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CHROMOSOME NUMBERS AND DNA CONTENT FROM *ILEX ARGENTINA (AQUIFOLIACEAE)*

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Summary Morphological and phytochemical relationships between *Ilex argentina* and *I. paraguariensis* («yerba mate») are strong. The first species was employed in the past as substitute of the authentical «yerba mate». In this paper chromosome numbers and DNA content are reported for first time for *I. argentina* (n = 40, 2C = 4.27 ± 0.07 pg). Previous data for *I. paraguariensis* were confirmed (n = 20) and DNA content was determined (2C = 2.23 ± 0.08 pg). In spite that differences in chloroplast number per guard cell was found to be statistically significant between both species, it has not proved to be an accurate alternative to assess ploidy level. The present results suggest that both taxa should be considered distinct species.

Resumen *Número cromosómico y contenido de ADN en* Ilex argentina (Aquifoliaceae). Las semejanzas morfológicas y fitoquímicas entre *Ilex argentina* e *I. paraguariensis* («yerba mate») son acentuadas. La primera especie fue empleada en el pasado como un substituto de la yerba mate genuina. En este artículo se presentan por primera vez datos del número cromosómico y del contenido de ADN para *I. argentina* (n=40 ,2C = 4.27 ± 0.07 pg). Se confirman datos previos de *I. paraguariensis* (n=20) y se determina el contenido de ADN (2C = 2.23 ± 0.08 pg) para esta especie. A pesar que entre ambas entidades, las diferencias en el número de cloroplastos por célula oclusiva son estadísticamente significativas, los recuentos de plástidos no han probado en estos taxa ser una alternativa exacta para determinar el nivel de ploidía. Los resultados aquí presentados sugieren la conveniencia de seguir considerando que *I. argentina* e *I. paraguariensis* son dos especies distintas.

INTRODUCTION

Ilex argentina Lillo *(Aquifoliaceae),* vernacularly known as «árbol de la yerba» or «palo de yerba» in Northwestern Argentina, is a dioecious native tree from the subtropical subandean rainforests, whose geographical range spreads from 17° S (Santa Cruz de la Sierra, Bolivia) to about 27° S (Catamarca, Argentina) —Figure 1—. It is a species which had a sporadic regional use —XVIII and XIX centuries— (Arenales, 1833; Grondona, 1953; Schleh, 1914) as a substitute for *I. paraguariensis* St. Hil., the authentic «yerba mate», an allopatic related taxon of widespread use in Southern South America. *Ilex paraguariensis* is nowadays widely exploited and/ or grown in NE Argentina, Southern Brazil and Eastern Paraguay, and the product of its industrialization is used to prepare drinks and infusions with stimulant properties because of its content in caffeine.

Even at the times of *I. argentina's* descriptio princeps (Lillo, 1911: 171), its resemblances to *I. paraguariensis* were noted. The same exomorphological and anatomical similarities were registered by several authors (Baas, 1973, 1975 ; Giberti, 1979, 1990); the first of them classified this species within the same taxonomic group to which *I. paraguariensis* belongs: subsection *Repandae* Loesener (Bass, 1973: 227; 1975: 362). It must be quoted here that Loesener (1942) did not place *I. argentina* anywhere within his infrageneric system for *Ilex*. In a similar way, leaf flavonoid analyses have also shown a high degree of similarity among both species (Ricco *et al.*, 1991).

However, some morphological, chemical and phenological differences among both species also occur, as well as their geographical discontinuity (Hueck, 1978).

Bearing in mind the economical importance of *Ilex paraguariensis* for this region of South America, and considering also that the biology of the genus *Ilex* is scarcely known, we are now presenting comparative cytological results of both species. Previous chromosome records for *I. paraguariensis* (Andrés & Saura, 1945; Grondona, 1954; Niklas,

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Fig. 1.—Distribution map of *I. argentina* and *I. paraguariensis* in South America.

1987; Saura, 1944) were corroborated, and the first results for *I. argentina* are reported in the present paper, as well as the total DNA content data for both taxa.

MATERIALS AND METHODS

Seedlings and seeds were obtained form plants grown at the INTA Experimental Agriculture Center of Cerro Azul, Misiones, Argentina. Flowers for cytological studies were collected at Tucumán, Argentina, in 1992.

Material studied:

Ilex argentina. ARGENTINA. *Prov. Tucumán: Dpto. Yerba Buena*, al SW de Anta Muerta, 8-IV-1991, Giberti *et al.* 330, 331, 333, 334 (BACP). *Dpto. Lules*, cumbres de San Javier, 8-IV-1991, Giberti *et al.* 332 (BACP). *Dpto. Monteros*, Quebrada de los Sosas, 9-IV-1991, Giberti *et al.* 335, 339 (BACP).

Ilex paraguariensis. ARGENTINA. *Prov. Misiones: Dpto. L. N. Alem*, INTA Cerro Azul, 24-X-1993, Giberti 480, 481, 482, 483, 484 (BACP).

Mitotic studies: have been performed on root tips cells of grafts obtained with an IBA solution (2000 ppm) and from shoot buds. Pretreatment was done with colchicine 0.05% during 2-3hr, at room temperature in the dark; fixation was performed in absolute ethanol-acetic acid (3:1) and the staining was done with acetic haematoxylin 2%.

Meiotic studies: young flowers were fixed in 3:1 and the anthers stained with 2% acetohaematoxylin.

Determination of DNA content: it was measured in telophase nuclei (2C) of the root apex, following the procedure described by Poggio and Hunziker (1986). The optimal hydrolisis time was 30 min. The amount of Feulgen staining per nucleus expressed in arbitrary units, was measured at a wavelength of 570 nm, using the scanning method with a Zeiss Universal microphotometer (UMSP30). The DNA content expressed in picograms was calculated using *Allium cepa* as a standard (2C = 33,55 pg). The differences in DNA content between means were evaluated using ANOVA and Scheffe's method.

Chloroplast number: A small piece of epidermis was peeled from the lower surface of each leaf and placed in a drop of water on a microscope slide. A coverslip was placed over the slide and chloroplasts in the guard cells were counted using a fluorescence microscopy (Zeiss, Axioplan), at a wavelength of 450-490 nm. Fourteen individuals of *I. argentina* and five of *I. paraguariensis* were analyzed. The differences between species were evaluated through the G (Williams) test.

RESULTS

The chromosome number of *I. paraguariensis* was confirmed (n = 20). All individuals studied from 20 bivalents at diakinesis, PMI and MI (Fig. 2A). In MI, about 5% of cells show a bivalent out of plate. Nor laggards neither micronucleus were observed at AI-TI. On the other hand, *I. argentina* showed ca. 80 chromosomes in mitosis and 40 bivalents at diplotene-diakinesis (Fig. 2B). No univalents out of plate or multivalents in MI were detected.

DNA content (2 C value) expressed in picogrammes, are summarized in Table 1 and Figure 3. For each species 4-5 individuals were measured. Since the ANOVA test and Scheffe method showed nonsignificant differences within the species, data were pooled for each species in Table 1. The analysis of variance showed the existence of significant differences between species ($F = 206$, $\alpha = 0,05$).

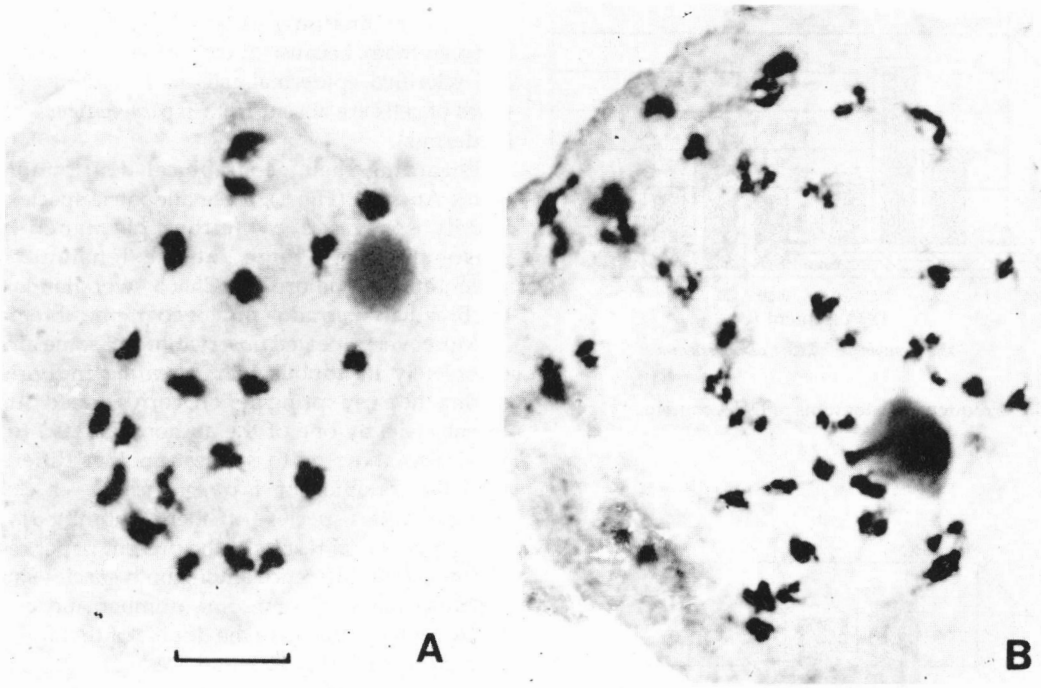

Fig. 2.— A: *I. paraguariensis*. Diakinesis 20 II. B: *I. argentina*. Diplotene 40 II. Bar represents 10 µm

Table 1.– Chromosome number and total DNA content of *Ilex paraguariensis* and *I. argentina*.

Species	2n	Nuclei studied (1)	DNA content (2C) pg X ± SE
I. paraguariensis	40	86(4)	2.23 ± 0.08
I. argentina	80	78(5)	4.27 ± 0.07

(1) Number of replicates between brackets.

In figure 4 the frequencies of chloroplast number per guard cell for the studied plants of both species are represented.

Although the range of chloroplast numbers of *I. paraguariensis* overlap with the range of *I. argentina*, the average values are different (*I. paraguariensis*, $\bar{x} \pm SE = 4.56 \pm 0.064$; *I. argentina*, $\bar{x} \pm SE = 5.50 \pm 0.061$). The difference between means is significant (test of homogeneity G (Williams) = 80.5, $P < 0.0001$). The frequencies of chloroplast numbers are different between both species. In *I. paraguariensis* the most frequent number of chloroplasts per guard cell was 4 and 5 (46% and 41% respectively). In *I. argentina*, the percentage of guard cells with 4 chloroplasts is rare (5,9%), being 5 and 6 the more frequent values (37,7 and 35,5% respectively). Moreover, the later species showed guard cells with 7 and 8 chloroplasts, which never appeared in *I. paraguariensis* (Fig. 4).

DISCUSSION

I. argentina and *I. paraguariensis* show several exomorphological, anatomical and biochemical

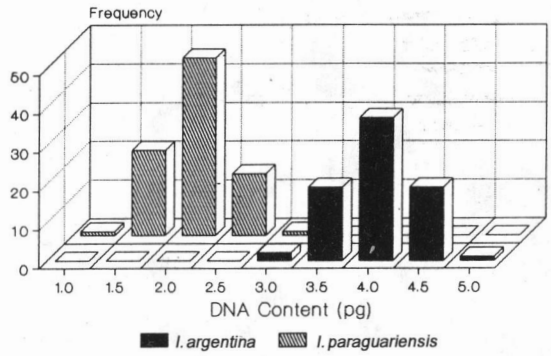

Fig. 3.— Frequency histograms of DNA content.

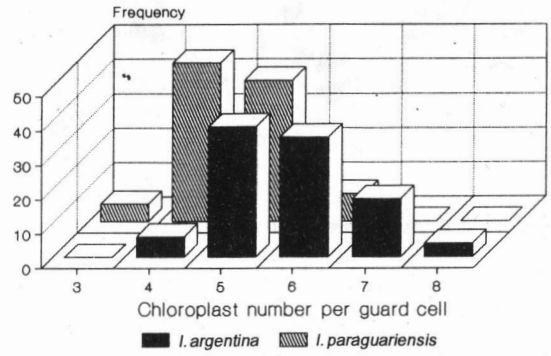

Fig. 4.— Frequency histograms of chloroplast number per guard cell in leaf epidermis.

(leaf flavonoid compounds) similarities (Baas, 1975; Giberti, 1979, 1990; Ricco *et al.*, 1991) in spite that some morphological, chemical and phenological differences among them also occur. Caffeine is a significative component of *I. paraguariensis*. Xanthine studies for *I. argentina* were not able to identify caffeine in this species; the same research has only reported theobromine for *I. argentina* (Filip *et al.* 1983, 1989). Additionally, the saponins occurring in both species are different (Grossman *et al.* 1989; Schenkel *et al.*, 1993). Giberti (submitted) reported some exomorphological, anatomical and phenological dissimilarities.

On morphological grounds, although *I. paraguariensis* often presents bigger leaves than *I. argentina*, female specimens of the first species show frequently more complex (fasciculated) inflorescences than the solitary female cymes of the second taxon. However, male trees often share the same type of inflorescences in both taxa (Giberti, 1979). Leaf anatomy is also slightly different between them, because of the presence of mucilage and sclerified epidermal cells in *I. argentina* (both types of cells are absent from *I. paraguariensis's* leaf epidermis).

Regarding their geographical distribution in South America (Fig. 1), these allopatric species are widely separated by (either permanently o seasonally dry) huge land extensions: the Argentinian xeromorphic «Chaco» woodlands and the Brazilian «cerrado» phytogeographical regions.

Moreover, repeated observations of some almost completely deciduous *Ilex argentina* individuals during the early spring period, corroborated during recent visits by one of the authors (G.C.G.) to the area, also allow us to suggest another difference with the condition of *I. paraguariensis* —a clearly non deciduous species— (Giberti, submitted).

Cytogenetical results of the present paper reveal an important difference among both species since *I. argentina* has a chromosome number and a total DNA content which are the double of that found in *I. paraguariensis* (Fig. 2,3; and Table 1).

The small size of the chromosomes of the species analized has made their detailed morphological studies impractical, but it was possible to determine 2n = 40 chromosomes in *I. paraguariensis* and 2n = ca 80 chromosomes in *I. argentina*. The chromosome number was confirmed through the meiotic studies. *I. paraguariensis* showed n = 20 bivalents while *I. argentina* showed 40 bivalents. The meiotic behaviour of both species seems to be regular, forming bivalents at Prophase I. Andres and Saura (1945) reported in *I. paraguariensis* an heteromorphic bivalent out of plate and suggested that it was a sexual pair of chromosomes. The individuals studies in the present paper do not show evidences of the occurrence of a sexual pair. Although there is a bivalent out of plate, its frequency was found to be very low (5%), and it did not look as heteromorphic.

The chromosome number of *I. paraguariensis* (2n = 40) was previously determined by Saura (1944), Andres & Saura (1945), Grondona (1954) and Niklas (1987). However, no data were recorded for *I. argentina*, being 2n = 80 the first reported for the species. The most common chromosome number for the genus is 2n = 40. So far, only three species were reported with high chromosome number: *I. anomala*, 2n = 80 (Carr, 1978); *I. verticillata* 2n = 72, and *I. pedunculosa*, 2n = 120 (Goldblatt, 1976, 1981).

I. argentina possess the double DNA content of *I. paraguarensis* (Table 1, Fig. 3). There are controversies about the basic chromosome number of the genus *Ilex* (x = 10 or x = 20). The data of the present work do not let answer this question. Anyway, the

DNA content per basic genome (considering x = 10) is similar in the three species studied so far: *I. argentina*, *I. paraguariensis* (Table 1) and *I. aquifolium* (Bennett and Smith, 1991).

There are several examples in which an observed increase in the number of chloroplasts in guard cells is in correspondence with increased polyploidy in several plants (Bingham, 1968; Chaudhari and Barrow, 1975; Dudley, 1958; Ho and Rayburn, 1991; Mochizuki and Sueoka, 1955; Najcevska and Spechmann, 1968; Nuesch, 1966).

The usefulness of using the chloroplast number in epidermal guard cells as an indirect ploidy indicator was evaluated in leaf epidermis of *I. paraguariensis* and *I. argentina*.

The results (Fig. 4) show that there is a significative difference in chloroplast number between *I. argentina* (2n = 80) and *I. paraguariensis* (2n = 40) but the relationship is not so direct as in maize or other plants. Although the differences are statistically significant this method is not a rapid and accurate way to assess ploidy level for *Ilex*. Several authors reported that there are various factors affecting the number of cloroplasts (Ellis and Leech, 1985; Pryywara *et al.* 1988; Pyke and Leech, 1987). One of these factors is the size of the cells, which is closely related with the ploidy level. *I. argentina* has a bigger area of guard cells (1677 ± 199 μm^2) than *I. paraguariensis* (977 ± 94 μm^2). Then, the relationship between the number of chloroplasts of guard cells and the ploidy level in these species of *Ilex* would be indirect and dependent, among other factors, of the size of the cells.

In spite of their morphological and phytochemical similarities, the occurrence of different ploidy levels in *I. argentina* and *I. paraguariensis* suggests that both belong to different biological species.

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