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GENOME SIZE VARIATION OF SOUTHERN SOUTH AMERICAN SPECIES OF *ILEX* (AQUIFOLIACEAE)

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Abstract. Garberoglio, M. J.; G. E. González, M. A. Kryvenki & A. M. Gottlieb. 2023. Genome size variation of southern South American species of *Ilex* (Aquifoliaceae). *Darwiniana*, nueva serie 11(1): 167-179.

The genus *Ilex* (Aquifoliaceae) has several species appreciated by their ornamental value and nutraceutical and medicinal properties. Southern South America is a major center of biodiversity, with ca. 13-15 species. However, they have been scarcely studied, particularly regarding cytogenetics. For instance, chromosome numbers have been solely documented in eight species, while the DNA content (genome size or *2C*-value) has been recorded just for three species. To contribute to the characterization of *Ilex* species from southern South America, here we established the genome size of *I. brasiliensis*, *I. brevicuspis*, *I. integerrima*, *I. microdonta*, *I. pseudobuxus*, *I. taubertiana* and *I. theezans* as novel data, and verified the DNA content for *I. argentina*, *I. dumosa* and *I. paraguariensis*. Our results indicate that the mean DNA content at the interspecific level ranges between 1.691 pg, as in *I. pseudobuxus* and 3.600 pg as in *I. theezans*. Yet, an individual of *I. theezans* from Paraguay showed about half the genome size. These unexpected outcomes for *I. theezans* lead us to propose the coexistence of diploid and polyploid cytotypes for this species. *I. brasiliensis* was determined to have $2n$=40 chromosomes and regular meiosis, for the first time. In sum, this work is a contribution to the knowledge of an understudied plant family. Gaining insight on genetic biodiversity is relevant for the potential use of non-industrialized species for the improvement of economically significant ones.

Keywords. Genome size; *Ilex*; ploidy level; South America.

Resumen. Garberoglio, M. J.; G. E. González, M. A. Kryvenki & A. M. Gottlieb. 2023. Variación en el tamaño del genoma de especies de *Ilex* (Aquifoliaceae) del sur de Sudamérica. *Darwiniana*, nueva serie 11(1): 167-179.

El género *Ilex* (Aquifoliaceae) posee especies muy apreciadas por su valor ornamental y sus propiedades nutracéuticas y medicinales. El sur de Sudamérica es un importante centro de diversificación, con ca. 13-15 especies registradas. Sin embargo, estas han sido escasamente estudiadas, particularmente en lo que respecta a la citogenética. Por ejemplo, el número de cromosomas se ha documentado únicamente en ocho especies, mientras que el contenido de ADN (tamaño del genoma o valor *2C*) se ha reportado solo en tres especies. Para contribuir a la caracterización de las especies de *Ilex* del sur de Sudamérica, en el presente estudio se determinó el tamaño del genoma de *I. brasiliensis*, *I. brevicuspis*, *I. integerrima*, *I. microdonta*, *I. pseudobuxus*, *I. taubertiana* e *I. theezans*, siendo estos datos novedosos, y se verificó el valor *2C* para *I. argentina*, *I. dumosa* e *I. paraguariensis*.

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Nuestros resultados indican que el contenido promedio de ADN a nivel interespecífico oscila entre 1,691 pg, como en *I. pseudobuxus*, y 3,600 pg como en *I. theezans*. Sin embargo, para un individuo de *I. theezans* de Paraguay se obtuvo un valor *2C* de aproximadamente la mitad. Estos resultados inesperados para *I. theezans* nos llevan a proponer la coexistencia de citotipos diploides y poliploides en esta especie. Además, se determinó por primera vez el número cromosómico de *I. brasiliensis* (2*n*=40) y se observó que la meiosis es regular. En suma, este trabajo contribuye al conocimiento de una familia de plantas poco estudiada. Conocer la biodiversidad genética en *Ilex* es básico para el potencial uso de las especies no industrializadas en el mejoramiento de las de importancia económica.

Palabras clave. *Ilex*; nivel de plodía; Sudamérica; tamaño del genoma.

INTRODUCTION

The genus *Ilex* L. (Aquifoliaceae Bercht. & J. Presl) comprises about 500 cosmopolitan species (Loizeau et al., 2005). A major center of species diversity is found in South America, with ca. 300 species (Loizeau et al., 2005); in the Southern Cone, from eastern Paraguay, southern Brazil, northeastern Argentina to southern Uruguay, 13-15 native species have been registered (Giberti, 1979, 1994a, b, 2001, 2008, 2011; Grela, 2004; Giberti & Gurni, 2008). Among the species occurring in this region are *I. affinis* Gardner, *I. argentina* Lillo, *I. brasiliensis* (Spreng.) Loes., *I. brevicuspis* Reissek, *I. chamaedryfoli*a Reissek, *I. dumosa* Reissek, *I. integerrima* (Vell.) Reissek, *I. microdonta* Reissek, *I. paraguariensis* A. St.-Hil., *I. pseudobuxus* Reissek, *I. taubertiana* Loes., and *I. theezans* Mart. ex Reissek. All species of *Ilex* are perennial, insect-pollinated (Ferreira et al., 1983; Carr, 1991; Tsang & Corlett, 2005) and functionally dioecious trees or shrubs, with unisexual flowers showing rudimentary reproductive organs of the opposite sex (Giberti & Gurni, 2008). Thus, the pistillate (or female) flower possesses staminodes (aborted stamens) and the staminate (or male) flower a pistillode (aborted gynoecium) (Giberti & Gurni, 2008). Among southern South American species, *I. paraguariensis,* known as the "yerba mate" tree, stands out by its medicinal and nutraceutical properties and major socio-economic importance (Filip et al., 2001; Bastos et al., 2007; Turner et al., 2011); their twigs and leaves are the raw materials for the popular "mate" or "tereré" infusions. Only recently, *I. dumosa*, commonly known as "yerba señorita", also became available on the market as an herbal infusion mix of low-caffeine content (Maiocchi et al., 2016).

There is little information on the biology of *Ilex* species, particularly regarding cytogenetics. Indeed, the chromosome number has been determined in only 5% of the species for the whole genus (Greizerstein et al., 2004). Among southern South American species, *I. brevicuspis*, *I. dumosa*, *I. integerrima*, *I. paraguariensis*, *I. pseudobuxus*, *I. taubertiana*, and *I. theezans* have been registered having a diploid chromosome complement of 2*n*=2*x*=40, while *I. argentina* is the single polyploid species (2*n*=4*x*=80) so far documented in the region (Andrés & Saura, 1945; Barral et al., 1995; Greizerstein et al., 2004). Information from public databases indicate that among the 39 records for worldwide accepted *Ilex* species, solely three are polyploids, namely the Hawaiian *I. anomala* Hook. & Arn. (2*n*=4*x*=80), the Asian *I. pedunculosa* Miq. (2*n*=6*x*=120) and the Sudamerican *I. argentina*; for *Ilex*, seven different gametic chromosome numbers are reported, being *n*=20 the most common (67%) (CCDB, Chromosome Count DataBase http://ccdb.tau.ac.il/). Then, a detailed karyotype analysis is currently known solely for *I. brevicuspis,* which has 40 small-sized chromosomes (0.93-2.63 μm), of which 36 are metacentric, 2 submetacentric and 2 subtelocentric (Daviña, 1998). In addition, the DNA content of the unreplicated, non-reduced chromosomic complement (known as the genome size or *2C*-value) (Greilhuber et al., 2005) has been estimated solely for three South American species. Barral et al. (1995) determined the DNA content of *I. argentina* (*2C*=4.27 pg) and *I. paraguariensis* (*2C*=2.23 pg) using Feulgen densitometry. Gottlieb & Poggio (2014) determined the *2C*-value of *I. paraguariensis* (*2C*=1.71 pg) and *I. dumosa* (*2C*=1.89 pg) by flow cytometry. On the other hand, there are records for the Eurasian taxon *I. aquifolium* L. (*2C*=1.93 pg; Loureiro et al., 2007), and for *I. mucronata* (L.) M. Powell, Savol. & S. Andrews (*2C*=2.2 pg) and *I. verticillata* (L.) A. Gray (*2C*= 4.1 pg) both from North America (Bai et al., 2012);

among the Asian species, there is information for *I. cornuta* Lindl. & Paxton (*2C*=1.31 pg; Zhang et al., 2013), *I. latifolia* Thunb. (*2C*=1.91 pg), *I. suaveolens* (H. Lév.) Loes. (*2C*=2.24 pg), *I. viridis* Champ. ex Benth. (*2C*=2.52 pg), and *I. micrococca* Maxim. (*2C*=3.05 pg) (Su et al., 2020). Recently, the genome size of *I. polyneura* (Hand.-Mazz.) S.Y. Hu has been obtained (*2C*=1.48 pg; Yao et al., 2022).

Present survey represents a contribution to the genetic knowledge of an understudied plant family that comprises many species which are highly appreciated worldwide as ornamentals, herbal teas, or even as medicinal plants. Gaining insight on genetic biodiversity is relevant for the potential use of non-industrialized species in the improvement of economically significant ones. Thus, to contribute to the characterization of *Ilex* species from southern South America, in the present study we established the genome size of 10 species and determined for the first time the chromosome number of *I. brasiliensis*.

MATERIALS AND METHODS

Fresh plant materials for *I. argentina, I. brasiliensis*, *I. brevicuspis, I. dumosa, I. integerrima*, *I. microdonta, I. paraguariensis, I. pseudobuxus*, *I. taubertiana* and *I. theezans* were obtained from the *Ilex* Germplasm Bank held at the "Estación Experimental Agropecuaria INTA Cerro Azul" (EEA-INTA-CA; Misiones, Argentina) and the Carlos Thays Botanic Garden of Buenos Aires City (Table 1). As no reliable living plants of *I. affinis* and *I. chamaedryfolia* were available to us, these species could not be studied.

DNA content was measured by flow cytometry following the procedure of Gottlieb & Poggio (2014), with some modifications. The fresh leaves from each plant sampled were kept in darkness for at least 24 h before processing. For each species, 1-3 individuals (plants) were assayed (that is, 34 individuals in total, from 27 accessions), and 2-3 measurements per individual plant were done using different leaves. Nuclear suspensions were prepared by simultaneously chopping 1 cm^2 of adult leaves of the target species and the internal standard (*Zea mays* ssp. *mays* cultivar CE-777 [*2C*=5.43 pg; Lysák & Doležel, 1998] or cultivar B73 [*2C*=4.6 pg; Schnable et al., 2009]) in 2 ml of Partec buffer (Partec GmbH, Münster, Germany). This suspension was treated with 10 µl RNase (20 mg/ml), incubated for 30 min at room temperature and then filtered through a 42 µm-nylon mesh. Next, 500 µl of propidium iodide (50 mg/ml) was added and the mix was incubated in darkness. The incubation time was set to 5 min and it was extended to 10, 15 or 20 min when necessary. The time was considered adequate when the histograms of fluorescence intensity vs. event counts showed well-defined peaks, and the minimum number of events collected was above 5,000. Only coefficients of variation below 5% were considered. Measurements were carried out in a CyFlow Ploidy Analyser (Partec GmbH, Münster, Germany) at the "Instituto de Floricultura del INTA" (Castelar, Buenos Aires, Argentina). For each individual plant surveyed, raw counts were obtained by means of histograms of event counts vs. fluorescence intensity using the Flowing Software version 2.5.1 (Cell Imaging Core, Turku Center for Biotechnology, Finland).

The *2C*-value was obtained by multiplying the known DNA content of the standard (in pg) by the quotient of the mean fluorescence intensity of the target species and that of the internal standard, which were deduced from the histograms (Doležel et al., 1989). The quotient between the mean *2C*-value of cultivar CE-777 and that of B73 was calculated to standardize measurements made for individuals of the same species when different internal standards were used. A correction factor (=1.167) was applied to measurements where cultivar B73 was the internal standard. An analysis of variance (ANOVA) was performed, followed by post-hoc Tukey test for multiple pairwise comparisons, using the *lme4* (Bates et al., 2015) and *emmeans* packages (Length, 2022) implemented in R (R Core Team, 2018).

Chromosome counts were made from secondary roots and staminate flower buds of individuals of *I. brasiliensis* from accessions EEA-INTA-CA 221 and EEA-INTA-CA 226, and to further explore our findings, individuals of *I. theezans* from accessions EEA-INTA-CA 101 and EEA-INTA-CA 225, were also inspected. For mitotic preparations, roots were treated with 8-hydroxyquinoline (0.002 M) for 2 h, fixed in an absolute ethanol:glacial acetic acid (3:1) solution for 72 h and then preserved in 70% ethanol until processing.

Table 1. Studied *Ilex* species, accession number, geographic origin (country, province-state, department) and herbarium number.

Species	Accession EEA-INTA-CA nº	Geographic origin	BACP number[a]
I. argentina	111	Argentina, Tucumán, Monteros	187
I. brasiliensis	221	Argentina, Misiones, Puerto Esperanza	402
"	226	Paraguay, Alto Paraná, San Alberto	429
I. brevicuspis	115	Brazil, Rio Grande do Sul, Camaqua	225
"	119	Brazil, Rio Grande do Sul, Veranopolis	229
I. dumosa	1048[b]	Argentina, Corrientes, Gdor. Virasoro	--
"	13	Brazil, Santa Catarina, Tres Barras	15/88, 37/89
"	44	Argentina, Misiones, Campo Viera	53/89-62/89
"	166	Brazil, Rio Grande do Sul, Ciriaco	360
"	48	Argentina, Misiones, Campo Viera	2/87
I. integerrima	69	Brazil, Paraná, San Mateo do Sul	114
"	62	Brazil, Paraná, Quatro Barras	108
"	73	Brazil, Paraná, Teixeira Soares	118
I. microdonta	121	Brazil, Rio Grande do Sul, San Francisco de Paula	230
I. paraguariensis	1846[b]	Argentina, Corrientes, Gdor. Virasoro	--
"	1844[b]	"	--
"	1848[b]	"	--
I. pseudobuxus	114	Brazil, Rio Grande do Sul, Campo Bon	224
"	131	Brazil, Rio Grande do Sul, Torres	239
"	132	Brazil, Rio Grande do Sul, Tramandaí	240
I. taubertiana	124	Brazil, Rio Grande do Sul, San Francisco de Paula	233
I. theezans	118	Brazil, Rio Grande do Sul, Veranopolis	228
"	46	Argentina, Misiones, San Antonio	--
"	167	Brazil, Rio Grande do Sul, Vacaria	361
"	169	Brazil, Santa Catarina, Lages	363
"	225	Paraguay, Alto Paraná, San Alberto	428
"	101	Brazil, Paraná, Laranjeiras do Sul	176

[a] Original BACP Herbarium numbers, stated when available; these vouchers are currently located at BA and BAA; Thiers (2022).
[b] Plants collected in Jardín Botánico Carlos Thays, Buenos Aires, Argentina.

Next, roots were washed in isocitrate buffer (1:10 citric acid:sodium citrate, pH 4.8) followed by incubation with an enzymatic solution of cellulase (2% w/v; Onozuka R10 Merck) and pectinase (20% v/v; Sigma P4716) for 2 h at 37 ºC. Root tips were dissected under a stereoscopic microscope and slides were prepared by the squash method, and stained with DAPI (4′,6-diamidino-2-phenylindole, 2 µg/ml) according to Sumner (1990).

For meiotic preparations, flower buds were fixed as described above. The anthers were squashed and stained with DAPI. All slides were examined and photographed using a fluorescence microscope (Leica DMLB) equipped with a digital camera Leica DFC 350 FX.

RESULTS

The mean DNA content among the species ranges between 1.691 and 3.600 pg (Table 2, Fig. S1); *I. theezans* shows the highest *2C*-value, while *I. pseudobuxus* has the lowest DNA content. In general, the 34 individuals analyzed show a slight variation in DNA content at the intraspecific level (Table S1). However, there are significant differences in *2C*-values among the nine individuals of *I. theezans*: eight of these (corresponding to five accessions) have a mean genome size of 3.600 ± 0.040 pg, while the single individual of accession EEA-INTA-CA 225 has a mean genome size 1.95 times lower (Table 2).

Table 2. DNA content of southern South American *Ilex* species. Abbreviations: **IT,** incubation time in propidium iodide (in min); **N,** number of plants; ***2C*,** mean *2C*-value ± SD; **TT,** Tukey's test: different letters indicate significant differences ($p < 0.05$) among species´ *2C*-value means. * accession EEA-INTA-CA 225 of *I. theezans*.

Species	IT (min)	N	*2C* (pg)	TT
I. argentina	5	2	3.322 ± 0.080	c
I. brasiliensis	20	3	1.879 ± 0.080	ab
I. brevicuspis	20	3	2.042 ± 0.065	b
I. dumosa	5	5	1.790 ± 0.051	a
I. integerrima	5	3	1.942 ± 0.065	ab
I. microdonta	5	1	1.733 ± 0.113	ab
I. paraguariensis	5	3	1.716 ± 0.065	a
I. pseudobuxus	5	3	1.691 ± 0.065	a
I. taubertiana	5	3	1.945 ± 0.065	ab
I. theezans	5-10	8	3.600 ± 0.040	c
"	5	1*	1.847 ± 0.113	ab

ANOVA showed significant differences ($p < 0.05$) in mean genome size among species. Tukey test identified the presence of three groups: (a) *I. dumosa*, *I. paraguariensis* and *I. pseudobuxus*, (b) *I. brevicuspis*, and (c) *I. argentina* and *I. theezans*. The species *I. brasiliensis*, *I. integerrima*, *I. microdonta* and *I. taubertiana,* and the accession EEA-INTA-CA 225 of *I. theezans* could not be assigned either to group (a) or (b) (Table 2).

The appropriate incubation time of nuclear suspensions in propidium iodide was 5 min for most species (Table 2); longer incubation times reduced fluorescence detection. In *I. theezans,* best results were achieved with incubations between 5 and 10 min, while in *I. brasiliensis* and *I. brevicuspis* the appropriate time was 20 min.

Cytological analysis of *I. brasiliensis* indicated that this species has $2n=40$ chromosomes (Fig. 1A) and regular meiotic behavior, with 20 bivalents (Fig. 1B). Given the variation in genome size among *I. theezans* individual plants, chromosome counts were carried out on individuals from accessions with contrasting *2C*-values (i.e., EEA-INTA-CA 101 and EEA-INTA-CA 225). Even though this was accomplished using flower buds because root tips were not available, all cells inspected from individuals of *I. theezans* showed 20 bivalents ($2n=40$) (Fig. 1C-E).

DISCUSSION

Genome size plays an important evolutionary role, and its variation has been associated with cytological, morphological, and developmental features, among others (Bennett & Leitch, 2005; Kron et al., 2007). Here we focused on the estimation of the genome size of most of the *Ilex* species from the Southern Cone of South America.

It is known that cytosolic metabolites such as caffeine and chlorogenic acids affect the accessibility of propidium iodide to nuclear DNA causing errors in the estimation of genome size, as previously observed in coffee by Noirot et al. (2003). Gottlieb & Poggio (2014) detected a strong interference in the fluorescence signal of nuclear suspensions of *I. paraguariensis* which was attributed to the high concentrations of the secondary compounds typically shown by the "yerba mate". In fact, the proportion of metabolites in the leaves of *I. paraguariensis* is up to 81 times higher than in the other southern South American species (Filip et al., 1998, 2000, 2001), and, even further, it is the only species with significant amounts of caffeine (Kim et al., 2010). The different incubation times required here to attain adequate measurements may be attributed to the particular combination and concentrations of the cytosolic compounds that characterize each species (Filip et al., 2001; Kim et al., 2010).

This is the first report of the DNA content of *I. brasiliensis*, *I. brevicuspis, I. integerrima*, *I. microdonta, I. pseudobuxus*, *I. taubertiana* and *I. theezans*. The species *I. brevicuspis*, *I. dumosa*, *I. integerrima*, *I. paraguariensis* and *I. taubertiana,* previously documented as diploids by Andrés & Saura (1945), Barral et al. (1995) and Greizerstein et al. (2004), showed here that their DNA content ranged between 1.69 and 2.04 pg. The Euroasian *I. aquifolium* and the Asian *I. cornuta* are the only species with both verified chromosome number (de Clavijo, 1991; Xu, 1992) and known *2C*-value (Loureiro et al., 2007; Zhang et al., 2013). Although both species were considered diploids, solely the former agrees with the DNA content range of our known diploid species.

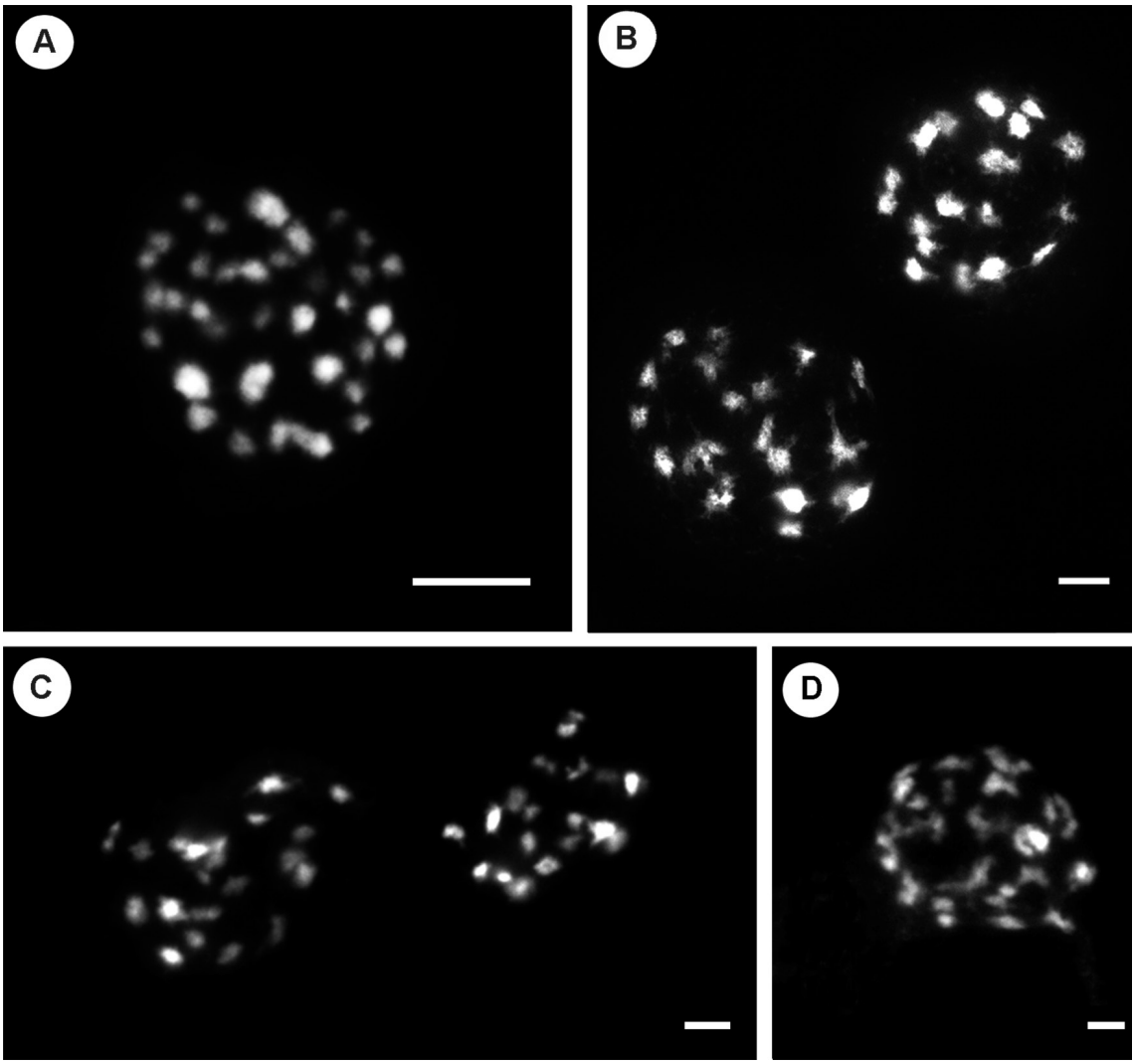

Fig. 1. Mitotic and meiotic chromosomes of *Ilex brasiliensis* and meiotic chromosomes of *I. theezans*. **A,** mitotic metaphases of *I. brasiliensis* EEA-INTA-CA 221 with 40 chromosomes. **B,** diakinesis of *I. brasiliensis* EEA-INTA-CA 221 showing 20 bivalents. **C-D,** diakinesis of *I. theezans* with 20 bivalents (**C,** accession EEA-INTA-CA 101; **D,** accession EEA-INTA-CA 225). Scale bars: 5 µm.

Prior to the present study, the genome size of southern South American *Ilex* species was known for *I. argentina, I. paraguariensis* and *I. dumosa* (Barral et al., 1995; Gottlieb & Poggio, 2014). The *2C*-values of *I. argentina* and *I. paraguariensis* recorded herein were ca. 20% lower than the data obtained by Barral et al. (1995), yet the relationship between the *2C*-values of both species remains similar between studies (1.94 and 1.91, respectively). These differences could be explained by the methodological approaches employed. Indeed, our measurements on *I. paraguariensis* and *I. dumosa* agree with those reported by Gottlieb & Poggio (2014), who also used flow cytometry.

Regarding *I. theezans,* our results indicate that the mean genome size obtained in eight out of the nine individuals analyzed (from

six accessions) was 1.6 to 2.2 times higher than that of the known diploids, and even 8% higher than that of *I. argentina*. It should be noted that the leaf morphology of *I. theezans* and *I. argentina* is quite distinct (Fig. S2), making species misidentification unlikely (Giberti, 1979, 1990, 1994). In contrast, the DNA content of the individual of EEA-INTA-CA 225 -from Paraguay- was similar to that of other *Ilex* species recognized as diploids (Table 2). Morphologically, *I. theezans, I. brasiliensis* and *I. integerrima* resemble and the literature consider they are closely related (Ricco et al., 2013), but the latter species does not occur in Paraguay (Giberti, 1989, 2001). Still, it cannot be completely ruled out that EEA-INTA-CA 225 had been inadvertently misidentified. Surprisingly, the individuals of *I. theezans* analyzed so far, and those previously reported (Andrés & Saura, 1945; Greizerstein et al., 2004), exhibited 20 bivalents, being thus diploids ($2n=2x=40$). In this context, the genomic sizes estimated for most accessions of *I. theezans* are unexpectedly high. It should be noted that, unfortunately, the materials available for meiotic analyses were a pool of individuals -from the same accession-, but not necessarily the same individuals used for genome size estimation. The existence of cytotypes due to intraspecific ploidy-level variation is known to occur in different plant species, including olive, elm, and poplar trees (Blonder et al., 2021). It is well established that the cytotypes could be distributed in different regions and may coexist in sympatry (Kolář et al., 2017). Assuming that the taxonomic identification of the plants held at the Germplasm Bank is correct, the DNA content variation observed in *I. theezans* could be attributed to the occurrence of cytotypes within Brazil, Argentina and Paraguay. There are other discordant results within the very few chromosome counts known in the genus. For instance, *I. verticillata* was established as diploid ($2n=36$) by Jensen (1944) and as a polyploid ($2n=72$) by Faasen & Nadeau (1976), suggesting the occurrence of diploid and polyploid cytotypes as well. On the other hand, the intraspecific genome size variation detected here in *I. theezans* may be explained by differences in heterochromatin content, as was documented in maize (Realini et al., 2016, González & Poggio, 2021). Chromosome bandings would aid in elucidating if highly repetitive sequences are involved in this variation. Southern South American species of *Ilex* yielded different groupings when scrutinized through AFLP genotyping (Gottlieb et al., 2005), metabolomics (Kim et al., 2010) and molecular phylogenetics (Gottlieb et al., 2005; Cascales et al., 2017). In this survey, we could not establish any association among the groupings based on genome sizes and those obtained before with other data type. Gottlieb et al. (2005) associated the greatest number of AFLP bands shown by *I. argentina* with its polyploidy. Yet, in that study, all the individuals of *I. theezans* showed banding patterns compatible with diploids. Thus, the evaluation of more individuals from diverse origins is required to further test the existence of cytotypes for *I. theezans*.

The chromosome number of *I. brasiliensis* ($2n=2x=40$) which is reported here for the first time, coincides with that of other diploid co-generic species, and agrees with its determination as a diploid through the *2C*-value estimated here. Regarding *I. microdonta*, although neither roots nor flower buds could be obtained for determining its chromosome number, the estimated *2C*-value may indicate that it is a diploid species as well.

CONCLUSIONS

Herein, the exploration of different incubation times in propidium iodide allowed obtaining, in a simple manner, adequate flow cytometry measurements; this could be a recommendable practice when dealing with non-model plants suspecting of producing secondary metabolites, as several *Ilex* species do.

Our study presented the genome sizes of most of the southern South American species of *Ilex* for the first time, and revealed, as well, an intraspecific variation of the DNA content in *I. theezans*. This leads us to propose the existence of cytotypes in this species.

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SUPPLEMENTARY MATERIAL

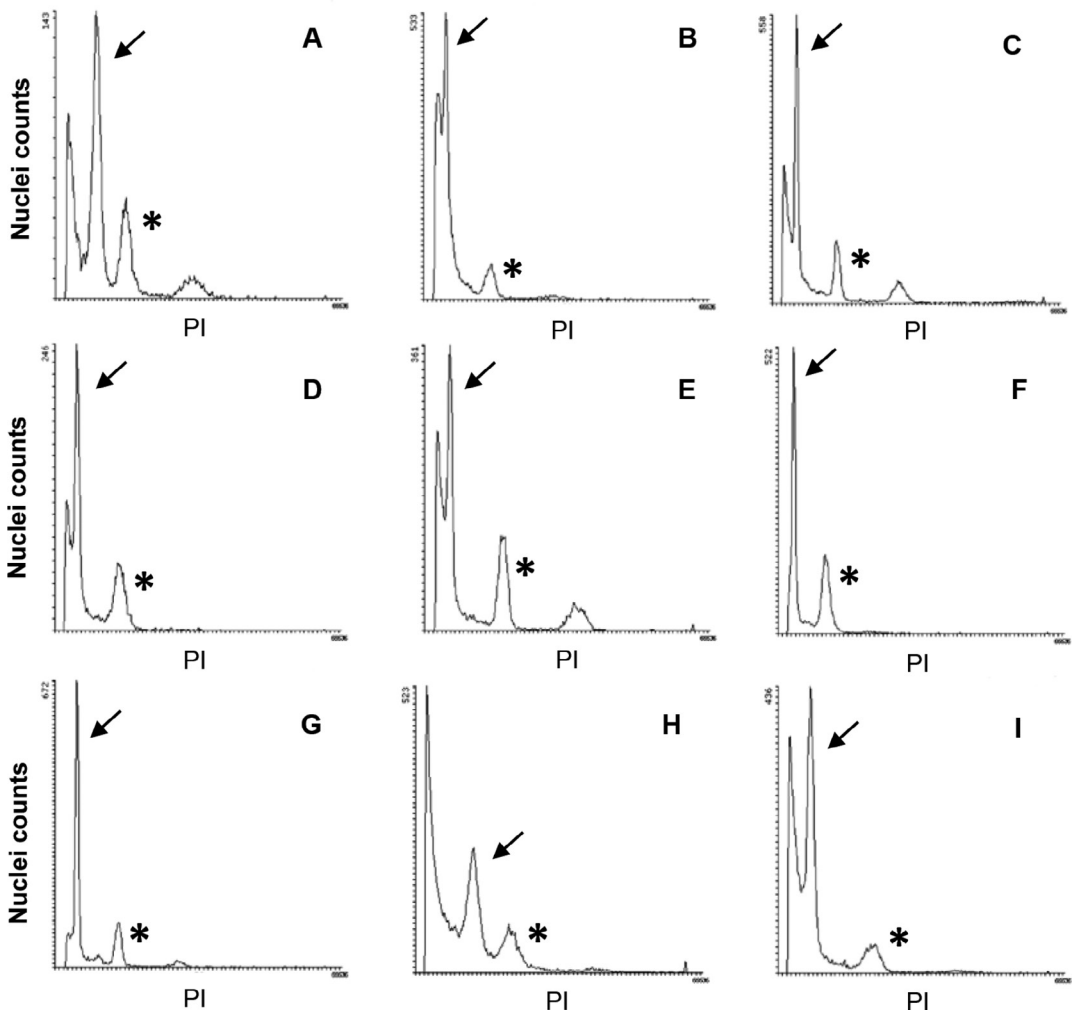

Fig. S1. Examples of flow cytometry histograms [nuclei counts vs. relative fluorescence (propidium iodide, PI) intensity] for eight southern south American *Ilex* species. For each histogram, the fluorescence peak of *Ilex* species is indicated with an arrow and the peak of the internal standard (in G_1 phase) is indicated with an asterisk. **A,** *I. argentina* (accession EEA-INTA-CA 111). **B,** *I. brasiliensis* (accession EEA-INTA-CA 221). **C,** *I. brevicuspis* (accession EEA-INTA-CA 115). **D,** *I. integerrima* (accession EEA-INTA-CA 62). **E,** *I. microdonta* (accession EEA-INTA-CA 121). **F,** *I. pseudobuxus* (accession EEA-INTA-CA 132). **G,** *I. taubertiana* (accession EEA-INTA-CA 124). **H,** *I. theezans* (accession EEA-INTA-CA 101). **I,** *I. theezans* (accession 225 EEA-INTA-CA).

Table S1. DNA content of southern South American *Ilex* species. Accession numbers and individuals measured per accession. For each individual and replicates, the internal standard used is indicated and the corrected *2C*-value is shown when the standard was B73. The replicates are indicated by rows.

Species	Accession	Individual #	Internal Standard	*2C* (pg)	Corrected *2C* (pg)
I. argentina	EEA-INTA-CA 111	1	CE777	3.412	
"	"	"	"	3.396	
"	"	"	"	3.44	
"	EEA-INTA-CA 111	2	"	3.14	
"	"	"	"	3.161	
"	"	"	"	3.383	
I. brasiliensis	EEA-INTA-CA 221	1	"	2.006	
"	"	"	"	1.822	
"	EEA-INTA-CA 226	2	"	1.875	
"	"	"	"	1.836	
"	"	"	"	1.847	
"	"	3	B73	1.612	1.882
"	"	"	"	1.613	1.883
I. brevicuspis	EEA-INTA-CA 115	1	CE777	2.024	
"	"	"	"	2.028	
"	"	"	"	2.043	
"	EEA-INTA-CA 115	2	"	1.955	
"	"	"	"	2.048	
"	"	"	"	2.113	
"	EEA-INTA-CA 119	1	"	1.999	
"	"	"	"	2.075	
"	"	"	"	2.095	
I. dumosa	JB 1048	1	"	1.763	
"	"	"	"	1.756	
"	EEA-INTA-CA 13	1	B73	1.411	1.647
"	"	"	"	1.571	1.834
"	"	"	"	1.595	1.862
"	EEA-INTA-CA 44	1	"	1.685	1.967
"	"	"	"	1.563	1.824
"	"	"	"	1.587	1.853
"	EEA-INTA-CA 166	1	"	1.522	1.776
"	"	"	"	1.505	1.757
"	"	"	"	1.506	1.758
"	EEA-INTA-CA 48	1	"	1.505	1.757
"	"	"	"	1.497	1.748
"	"	"	"	1.502	1.753
I. integerrima	EEA-INTA-CA 69	1	CE777	1.966	
"	"	"	"	1.881	
"	"	"	"	1.92	
"	EEA-INTA-CA 62	1	B73	1.714	2.001
"	"	"	"	1.578	1.842
"	"	"	"	1.656	1.933
"	EEA-INTA-CA 73	1	"	1.711	1.998
"	"	"	"	1.684	1.966
"	"	"	"	1.685	1.967
I. microdonta	EEA-INTA-CA 121	1	CE777	1.683	
"	"	"	"	1.765	
"	"	"	"	1.751	

Table S1. (Continuation). DNA content of southern South American *Ilex* species. Accession numbers and individuals measured per accession. For each individual and replicates, the internal standard used is indicated and the corrected *2C*-value is shown when the standard was B73. The replicates are indicated by rows.

Species	Accession	Individual #	Internal Standard	*2C* (pg)	Corrected *2C* (pg)
I. paraguariensis	JB 1846	1	"	1.698	
"	"	"	"	1.682	
"	"	"	B73	1.447	1.690
"	"	"	"	1.467	1.712
"	JB 1848	1	CE777	1.693	
"	JB 1844	1	"	1.658	
"	"		B73	1.566	1.829
"	"		"	1.515	1.769
I. pseudobuxus	EEA-INTA-CA 114	1	CE777	1.758	
"	"	"	"	1.665	
"	"	"	"	1.757	
"	EEA-INTA-CA 132	1	B73	1.480	1.728
"	"	"	"	1.395	1.628
"	"	"	"	1.409	1.645
"	EEA-INTA-CA 131	1	"	1.524	1.779
"	"	"	"	1.411	1.647
"	"	"	"	1.377	1.608
I. taubertiana	EEA-INTA-CA 124	1	CE777	2.038	
"	"	"	"	1.883	
"	"	"	"	2.052	
"	EEA-INTA-CA 124	2	"	1.929	
"	"	"	"	1.889	
"	"	"	"	1.894	
"	EEA-INTA-CA 124	3	"	1.949	
"	"	"	"	1.912	
"	"	"	"	1.956	
I. theezans	EEA-INTA-CA 118	1	"	3.683	
"	"	"	"	3.643	
"	"	"	"	3.768	
"	EEA-INTA-CA 118	2	"	3.775	
"	"	"	"	3.672	
"	"	"	"	3.716	
"	EEA-INTA-CA 46	1	B73	3.090	3.607
"	"	"	"	3.241	3.783
"	"	"	"	3.152	3.679
"	EEA-INTA-CA 167	1	"	3.298	3.850
"	"	"	"	3.273	3.821
"	"	"	"	3.199	3.734
"	EEA-INTA-CA 167	2	"	2.977	3.476
"	"	"	"	2.872	3.353
"	EEA-INTA-CA 169	1	"	2.888	3.372
"	"	"	"	2.766	3.229
"	"	"	"	3.151	3.679
"	EEA-INTA-CA 169	2	"	3.093	3.611
"	"	"	"	3.115	3.637
"	EEA-INTA-CA 101	1	"	2.795	3.263
"	"	"	"	2.781	3.246
"	EEA-INTA-CA 225	1	"	1.576	1.840
"	"	"	"	1.588	1.854

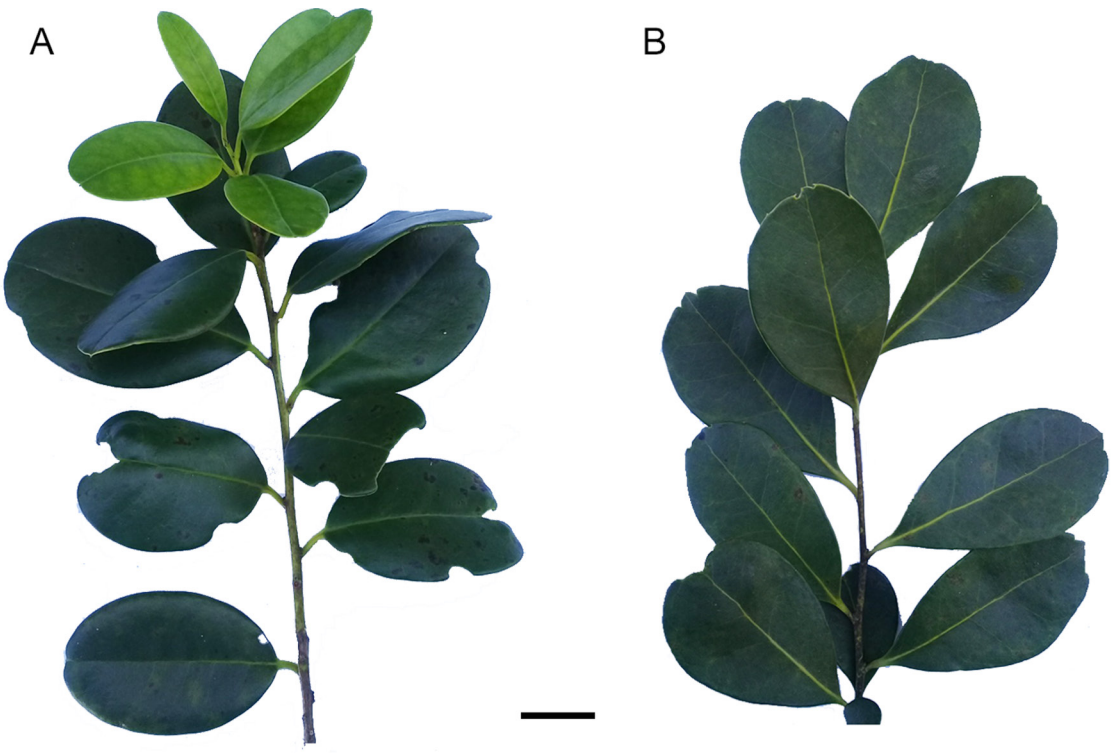

Fig. S2. Representative leaf morphology of *Ilex theezans*. **A,** accession EEA-INTA-CA 225. **B,** accession EEA-INTA-CA 101. Scale bar: 2 cm. Color version at http://www.ojs.darwin.edu.ar/index.php/darwiniana/article/view/1106/1295