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Determination of genome size variation among varieties of *Ilex cornuta* (Aquifoliaceae) by fow cytometry

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Abstract. *Ilex cornuta* Lindl. & Paxton is a commercially important horticultural species worldwide, and extensive cultivation and hybridization have produced many varieties. Despite the considerable breeding, selection, widespread cultivation and domestication, which may have a significant role in the composition of genomes, there are no other previous reports of intraspecific genome size variation in the different cultivars or hybrids of this species. In the present work, genome size of 12 varieties of *I. cornuta* was assessed and analyzed through high-resolution flow cytometry (FCM). Nuclear DNA was analyzed using nuclei isolated from young leaves, which used propidium iodide (PI) staining, with rice (*Oryza sativa* cv. Nipponbare) as internal reference. As a result, statistically significant differences in genome size were detected among all diploid *I. cornuta* varieties considered. The estimated genome size (2*C* value) of *I. cornuta* varieties ranged from 1.47 to 1.80 pg, with 1.22-fold variation and an average size of 1.65 pg. The domestication and interspecific hybridization induced variation of genome size in *I. cornuta*, and the genome size of hybrids exhibited a wider range of variation compared with that of cultivars. In summary, flow cytometry is a useful tool to analyze the genome size of *I. cornuta*. The first report of the genome sizes of varieties of this species would provide useful data for further research on *I. cornuta*, and enrich the *C* value database of *Ilex* L. What's more, our findings could be the foundation in the future of *I. cornuta* genome sequencing and breeding programs.

Keywords: *Ilex cornuta*, Genome size, DNA content, Flow cytometry, Hybrids, Cultivars.

INTRODUCTION

The *Ilex* L. (holly) is the only living woody dioecious angiosperm genus, accounting for approximately 600 species with a broad distribution from tropics to temperate regions within the monogeneric family of Aquifoliaceae (Loizeau et al. 2016). The *Ilex* species are prized for their glossy evergreen

foliage and abundant showy fruits that can bloom from autumn to early spring, when many other plants in the landscape are dormant (Yao et al. 2021). *Ilex cornuta* Lindl. & Paxton, one of very important native landscape woody materials and distributed only in eastern China and Korea (Hu 1949), has been utilized as a horticultural species because its leaves are distinctive rectangular foliage (one or two spines per side) and its fruits are red berries (Park et al. 2019). *I. cornuta*, the most speciose and commercially significant specie of the diverse genus *Ilex*, has a long and complex horticultural history. Together with *I. aquiforlium* usually distributed in Europe, it is a typical species for Christmas tree inside home. In addition, it has been utilized as medical plant in China so that it contains several useful compounds (Zhang et al. 2012; Kang et al. 2014). Extensive cultivation and hybridization have produced many varieties of *I. cornuta*, including commercially important horticultural species such as cultivated tea and iconic flowering shrubs (Hodges et al. 2001; Park et al. 2019).

Genome size (*C* value/haploid nuclear DNA content) is an important attributes of living organisms, which is correlated with size of nucleus/cell, cellular process including DNA synthesis rate and ecological traits, etc. Genome size has fundamental significance in a wide range of applications including molecular biology, ecology, systematic biology, cytology, evolutionary biology and genomics (Jatt et al. 2019). Genome sizes of more than 7500 plant species have been estimated (Bennett and Leitch 2012), but the genome sizes of higher species are still poorly understood (Bennett and Leitch 2011; Doležel and Bartoš 2005). Flow cytometric analysis in plants has proved to be useful to determine DNA content and ploidy level in different species and accessions (Sliwinska 2018; Pellicer et al. 2014; D'hondt et al. 2011).

The genus *Ilex* is one of the largest plant genera, but of which only 6 species of genome size have been determined (Bennett and Leitch 2012). As the most speciose member of this genus, *I. cornuta* has become widely cultivated throughout Asia, Europe and America. Despite the considerable breeding, selection, widespread cultivation and domestication of *I. cornuta*, which may have a significant role in the composition of genomes, except for *I. cornuta* (Zhang et al. 2013), there are no other previous reports of intraspecific genome size variation in the different cultivars or hybrids of this species. Improved knowledge of genome size of key cultivars and complex hybrids would be a valuable resource for further breeding and improvement of *I. cornuta*. Therefore, in this study, the genome size of cultivars and hybrids of *I. cornuta* were identified and analyzed by FCM. The genome size variation of different *I. cornuta* varieties were explored to provide a basis for the development and utilization of *I. cornuta* germplasm resources.

MATERIALS AND METHODS

Plant materials

The sampling site was located in the National Holly Germplasm Resources Repository of the Jiangsu Academy of Forestry, which is located at Jiangning District, Nanjing City, Jiangsu Province, China. The tested materials were 12 *I. cornuta* varieties in consideration of its commodity value; the age of trees is 5-7a, each was healthy and without pests and diseases. Diploid rice, *Oryza sativa* subsp. japonica cv. Nipponbare (1*C* = 389 Mb, GC = 43.6 %; International Rice Genome Sequence Project 2005), were used as an internal standard, which was provided by Nanjing Agricultural University and germinated in Petri dishes in the laboratory. Leaves were collected from 12 healthy *I. cornuta* varieties and rice.

Flow cytometry analysis

Experimental design

Prior to FCM measurement, flow cytometer parameters were determined, based on external analyses of sample and primary standard. Subsequently, internal FCM procedure was performed.

Preparation of plant nuclei suspensions for flow cytometry

(1) Sampling: 0.05 g of young leaves of *I. cornuta* and 0.05 g of young leaves of rice was collected, washed in distilled and deionized water successively to remove surface dirt, and dried on filter paper.
(2) Dissociation: 1 mL of pre-cooled Tris dissociation solution was added to a pre- cooled culture dish, and the cut leaf tissues were immersed in this solution and then chopped quickly with a razor blade. After chopping, 1 mL Tris dissociation solution was added, well mixed and allowed to stand for 1-3 min at 4 °C.
(3) Filtration: The liquid mixture was drawn from the culture dish, filtered once through a 400 mesh membrane and placed into a centrifuge tube. The mixture was incubated at 4 °C for 5 min.
(4) Centrifugation: The cell nuclear suspension was obtained by centrifugation at 4 °C at 1000 r/min for 5 min.
(5) Dyeing: The supernatant was discarded. The nuclei were stained with propidium iodide (PI), and RNase

was added to a final concentration of 50 μg/mL. The mixture was dyed at 4 °C for 5-10 min in the dark environment before being analyzed.

Settings of flow cytometer and calculations

Samples were run on a BD Influx™ cell sorter (BD, Piscataway, NJ, USA) with an argon laser exciting at 488 nm. Pulse area was detected using 670 mean/30 bandwidth detector, as well as with side (SSC) and forward (FSC) scatters. Prior to analysis, the instrument was checked for linearity and the amplification was adjusted so that the peak corresponding to rice was positioned approximately at channel 10000. The voltage was maintained at a constant high level throughout each experiment. Each plant was measured at least three times by the same operator to eliminate potential artefacts. If the difference among the three measurements exceeded 2%, the most deviating value was discarded and the sample was re-analyzed. Coefficient of variation values (CV) was used to evaluate the results. Nuclear genome size was calculated as a linear relation between the ratio of G_0/G_1 peak of the samples and the standard according to the following formula (Dolezel and Bartos 2005): Sample genome size = [(sample G_0/G_1 peak mean)/(standard G_0/G_1 peak mean)] × standard genome size. Genome size data are presented in absolute terms in pg (1*C* and 2*C* value) and Mbp (1 pg DNA = 978 Mbp; Doležel 2003).

Statistical analyses of genome size

FCM detection results were edited and analyzed by BD FACS sortware1.0.0.650, and a flow histogram was obtained. Variance analysis was carried out using Excel 2003 and SPSS 13.0 with convective detection parameters. A one-way ANOVA (analysis of variance) was used to compare genome sizes among individuals of the same varieties and among 12 sampled varieties respectively. Fisher's least significant difference (LSD) test ($P<0.05$) was used for the multiple comparison. A t test was used to compare genome size values for four cultivars and eight hybrids to determine whether differences were significant between two groups. Genome size data were $\log_{10}$ transformed prior to analyses.

RESULTS

Optimization of flow cytometry for I. cornuta

Based on recommendations from specialized FCM bibliography and small genome size values reported in Aquifoliaceae (Bennett and Leitch 2012), we chose rice as primary standard by internal standardization to lower the bias (Noirot et al. 2003; Lysák et al. 2000). The fluorescence intensity range of standard and sample was determined by observing the position of peak in the flow cytometric histogram, when they were analyzed separately on the machine. As shown in Figure 1 and Figure 2, the debris peak and nuclei peak were effectively separated, and the sample peak had good linearity, indicating that the nuclear dissociation solution was suitable. The G_0/G_1 peak of rice was tuned to fluorescence channel 10000 (Figure 1b), and followed the same protocol, the G_0/G_1 peak of *I. cornuta* sample was positioned at channel number around 18000 (Figure 2b). When rice and *I. cornuta* being chopped simultaneously, the SSC (Figure 3a) showed that the particles of the target species and the internal standard are clearly concentrated with good discrimination, and it was easy to distinguish two dominant G_0/G_1 peaks in histogram (Figure 3b). Nuclear DNA content was calculated as a linear relationship between the ratio of G_0/G_1 peaks of the sample and standard, indicating that the *I. cornuta* nuclei contained more DNA than rice nuclei. Therefore, in the flow cytometry histograms of mixed samples, the peak reflecting the nuclei isolated from rice should be positioned at the left side of the histogram, while the peak reflecting the nuclei isolated from *I. cornuta* sample should be positioned at the right side of the histogram (Figure 3b).

Table 1. Variance analysis of FCM detection parameters of different varieties in *I. cornuta*.

Index	Variation source	Sum of Squares	Degree of freedom	Mean Square	*F* value	Significance level
2*C* value	Between varieties	0.390	11	0.035	17.631	0.000
	Within varieties	0.058	29	0.002		
	Total variation	0.448	40			
1*C* value	Between varieties					
	Within varieties	0.058	29	0.002		
	Total variation	0.448	40			

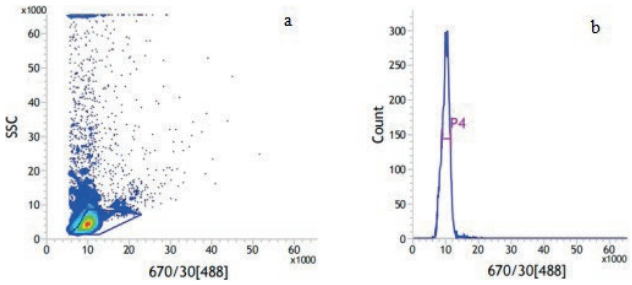

Figure 1. FCM detection analysis result for rice that was separately processed. (a) scatterplot on side scatter (SSC) versus PI fluorescence with manually drawn polygon gate; (b) histogram of relative fluorescence intensity derived from nuclei isolated from rice only. Peak 4 represent G_0/G_1 nuclei of rice.

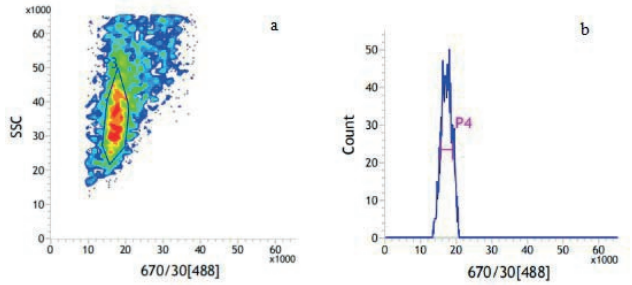

Figure 2. FCM detection analysis result for *I. cornuta* sample that was separately processed. (a) scatterplot on side scatter (SSC) versus PI fluorescence with manually drawn polygon gate; (b) histogram of relative fluorescence intensity derived from nuclei isolated from *I. cornuta* only. Peak 4 represent G_0/G_1 nuclei of *I. cornuta* sample.

FCM histograms of I. cornuta varieties

The peak histograms of different classified *I. cornuta* varieties obtained with FCM were shown in Figure 4. Mean fluorescence values from 12 *I. cornuta* varieties showed G_0/G_1 nuclei peaks in a fluorescence range from 17240 to 23090. Flow cytometry analyses produced high-resolution histograms with CV values for the internal standard and sample peaks varying between 2.77% and 5.10% (mean 4.15%) and between 1.82% and 5.15% (mean 3.95%), respectively, which suggested that the resolutions of the high quality histograms were appropriate for genome size analysis (Table 2).

Assessment of genome size of I. cornuta varieties

The mean 2*C* value was determined for each varieties of *I. cornuta* by comparing the relative G_0/G_1 nuclei PI-fluorescence peak of rice (primary standard) to that of each *I. cornuta* sample. Analysis of variance (ANO-

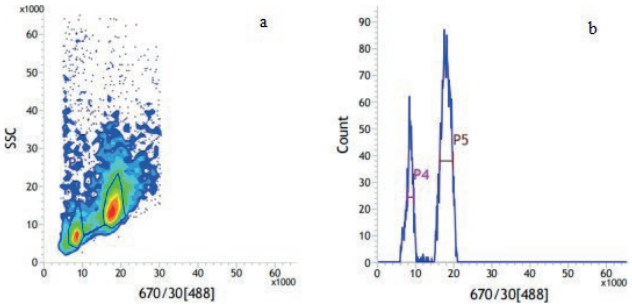

Figure 3. FCM detection analysis result for mixed samples of rice and *I. cornuta* sample that were simultaneously processed (co-chopped). (a) scatterplot on side scatter (SSC) versus PI fluorescence with manually drawn polygon gate, (b) histogram of relative fluorescence intensity derived from nuclei isolated from rice and *I. cornuta* processed simultaneously. Peak 4 represent G_0/G_1 leaf nuclei of rice, peak 5 represent G_0/G_1 nuclei of *I. cornuta* sample.

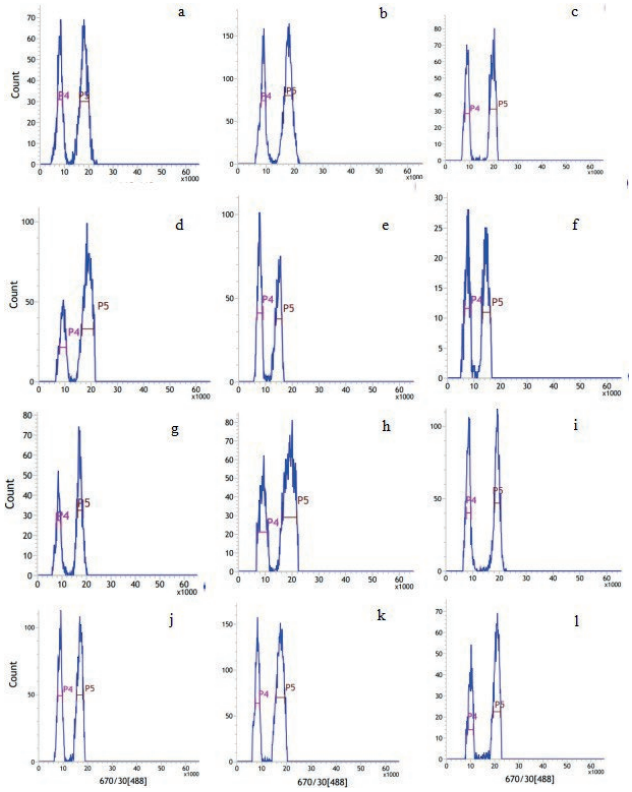

Figure 4. FCM histograms obtained after analyses of propidium iodine-stained nuclei isolated *I. cornuta* varieties 'Burfordii' (a), 'Dwarf Burford' (b), 'Luteocarpa' (c), "O'Spring' (d), 'Emily Bruner' (e), 'James Swan' (f), (g) '*Ilex dabieshanensis* No.1'; 'Mary Nell'(h), 'Nellie R. Stevens' (i) , 'Edward J. Stevens' (j), 'Golden Nellie R Stevens' (k) and 'China Girl'(l) with internal standard rice. Peak 4 represent G_0/G_1 nuclei of rice, peak 5 represent G_0/G_1 nuclei of *I. cornuta* varieties.

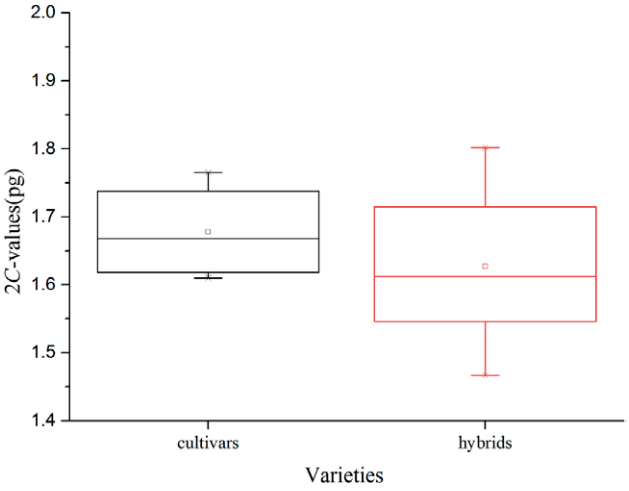

Figure 5. Boxplot of genome sizes in cultivars and hybrids. The horizontal black line within each bar represents the median value of the genome size, while a black dot within the bar denotes the average value of genome size.

VA) of genome sizes variation in different varieties of *I. cornuta* was significant (Table 1). The 2*C* genome sizes (2*C* value) varied 1.22-fold among all *I. cornuta* varieties, ranging from 1.47±0.0217 to 1.80±0.0148 pg, thus with 1*C* genome size estimates for *I. cornuta* ranging from 717 to 881 Mb (0.733–0.901 pg) with an average of 805 Mb (0.823 pg). 'Emily Bruner' had the smallest genome size, whereas 'Nellie R. Stevens' had the largest genome size (Table 2).

Comparison of genome sizes between cultivars and hybrids

The *I. cornuta* varieties measured were distinguished into two groups based on genetic origin, which were significantly different ($P < 0.05$). The first represents four

I. cornuta-related cultivars, whereas the second includes eight interspecific hybrids developed based on *I. cornuta*. The 2*C* value of genome size was estimated between 1.61±0.0048 and 1.77±0.0263 pg for cultivar family and 1.47±0.0217 and 1.80±0.0148 pg for hybrids family (Table 2).

Box and whisker plot showing differences in the 2*C* values assessed with different germplasm sources (Figure 5). The median and average 2*C* value of genome sizes were 1.688 pg and 1.678 pg for cultivars and 1.634 pg and 1.627 pg for hybrids, respectively. The variation in genome size of hybrids (1.22-fold) was somewhat larger than that of cultivars (1.10-fold), possibly due to those hybrid nature.

DISCUSSION

Genome size estimation of I. cornuta

In the present work, the genome sizes of 12 *I. cornuta* varieties were measured, according to their global commodity value in different countries. Results of flow cytometric analysis showed that the mean 2*C* genome size estimated of *I. cornuta* in this study ranged from 1.47 pg to 1.80 pg (1.63 pg on average), corresponding to the 1*C* genome size of 805 Mb or 0.823 pg, which indicated that genome size estimates of *I. cornuta* varieties were very small (1*C*≤1.4 pg; Leitch et al. 1998; Soltis et al. 2003) and considerable variations were found among all varieties. Therefore, caution should be taken when cultivars/genotypes are selected for genome sequencing and other genome-based studies.

Nevertheless, these new data on the genome sizes of both cultivars and complex hybrids are larger than the previous result of *I. cornuta* (2*C*=1.31 pg; Zhang et al. 2013). The possible reasons for this difference might be due to the use of different origins of plant materials, reference plants, methods for lysates, staining protocols and instruments (Wang et al. 2019; Jatt et al. 2019; Kolano et al. 2012). Given the large variation in genome sizes of *I. cornuta* germplasm resources, it is essential to investigate a large number of varieties before a more accurate estimation of their average genome sizes can be achieved.

Optimization of internal reference to estimate genome size accurately

Due to its indirect nature, one of the important steps in FCM analysis was to choose the reference plant species used as an internal standard, which can reveal significant differences in DNA contents among the same cultivar or plant species (Ortega-Ortega et al. 2019; Jatt et al. 2019; Doležel et al. 2003). To be useful as a primary standard, a plant must have similar, but not identical, genome size to the analyzed plant, and the G_0/G_1 peaks of the standard should not overlap to the peaks of the sample and is located relatively at a distance from the samples that can help to decrease the errors in measuring DNA content (Doležel and Greilhuber 2010; Jatt et al. 2019). Ideally, the 2*C* peak of the target species should be located between the 2*C* and 4*C* peaks of the internal reference standard and the genome size of the target and internal standards should not differ more than fourfold (Suda and Leitch 2010). In addition, the standard must be easy to use, genetically stable, nuclei must be obtained in enough amounts for analysis (Ortega-Ortega

Table 2. Genome size of the varieties of *I. cornuta* analyzed in this work.

No	Varieties	Genome size						CV (%) of samples	CV (%) of standard
		2*C* (pg)				1*C* (pg) Mean	1*C* (Mpb) Mean		
		Mean ± SD	Min.	Max.	Significance*				
Ilex cornuta -related cultivars									
1	'Burfordii'	1.77±0.0263	1.72	1.81	ab	0.883	863	4.55	4.40
2	'Dwarf Burford'	1.61±0.0048	1.60	1.62	def	0.805	787	3.50	2.77
3	'Luteocarpa'	1.71±0.0351	1.63	1.81	bc	0.855	836	3.49	4.09
4	'O Spring'	1.63±0.0282	1.57	1.67	de	0.813	794	5.15	4.94
	Average	1.68				0.839	820		
Interspecific hybrids									
Ilex (cornuta x latifolia)									
5	'Emily Bruner'	1.47±0.0217	1.42	1.53	g	0.733	717	4.56	4.81
6	'James Swan'	1.55±0.0294	1.50	1.60	ef	0.774	757	4.85	4.90
Ilex									
7	'*dabieshanensis* No.1'	1.56±0.0355	1.50	1.62	ef	0.781	763	2.81	3.76
Ilex [(cornuta x pernyi) x latifolia]									
8	'Mary Nell'	1.71±0.0048	1.70	1.72	bc	0.856	837	4.57	4.97
Ilex (aquifoilium x cornuta)									
9	'Nellie R. Stevens'	1.80±0.0148	1.78	1.83	a	0.901	881	1.82	3.60
10	'Edward J. Stevens'	1.54±0.0039	1.54	1.55	f	0.772	754	3.98	4.26
11	'Golden Nellie R Stevens'	1.72±0.0146	1.69	1.76	bc	0.859	839	5.08	3.58
Ilex (rugosa x cornuta)									
12	'China Girl'	1.66±0.0093	1.64	1.68	cd	0.831	812	3.09	3.78
Average		1.63				0.813	795		
Overall average		1.65±0.0165	1.42	1.83		0.823	805	3.95	4.15

et al. 2019). Rice has all these characteristics. Initially in this study, rice was selected as internal standard. The G_0/G_1 peak of *I. cornuta* was about twice that of the diploid cultivated rice and they don't overlap each other (Figure 3), which proved the rice was a advisable standard for *I. cornuta* flow cytometric analysis.

Performance of flow cytometry for I. cornuta

The CV has been considered an important FCM parameter, indicating the quality of nuclei suspensions (Favoreto et al. 2012). CV within 9% indicated that test results were relatively reliable (Georgiev et al. 2009); CV below 5% indicated the highest accuracy for FCM assessments in plants (Doležel and Bartoš 2005). In the present work, for 'O Spring' and 'Golden Nellie R Stevens', it was very difficult to obtain CV values at the level of below 5%, which mainly due to the high amount of autofluorescence and phenolics in the gold leaves hampering the dyeing and analysis of the nuclei (Choudhury et al. 2014). Except for these two varieties, the FCM procedure used here provided fluorescence peaks of G_0/G_1 nuclei showing CV are all below 5%, which indicated that the extraction and staining procedure using Tris dissociation solution combined with a centrifugation step can result in the accepted histograms. Thus, the FCM procedure in this work is adequate for determination of genome size for *I. cornuta* and can be applied in other FCM studies of *Ilex*.

Intraspecific variations in genome size

Statistical analysis can help assess the extent of genome size variation among varieties of related species or cultivars. In the case of *I. cornuta* it is important to determine the genetic variability between different cultivars (including genome size) since most of them are not biologically defined species, but rather the result of somatic mutations and artificial hybridization (Hodges et al. 2001). Thus, FCM analysis applied to estimate total nuclear DNA content in *I. cornuta* cultivars can help to identify those cultivars with higher possibilities of hav-

ing sexual compatibility and therefore hybridization via conventional breeding.

Among Angiosperms, there is a great variation in genome sizes, ranging from 0.065 pg/1*C* of DNA in *Genlisea margaretae* Hutch. to 152.23 pg/1*C* in *Paris japonica* Franch (Kolano et al. 2012). Also, numerous studies revealed the existence of considerable variation in genome size at the interspecific level, e.g. *Coffea arabica* (Ortega-Ortega et al. 2019; Noirot et al. 2003), *Phoenix dactylifera* (Jatt et al. 2019), *Chenopodium quinoa* (Kolano et al. 2012), several *Pisum* species (Baranyi et al. 1996), three *Saccharum* species (Zhang et al. 2012), *Agave tequilana* (Palomino et al. 2003) and *Arabidopsis thaliana* (Schmuths et al. 2004). Amongst the cultivars of *I. cornuta* there was a 1.16-fold range of variation, and although some of this might be attributable to methodological variation, it is possible that not all can be explained in this way. Murray (2005) has suggested that intraspecific variation in *C*-value may be indicative of taxonomic heterogeneity and there is no doubt that *I. cornuta* is a highly variable species that exhibits a wide range of morphological variation.

In general, these cultivars, resulting from spontaneous mutations and being thus related, have remarkably similar genome sizes and a different DNA content relative to their progenitors. This is the case of the *I. cornuta* cultivar that gave rise to 'Burfordii', 'Dwarf Burford', 'Luteocarpa' and 'O'Spring'. In contrast, a wider range of variation in the genome size were exhibited among the hybrids, one possible explanation for which was that varieties of hybrid origin have undergone substantial genome size changes as compared with natural mutations (Ortega-Ortega et al. 2019). Other varieties have different genetic origin and yet possess similar DNA content, i.e., 'Luteocarpa' and 'Golden Nellie R Stevens'. It is known that variations in genome size has been primarily attributed to fluctuation within highly repetitive DNA, variation in chromosome number, amplification/deletion of DNA sequences (Wang et al. 2017; Kolano et al. 2012; Sharma et al. 2019). Mechanisms underlying intraspecific and interspecific genome size variation in plants still remain controversial; thus, more research is fairly required in this regard (Ortega-Ortega et al. 2019; Huang et al. 2013).

CONCLUSION

In conclusion, genome size of 12 commercially important *I. cornuta* varieties was analyzed in picograms by flow cytometry technique for the first time. This study can fill a gap in the literature by providing information about the genome size of *I. cornuta* varieties and enrich the *C* value database of *Ilex* L. It also provides a valuable reference for other *Ilex* L. species to determine genome size by flow cytometry. This information can be helpful for *I. cornuta* breeding programs, give the paucity of genome size studies with FCM in different important *I. cornuta* cultivars. Additionally, these results may be relevant for genomic analysis as well as for a better understanding of *I. cornuta* evolutionary relationships, diversification, hybridization, and polyploidy.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.Z. and M.Z.; formal analysis, Q.Z.; investigation, J.L. and P.Z.; data curation, J.H.; writing—original draft preparation, P.Z.; writing—review and editing, Q.Z. and M.Z.; visualization, F.L. and J.H.; funding acquisition, M.Z. and P.Z. All authors have read and agreed to the published version of the manuscript.

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