mungo	India		*Narenga* Bor	GXN1	Guangxi
		Pansahi	India			GXN2	Guangxi
	S. sinense	Uba	Unknon			GXN5	Guangxi
		Luzhe	Sichuan			HJCN	Guangxi
	S. spontaneum	GSM56	Guangxi	Variety		POJ2878	Jawa
		GSM84-24	Guangxi			Co281	India
		GSM85-2	Guangxi			F134	Taiwan
		GSM79-9	Guangxi			CP49-50	America
		YCGSM12	Hainan			ROC22	Taiwan
		FJGSM2	Fujian			GT11	Guangxi
						GT21	Guangxi
	S. robustum	*S. Robustum*	Unknon			GT28	Guangxi
Related genera	*Erianthus*	GXBM32	Guangxi			GT29	Guangxi
	Michx.	XJZSBM3	Guangxi			GT42	Guangxi
		PXBM6	Guangxi			GT43	Guangxi
		WMBM09-11	Guangxi			GT45	Guangxi
		BM85-48	Guangxi			GT46	Guangxi
		BM52	Guangxi			LC05-136	Guangxi
		YFBM11	Guangxi			YT93-159	Guangdong
		SHBM4	Guangxi			GF1	Fiji
		DZM1	Yunnan			GF2	Fiji
		DZM2	Yunnan				

100 mL by ddH_2O and the pH was adjusted to 8.0. Solution B: 375 μL Triton X-100 was added to 14.3 mL A buffer solution, and then 300 μL PI and 15 mg DTT were added to the mixture. Solution C: 7.5 μL RNAase was added to and mixed with 3 mL solution B, which was then stored at 4℃ for use. The main instrument used was flow cytometry BD Accuri C6 plus.

3.2 Ploidy detection

Pull out the outer leaves of sugarcane slightly and take 50 mg of the inner tip tissue; Add 1 mL solution B and cut up the tip (<0.5 mm); The mixture was screened to EP tube through 33 μm screen mesh; 13 000 r/min centrifugation for 20 s, discarding supernatant; Resuspension with 200 μL solution C and incubation at 37℃ for 15 min. Flow cytometry BD Accuri C6 plus was used to detect and the threshold was 5 000. FSC-SSC was used to construct scatter diagram, and main cell mass was circled. FL2-A-FL2-H was used to construct scatter diagram, and PI-positive and non-adherent cells in the upper right corner were circled. Then FL2-A was used to construct histogram and abscissa was changed to logarithmic form. Set Badila (octaploid) as the control, and the ploidy of the tested materials was calculated according to sample ploidy = (Control ploidy × Peak fluorescence intensity of samples)/Peak fluorescence intensity of internal reference (Oliveira et al., 2015; Mondal et al., 2017).

Authors' Contributions

LXH was the designer and executor of the experiment in this study; ZRH, GYY, WJJ and QHP participated in experiment implement and results analysis; DWX was involved in materials planting; HDL and LYR were responsible for the project conception, test design and paper revision. All authors read and agreed to the final manuscript.

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