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Determination of deoxyribonucleic acid content and relative 2C genome sizes of some promising commercial varieties of sugarcane using flow cytometer

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ABSTRACT

In this study, 2C deoxyribonucleic acid (DNA) content and the genome sizes (in pictograms [pg] and megabase pairs [Mbp], respectively) of 19 promising commercial varieties of sugarcane, the derivatives of man-made interspecific hybrids between cultivated and wild species were analyzed using flow cytometry. In this work, 2C nuclear DNA content was determined. Knowing the 2C nuclear DNA content, the unknown chromosome numbers of the varieties could be predicted. Large differences (65% variation) in DNA content (2C) of 19 varieties were detected, ranging, from 3.80 to 10.96 pg, which corresponds to a genome size ranging from 3724.00 to 10740.80 Mbp due to the variation of ploidy level and are considered the most complex genomes among crop plants. However, the relationship between chromosome number and genome size was highly significant ($P < 0.001$). In this study, internode diameter, sugar juice content and cane yield/ha are also positively correlated with DNA content. The estimated genome sizes would also yield information critical for sugarcane breeding and genome sequencing programs.

KEY WORDS: Deoxyribonucleic acid content, genome size, flow cytometry, sugarcane varieties

INTRODUCTION

Sugarcane (*Saccharum* spp., Poaceae), is a large perennial vegetatively propagated crop mostly grown in the tropical and subtropical environments globally for its sugar-rich stalks and accounts for 80% of world sugar production (glycophyte). It is one of most efficient crops in converting solar energy into chemical energy because sugarcane is utilizing the C_4 pathway of photosynthesis. Sugarcane is a first generation biofuel crop used for ethanol production as an alternative source of energy (Lam *et al*., 2009), and has proven to be an efficient feedstock for generating biofuel.

Species within the genus *Saccharum* are well delineated by cytogenetics and well characterized by morphological characters (Bremer, 1961; Daniels and Roach, 1987; Nair, 1975, Price, 1960; Roach, 1969; Sreenivasan *et al*., 1987). Variation in chromosome numbers ranges from 2n = 40 (*Saccharum spontaneum*) to 2n = 130 (interspecific hybrids) with a basic number(x) set at 8-10 (d'Hont *et al*., 1998). Commercial sugarcane cultivars are complex alloploids or interspecific hybrids that originated from crosses between a few parents belonging mostly to the *Saccharum officinarum* and *S. spontaneum* species (Sreenivasan *et al*., 1987). The genome of sugarcane is large and complex originating from hybrids between two wild polyploidy relatives, *S. officinarum* and *S. spontaneum*. The application of genomic techniques in such a complex genome has been more challenging than in simpler (e.g., diploid) genomes. However, little information is available on the nuclear genome size and deoxyribonucleic acid (DNA) content in sugarcane. Such information can be valuable in understanding the cytogenetic phenomena in wide crosses (Burner, 1997) and complement conventional and molecular germplasm development programs aimed at increasing genetic diversity and gene exchange. Knowing the genome sizes of various *Saccharum* varieties may help in the utilization of sugarcane genetic resources for breeding program and improvement of sugarcane. Sugarcane is vegetatively propagated, and most commercial sugarcane

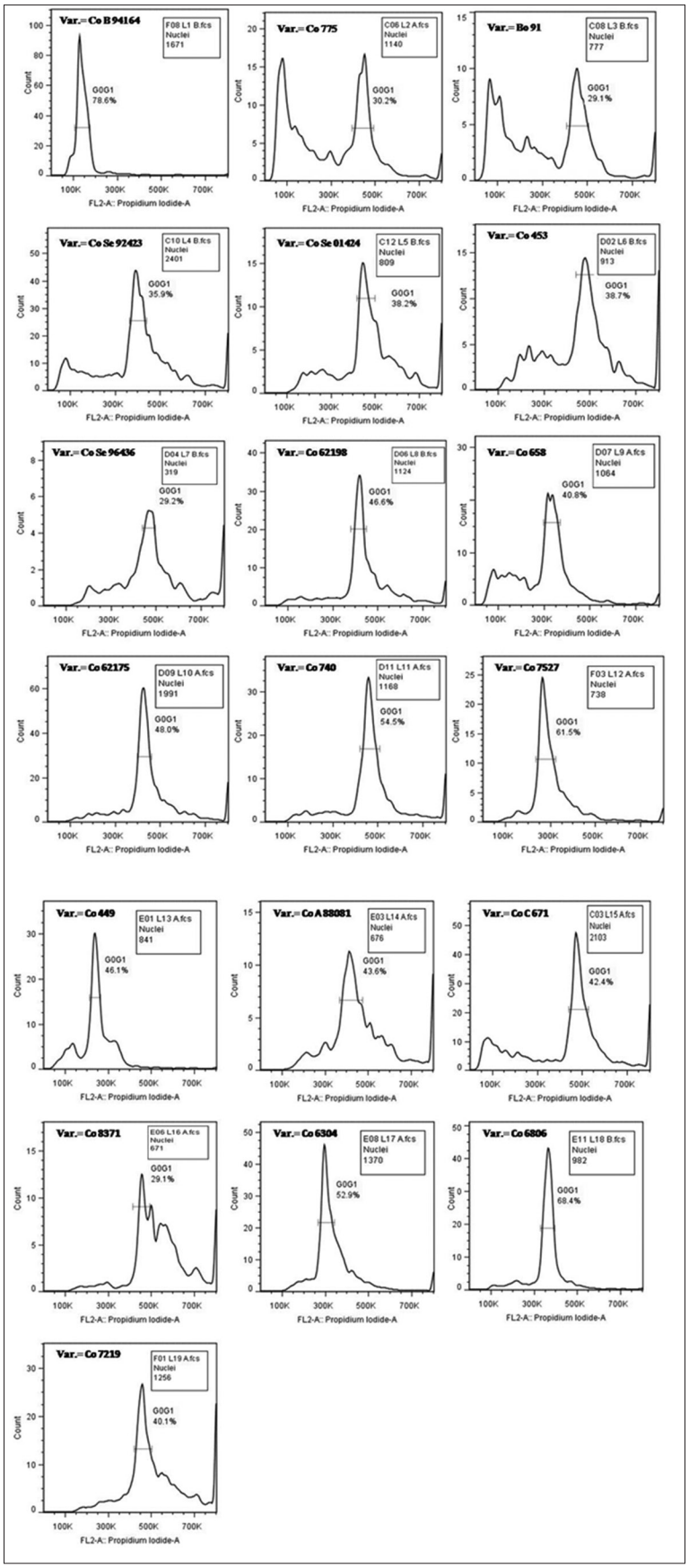

Figure 1: Flow cytometric analyses of 19 varieties of sugarcane showing the graphical profiles of peak that corresponds to the gated G0/G1 phase (2C level) of the cell cycle and cells are stained with propidium iodide

varieties are probably only a few sexual generations from wild plants suggesting significant potential for further genetic improvement.

Flow cytometry is a simple and efficient technique that is used more commonly to determine ploidy levels (De Laat *et al*., 1987), nuclear DNA content and genome size (Arumuganathan and Earle, 1991b; Bennett and Leitch, 1995; Dolezel, 1997). Cytometric determination of nuclear DNA content has proved to be useful in studying the variation in DNA content in plants (Dolezel *et al*., 1989; Hammat *et al*., 1991). The benefits of this technique have not been exploited yet in sugarcane. Arumuganathan and Earle (1991a) is the only report to list the DNA content (2C) and genome size of four *Saccharum* species.

Sugarcane breeders assume that an increase in DNA content in hybrids would be accompanied by an increase in cane and sugar yields (Burner, 1997). The objectives of this study were to estimate nuclear DNA content and genome size in hybrids and their parents.

In this study, 19 commercial varieties of *Saccharum* were selected to estimate the genome sizes using flow cytometry. Our main objective was to assess the degree of genome size variation within and among the selected *Saccharum* varieties.

MATERIALS AND METHODS

About 19 commercial varieties of *Saccharum* were collected from the National Hybridization Garden Sugarcane Breeding Institute, Coimbatore (11°1′6″N 76°58′21″E, elevation = 411.2 m [1349.1 ft above mean sea level]), Tamil Nadu, India, and also used as experimental materials for analyzing nuclear DNA content. Name of the selected varieties of sugarcane, their morphological district-uniform-stable characters, and flowering status are given in Table 1.

About 200 mg of tender first leaf tissue of each variety was used for flow cytometry analysis. Nuclear suspensions were prepared by finely chopping the leaf samples on a Petri dish placed on ice according to the protocol of Galbraith *et al*. (1983) using the Galbraith's Buffer (45 mM $MgCl_2$, 20 mM MOPS, 30 mM sodium citrate, 0.1 % (v/v) Triton X-100, and pH adjusted to 7.0). About 1 ml of nuclear suspension was recovered and filtered through a 50 mm nylon mesh to remove cell fragments and large debris. Nuclei were stained with 50 µg/ml propidium iodide (PI; Sigma) and 20 µg/ml RNase A (Sigma) was added to it to prevent staining of double-stranded RNA. Samples were incubated for 60 min in the dark on ice before analysis. The suspensions of samples containing stained nuclei were finally analyzed in a BD Accuri™ C6 Flow Cytometer (Becton, Dickinson and Company, NJ, USA). PI-stained nuclei of *Zea mays* cv. CE-777 with genome size 5.43 pictograms (pg), were used as an internal standard to calculate nuclear DNA content. The total number of base pair present in 2C nuclear DNA content of each sample of *Saccharum* was calculated. The standard 1 pg of DNA contains 980 megabase pairs (Mbp) (Bennett and Smith 1976; Suda *et al*., 2003). Therefore, the total number of base pair present in 2C nuclear DNA was calculated by multiplying the total DNA content obtained for each sample in pg by 980 Mbp.

RESULTS

Flow cytometric analyses of nuclei isolated from sugarcane leaves show one peak that corresponds to the G_0/G_1 phase (2C level) of the cell cycle (Figure 1). Peaks corresponding to the G_2 + M (M = mitosis) phase (4C level) or beyond were not detected, indicating the absence of dividing cells or of endo ploidy (an increase in the number of chromosome sets caused by replication without cell division) in sugarcane leaves. Figure 2 shows the dot plot of FL3-A/FL2-H for 2C DNA content. Large differences (65% variation) in DNA content (2C) were detected, ranging from 3.80 pg (Co B 94164) to 10.96 pg (Co 453), which corresponds to a genome size ranging from 3724.00 to 10740.80 Mbp (Table 2).

Bennett and Smith (1976) suggested that variation in nuclear DNA content has an impact on many plant traits. In this study, genome size or DNA content was negatively correlated ($r = -0.1095$, $P < 0.0001$) with plant height. However, low but positive correlations were found between DNA content and sugar juice analyses ($r = 0.2209$, $P < 0.0001$ for sucrose content). Similarly, internodes diameter and cane yield/ha were weakly positive correlated with DNA content ($r = 0.07199$, $P < 0.0001$ for internodes diameter and $r = 0.2844$, $P < 0.0001$ for cane yield/ha).

The regression of chromosome number on genome size was highly significant ($P < 0.001$; $r^2 = 0.999$) (Figure 3). Knowing the 2C nuclear DNA content, chromosome numbers were predicted for the varieties by the following equation: Chromosome number (y) = 0.60902484829371 + 10.34910205645x DNA content (pg). Based on the prediction equation, chromosome numbers in the varieties ranged from 2n = 40 to 114.

Table 1: The sugarcane varieties used for analyzing nuclear DNA content

Name of commercial Indian varieties of sugarcane	DUS character of varieties
Co B 94164 (Madhuri)	Stem medium thick yellowish green, cylindrical cane, long internodes, ovate buds, bud cushion, and bud groove absent. Cane height≈212 cm
Co 775	Stem grayed brown, cane with strong growth ring, rind surface appearance smooth, bud shape ovate, size medium, bud groove shallow, and bud cushion absent. Cane height≈230 cm
Bo 91	Stem medium thick, yellowish/purplish green cane with prominent growth ring, bud shape oval, prominent bud groove extending the entire length of the node, and bud cushion absent. Cane height≈240 cm
Co Se 92423 (Rajbhog)	Stem purple green, cane with moderate growth ring, ivory marks present only, bud shape pentagonal, size medium, bud groove shallow, and bud cushion absent. Cane height≈203 cm
Co Se 01424	Stem greenish purple, cane with moderate growth ring, smooth, bud shape obovate, size large, bud groove shallow, and bud cushion absent. Cane height≈216 cm
Co 453	Stem greenish yellow, cane with strong growth ring, corky patches and ivory marks present, bud shape ovate, size medium, bud groove shallow, and bud cushion present. Cane height≈198 cm
Co Se 96436 (Jalpari)	Stem green, cane with not swollen growth ring, smooth, bud shape oval, size medium, bud groove shallow, and bud cushion absent. Cane height≈227 cm
Co 62198	Stem yellow-green, cane with strong growth ring, smooth surface, bud shape ovate, size medium, bud groove shallow, and bud cushion absent. Cane height≈240 cm
Co 658	Stem greenish purple, cane with strong growth ring, corky patches only, bud shape obovate, size small, bud groove absent, and bud cushion present. Cane height≈230 cm
Co 62175	Stem yellow-green, cane with strong growth ring, ivory marks present only, bud shape triangular pointed, size large, bud groove absent, and bud cushion present. Cane height≈223 cm
Co 740	Stem yellow-green, cane with strong growth ring, corky patches and ivory marks present, bud shape ovate, size medium, bud groove shallow, and bud cushion absent. Cane height≈265 cm
Co 7527	Stem yellow-green, cane with strong growth ring, corky patches only present, bud shape round, size medium, bud groove shallow, and bud cushion absent. Cane height≈271 cm
Co 449	Stem yellow, cane with strong growth ring, corky patches and ivory marks present, bud shape ovate, size small, bud groove absent, and bud cushion absent. Cane height≈270 cm
Co A 88081	Stem yellow-green, cane with strong growth ring, corky patches present only, bud shape ovate, size medium, bud groove shallow, and bud cushion absent. Cane height≈210 cm
Co C 671	Stem grayed orange, cane with strong growth ring, corky patches present only, bud shape obovate, size medium, bud groove shallow, and bud cushion absent. Cane height≈245 cm
Co 8371 (Bhima)	Stem yellow-green, cane with strong growth ring, corky patches and ivory marks present, bud shape oval, size medium, bud groove shallow, and bud cushion present. Cane height≈242 cm
Co 6304	Stem yellowish green, cane with strong growth ring, corky patches and ivory marks present, bud shape ovate, size medium, bud groove shallow, and bud cushion present. Cane height≈201 cm
Co 6806	Stem grayed brown, cane with strong growth ring, corky patches only present, bud shape ovate, size medium, bud groove shallow, and bud cushion absent. Cane height≈210 cm
Co 7219 (Sanjeevani)	Stem yellow-green, cane with strong growth ring, corky patches present only, bud shape ovate, size medium, bud groove deep, and bud cushion absent. Cane height≈200 cm

Co: Coimbatore (Tamil Nadu), B: Bethuadahari (West Bengal), Bo: Bihar-Orissa, Se: Seorohi (Uttar Pradesh), A: Anakapalle (Andhra Pradesh), C: Cuddalore (Tamil Nadu). For example, 94164=94 stands for year of release of variety 1994 and 164 denotes the genotype number.
DNA: Deoxyribonucleic acid, DUS: District-uniform-stable

DISCUSSION

Estimates showed that sugarcane has a variable genome size (3.80-10.96 pg/2C). These values are comparable to the DNA content estimates published by Arumuganathan and Earle (1991a). Large variations of genome size might be attributed to amplification and deletions of chromosome segments leading to variations in the copy number of repeated sequences. Rayburn *et al*. (1993) suggested that variation in nuclear DNA content of maize F_1 hybrids was due to instability in DNA sequence copy number which depended on the parental combinations. Knight *et al*. (2005) provided a literature review, covering many of the factors that have been linked to DNA content variation in plants such as species diversity, altitude, latitude, temperature, precipitation, seed mass, various leaf anatomical traits, generation time, and growth rate. The increase in DNA content in sugarcane did not always necessarily affect phenotype in these varieties. Plant height an important phenotypic character is negatively correlated with DNA content or genome size. Biradar *et al*. (1994) observed the similar result in maize. This means that there is little support for the effect of genome size on plant growth rate on plant ultimate height (Knight and Beaulieu, 2008). Root/shoot apical meristem (SAM) established in the embryonic stage is not dependent on genome size or *vice versa* (Francis *et al*., 2008). Therefore, the plant height controlled by the activity of SAM has no correlation with the DNA content or genome size.

However, internode diameter is low but positively correlated with DNA content. Since sugarcane is a monocot plant, so it has no vascular cambium. Therefore, the growth of internode diameter is not controlled by any

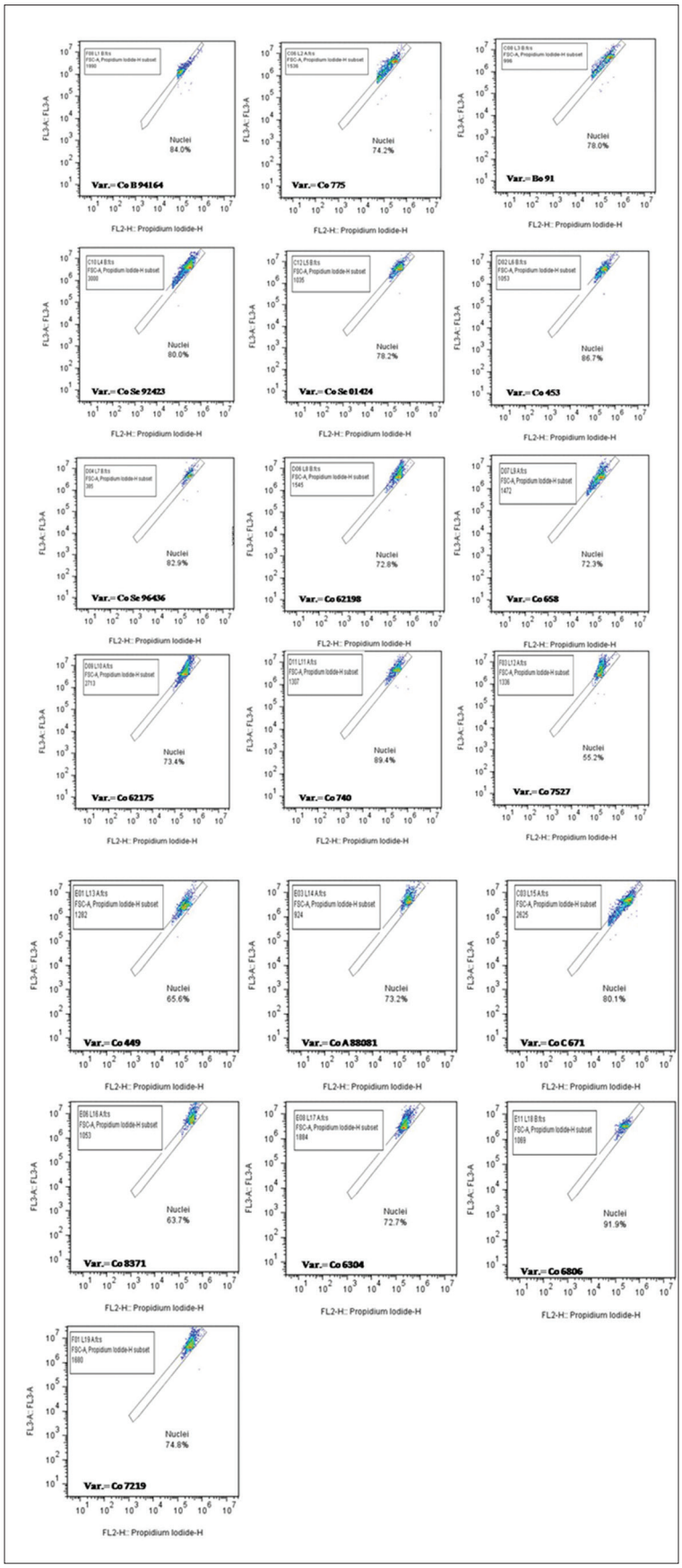

Figure 2: Dot plot of FL3-A/FL2-H for 2C deoxyribonucleic acid content of 19 varieties of sugarcane and the G0/G1 cell population is gated

Table 2: The nuclear 2C DNA content (in pg) and the estimated genome sizes (in Mbp) of the 19 varieties of *Saccharum* using flow cytometer

Name of variety	G_0/G_1 Median	2C DNA content (in pg)	Total no. of base pairs (in Mbp)/genome size
Co B 94164 (Madhuri)	170000	3.80±0.980774506	3724.00±961.1590162
Co 775	445000	9.96±0.00	9760.80±0.00
Bo 91	468500	10.48±0.395473591	10270.40±387.5641194
Co Se 92423 (Rajbhog)	424500	9.50±0.838404013	9310.00±821.6359332
Co Se 01424	454000	10.16±0.063275775	9956.80±62.01025911
Co 453	490000	10.96±0.253103098	10740.80±248.0410364
Co Se 96436 (Jalpari)	471500	10.55±0.110732606	10339.00±108.5179534
Co 62198	426500	9.54±0.332197817	9349.20±325.5538603
Co 658	312500	6.99±0.616938802	6850.20±604.6000263
Co 62175	415500	9.30±0.363835704	9114.00±356.5589899
Co 740	456500	10.21±0.17400838	10005.80±170.5282126
Co 7527	265000	5.93±0.284740986	5811.40±279.046166
Co 449	230500	5.16±0.268922042	5056.80±263.5436012
Co A 88081	301500	6.74±3.654175983	6605.20
Co C 671	463333	10.37±0.303734703	10162.60±297.6600092
Co 8371 (Bhima)	460500	10.30±0.079094718	10094.00±77.51282389
Co 6304	288000	6.59±0.205646267	6458.20±201.5333421
Co 6806	367500	8.22±0.110732606	8055.60±108.5179534
Co 7219 (Sanjeevani)	448500	10.03±0.268922042	9829.40±263.5436012

Co: Coimbatore (Tamil Nadu), B: Bethuadahari (West Bengal), Bo: Bihar-Orissa, Se: Seorohi (Uttar Pradesh), A: Anakapalle (Andhra Pradesh), C: Cuddalore (Tamil Nadu). DNA: Deoxyribonucleic acid, pg: Pictograms, Mbp: Megabase pairs

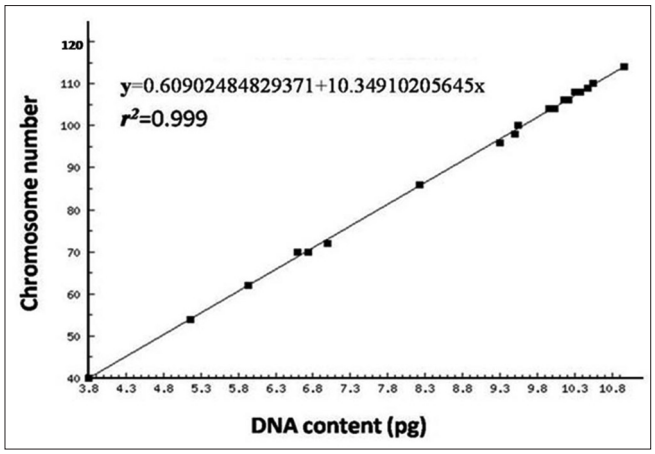

Figure 3: Relationship between deoxyribonucleic acid (DNA) content and chromosome numbers of 19 varieties of sugarcane (y = chromosome number and x = 2C DNA content in pictograms; $P < 0.001$)

activity of cambium. Increase or decrease of internode diameter is proportional to sucrose content which is stored within soft internal culm tissues made of parenchyma cells. Storage of exceptionally high concentration of sucrose within the cell increases cell volume because sucrose is an osmotically active solute and absorbs a huge amount of water (McCormick *et al.*, 2009). The diameter of culms is usually a varietal character. The growth of internode is obviously controlled by different cellular parameters such as nuclear volume, cell volume, and mitotic cycle time. The effect of such different cellular parameters due to nuclear DNA contents is referred to as nucleotypic effect (Bennett, 1972; Ho and Rayburn, 1991). Cuadrado *et al.* (2004) provided evidence for cell volume increase in relation to genomic DNA content in three modern sugarcane cultivars studied. The rates of development and the amount of growth at the cellular level also determine the rate of development and amount of growth of the whole plant. In higher plants, the nucleotypic effects are additive at successive cell cycle so that they could have a major impact on parameters of agronomic importance.

In this study, cane yield/ha is also correlated with DNA content. Yield in a crop is a function of different growth and yield components, any correlations of genome size with growth and yield components will ultimately reflect a correlation of genome size on the final yield of sugarcane.

Sugar juice analyses of the commercial varieties of sugarcane revealed that there is a low but positive correlation with DNA content. Molecular studies showed that they can exhibit "enzyme multiplicity" (Soltis and Soltis, 1993) and can produce all the enzymes of two different parental genomes, and also new hybrid enzymes that will lead to greater biochemical flexibility even in relation to sucrose synthesis.

Genome size was highly correlated with chromosome number, indicating that chromosome numbers can be derived from estimates of genome size. Correlation between genome size and ploidy level is common in plants (Palomino *et al.*, 2003) and similar highly significant correlations were also found in buffalograss (*Bouteloua dactyloides*) (Johnson *et al.*, 1998) and fine fescues (*Festuca* spp.) (Huff and Palazzo, 1998). A quick and reliable

determination of the ploidy level via flow cytometry would greatly alleviate tedious slide preparations for error-prone microscopic counting of chromosomes. Genome size estimation using flow cytometry provides an alternative approach to verify chromosome information where these are not available.

CONCLUSION

This study used flow cytometry to estimate DNA content and genome size in 19 promising commercial varieties of Indian sugarcane and revealed that sugarcane has a large genome (3.80-10.96 pg). Analysis of the nuclear DNA content and genome size showed large differences among the sugarcane varieties. Flow cytometry offers the potential, through determination of DNA content and genome size, to predict the unknown chromosome numbers of the varieties and consequently make genetic and genome analyses more efficient. The technique may help facilitate a better utilization of interspecific crosses aimed at transferring genes among different ploidy levels and at finding species or chromosome-specific markers for marker-assisted selection and breeding in sugarcane.

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