



First cytomolecular characterization of three Neotropical woody bamboos (Bambusoideae, Poaceae) suggests ancient diploidized karyotypes

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ABSTRACT

Although karyotype features are useful data for evolutionary studies, cytogenetic data in Bambusoideae are mainly based only on chromosome counts. The first comparative cytogenetic analysis of three Neotropical woody bamboo species — *Guadua chacoensis*, *G. angustifolia* and *Chusquea tenella* — was undertaken based on new and reviewed chromosome counts, CMA/DAPI double staining, fluorescent *in situ* hybridization (FISH) with 35S and 5S rDNA probes, and genome size estimation. The two species of *Guadua* were found to have $2n = 46$ chromosomes, while the first record for *C. tenella* was $2n = 44$. Only one pair of CMA⁺/DAPI was detected on the terminal region of metacentric chromosomes in all three species. Likewise, one pair of 5S and 35S rDNA sites was detected in all three species, with the 35S rDNA sites always collocated with the CMA⁺ bands. Genome sizes ranged from $2C \approx 3.99$ pg for the species of *Guadua*, to $2C = 4.77$ pg for *C. tenella*. Considering the Miocene origin of Neotropical woody bamboos, the observed karyotype stability suggests that the analyzed species are diploidized paleopolyploids. The results reveal the conservative cytomolecular organization of Neotropical woody bamboo karyotypes, which helps to improve our understanding of the evolution of this group.

Keywords: *Chusquea*, cytogenetics, diploidization, evolution, *Guadua*, polyploidy

Introduction

Representing the major clade of grasses with diversification in forest habitats, the bamboos (subfamily Bambusoideae) are perennial and multipurpose plants and one of the major non-timber forest products worldwide (Clark *et al.* 2015). The monophyletic Bambusoideae (127 genera/1,680+ species) is currently classified in three tribes, encompassing the herbaceous (Olyreae) and woody bamboos (Arundinarieae and Bambuseae) (Kelchner &

BPG 2013; Soreng *et al.* 2017; Clark & Oliveira 2018). Within Bambuseae tribe, two major lineages are recognized [Paleotropical and Neotropical], with Brazil as one of the main centers of diversity and endemism of Neotropical bamboos (Greco *et al.* 2015; Soreng *et al.* 2017).

Both belonging to the Bambuseae tribe, the genera *Guadua* and *Chusquea* occur from Mexico to Argentina and represent relevant ecological component with economic potential within the Neotropical lineage (Clark *et al.* 2015). The genus *Guadua* (33 species) comprises species of greater height (up to 30 m) within the group, such as

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G. chacoensis and *G. angustifolia*, which has great suitability for construction (Clark *et al.* 2015; Vorontsova *et al.* 2016). The most diverse genus among woody bamboos (more than 180 species), *Chusquea* occurs primarily in montane forests and high-elevation grasslands, where they tend to occupy specialized habitats (Fisher *et al.* 2014; Clark & Mason 2019; Ruiz-Sanchez *et al.* 2020). *Chusquea tenella* is a common species of Brazilian Atlantic forest's understory, frequently associated with altered and endangered environments (Schmidt & Longhi-Wagner 2009).

Woody bamboos are complex plants, with a tree-like habit with highly lignified culms and peculiar flowering behavior, displaying a long vegetative phase and typically gregarious and monocarpic cycles (Clark *et al.* 2015). Their evolutionary history involves independent rounds of polyploidy preceding the diversification of the main current lineages (Triplet *et al.* 2014; Guo *et al.* 2019). Assuming the basic chromosome number $x = 12$ proposed for Bambusoideae (Hilu 2004; Zhou *et al.* 2017), tetraploids are observed in Arundinarieae and Neotropical Bambuseae, while Paleotropical Bambuseae are considered hexaploids (Hilu 2004; Clark *et al.* 2015).

Polyploidy has been recognized as one of the major evolutionary forces in Angiosperms diversification and evolution, which is frequently associated with the process of cytological and genetic diploidization (Wendel 2015; Dodsworth *et al.* 2016). In Poaceae, it is estimated that all the members share at least two events of polyploidization followed by diploidization evolution (paleopolyploids) in some species, such as *Sorghum bicolor* and *Oryza sativa* (Jiao *et al.* 2011; Paterson *et al.* 2012). Thus, considering the ancient polyploid origin of Bambuseae tribe (ca. 33 Mya; Ruiz-Sanchez 2011; Guo *et al.* 2019), Neotropical lineages represent excellent models for investigating the cytogenetics consequences of diploidization processes in paleopolyploids (see Figueredo *et al.* 2016).

Karyotype analyses are proven to be useful tools for detailed genome characterization, providing relevant information such as chromosomal rearrangements, allopolyploid origin, and phylogenetic and evolutionary relationships of many plant groups (Berjano *et al.* 2009; Guerra 2012; Kolano *et al.* 2013; Souza *et al.* 2015; Carvalho *et al.* 2017). However, especially due to their long-life cycle and unpredictable flowering in woody bamboos, karyotype evolution is a complex and neglected subject, relying basically on chromosome counts (Nirmala *et al.* 2014).

The present work analyzed the karyotypic diversity of the three Neotropical woody bamboo species based on chromosome counts, morphology, double staining with the fluorochromes chromomycin A₃ (CMA) and 4',6-diamidino-2-phenylindole (DAPI), and fluorescent *in situ* hybridization (FISH) with 5S and 45S rDNA. Besides that, we estimated the genome size of these species by flow cytometry. By the new data obtained here, we aimed to address three questions in the woody bamboos: (1) Does

cytomolecular data corroborate a paleopolyploid origin of this lineage? (2) What is the degree of diploidization of the analyzed karyotypes? (3) Did the species of *Guadua* and *Chusquea* show similar trends in the evolution of the numbers of rDNA sites?

Materials and methods

Plant materials

Specimens of *Guadua chacoensis* (Rojas) Londoño & P.M. Peterson and *G. angustifolia* Kunth were obtained from the collection of bamboo species from "Ressacada" Experimental Farm of the Federal University of Santa Catarina (UFSC) (27°41.1' S; 48°32.63' O), while *Chusquea tenella* Nees samples were collected from a natural population at "Ponta do Goulart" locality (27°33.68' S; 48°31.33' O), both in Florianópolis, Santa Catarina state (SC), Brazil. Voucher specimens were deposited in the Herbarium FLOR (UFSC) with the following numbers: *G. chacoensis* (FLOR 58620; Rossarolla MD & Venturi M., 16); *G. angustifolia* (FLOR 58626, Rossarolla MD, Venturi M., 26) and *C. tenella* (FLOR 58638; Rossarolla *et al.* 5).

Root tips were pretreated with 2 mM 8-hydroxyquinoline for 24 h at 10 °C, fixed in a solution of ethanol:acetic acid (3:1, v/v) for 2 to 24 h at room temperature, and stored at -20 °C.

Cytogenetic analyzes

Chromosome banding

After washing with distilled water, root tips were digested in a solution of 2 % (w/v) cellulase (Onozuka)/20 % (v/v) pectinase (Sigma) for 1 h at 37 °C. For slide preparation of chromosomes spread, the root was squashed in a drop of 45 % acetic acid (v/v) and the coverslip was later removed in liquid nitrogen.

For the CMA/DAPI double staining technique, the slides were aged for three days, stained with 10 µL CMA (0.1 mg mL⁻¹) for 1 h, and restained with 10 µL DAPI (2 µg mL⁻¹) for 30 min (Barros-e-Silva & Guerra 2010). The slides were mounted in glycerol:McIlvaine buffer pH 7.0 (1:1, v/v), and aged for three more days before analysis in an epifluorescence Leica DMLB microscope. Images were captured with Cohu CCD video camera using Leica QFISH software and then edited using Adobe Photoshop CS3 version 10.0 for better brightness and contrast.

Fluorescent *in situ* hybridization (FISH)

For the subsequent FISH, selected slides were destained in a solution of ethanol:acetic acid (3:1, v/v) (for 30 min) and absolute ethanol (for 1 h), and pretreated as described by Pedrosa *et al.* (2001). In order to localize rDNA sites, a 500 bp 5S rDNA clone (D2) of *Lotus japonicus* (Regel) K. Larsen labeled with Cy3-dUTP (Amersham) and a 6.5 kb



18S-5S, 5S-25S rDNA clone (R2) from *Arabidopsis thaliana* (L.) Heynh. labeled with digoxigenin-11-dUTP (Roche) were used as probes (Pedrosa *et al.* 2002). Both labeling techniques were performed by nick translation. The 35S rDNA probe was detected with sheep anti-digoxigenin FITC conjugate (Roche), and amplified with donkey anti-sheep FITC conjugate (Vector).

FISH analysis was performed according to Pedrosa *et al.* (2002), with minor modifications. The hybridization mix contained 50 % (v/v) formamide, 10 % (w/v) dextran sulfate, 2 × SSC and 5 ng μL⁻¹ of each probe. The slides were denatured at 75 °C for 5 min and hybridized for up to 48 h at 37 °C. Post-hybridization washes were performed in 0.1 × SSC at 42 °C, reaching a final stringency of approximately 76 %. Afterward, the slides were counterstained and mounted in DAPI (2 μg mL⁻¹):Vectashield (Vector) (1:1, v/v) solution, and images of the cells were acquired as described above.

Chromosome morphometry and idiograms

For each species, at least 10 well-spread metaphases were analyzed using Adobe Photoshop CS3 version 10.0. The chromosome arm ratio (AR = length of the long arm / length of the short arm) was used to classify the chromosomes as metacentric (AR = 1-1.4), submetacentric (AR = 1.5-2.9), or acrocentric (AR > 3.0), following Guerra (1986). Karyotype symmetry was evaluated by the intrachromosomal (A₁) and interchromosomal (A₂) asymmetry indices (Zarco 1986). Mean lengths of total chromosome complement (T) of each chromosome pair, of short (SA) and long arms (LA), as well as number and position of heterochromatic bands and of the 35S and 5S rDNA sites were used to construct idiograms representing the haploid complement of each analyzed species, using CorelDRAW version X6 software.

Flow Cytometry

DNA content of three independent specimens was estimated by flow cytometry (FCM) using *Glycine max* var. Polanka (L.) Merr. as internal standard (2C = 2.5 pg; Doležel *et al.* 1994). Nuclei suspensions were obtained by co-chopping leaf fragments (30 mg) of each bamboo species and the internal standard in a Petri dish containing 1.5 mL of Woody Plant Buffer (Galbraith *et al.* 1983; Loureiro *et al.* 2007). Afterward, the nuclei suspension was stained with 20 μL propidium iodide (1 mg mL⁻¹) and analyzed on a flow cytometer PARTEC CyFlow. Histograms of relative fluorescence intensity of the G₀/G₁ peak were analyzed using FlowMax software version 2.4 (Partec). The genome size, or nuclear DNA content (pg), of each species was calculated according to the formula:

$$\text{Nuclear DNA content} \left(\frac{\text{pg}}{2C} \right) = \left[\frac{\text{sample G1 peak mean}}{\text{standard G1 peak mean}} \right] \times \text{standard nuclear DNA content} \left(\frac{\text{pg}}{2C} \right)$$

Results

The cytogenetic data of *Guadua chacoensis*, *G. angustifolia*, and *C. tenella* revealed similar and symmetrical karyotypes (see A₁ and A₂ index), regarding length of total chromosome complement (T) and predominance of metacentric (M) and submetacentric (SM) chromosomes. *Guadua chacoensis* and *G. angustifolia* equally showed 2n = 46 chromosomes (Figs. 1A-D, 2A-B), with the karyotype formula 14SM + 9M and 12SM + 11M, and T = 72.86 μm and T = 73.97 μm, respectively. In turn, *C. tenella* showed 2n = 44 (Figs. 1E-F, 2C) with 9SM + 13M, and similar mean length of total chromosome complement (T = 73.68 μm). Concerning the karyotype symmetry, *C. tenella* was slightly more symmetric (A₁ = 0.249; A₂ = 0.203) compared to *G. chacoensis* (A₁ = 0.323; A₂ = 0.342) and *G. angustifolia* (A₁ = 0.308; A₂ = 0.319).

Chromosome banding with CMA/DAPI fluorochromes showed only one pair of CMA⁺/DAPI bands for each species, revealing a remarkably stable heterochromatic pattern (Fig. 1A, C, E). The single pair of CMA⁺/DAPI bands was located on the terminal region of the long arm in *G. chacoensis* (Fig. 2A) and *G. angustifolia* (Fig. 2B), and short arm in *C. tenella* (Fig. 2C). No CMA⁺/DAPI bands were detected in all three species. The 5S and 35S rDNA sites, detected by FISH, were located in different chromosomes: the 35S rDNA sites were collocated with CMA⁺/DAPI bands (Fig. 2A-C), whilst the 5S were on the interstitial region of the long arm in both *Guadua* species (Fig. 2A, B) and on the short arm of *C. tenella* (Fig. 2C).

The nuclei suspensions of all bamboo species and *G. max* var. Polanka (internal standard) resulted in histograms of G₀/G₁ peak showing coefficients of variation below 5 %, providing reliable and high quality of flow cytometric data (Fig. 3). The mean nuclear DNA content was estimated as 2C = 3.98 pg for *G. chacoensis*, 3.99 pg for *G. angustifolia*, and 4.77 pg for *C. tenella*. This is the first genome size estimation for *G. chacoensis*, as well as for the genus *Chusquea*.

Discussion

In woody bamboos, the prevailing basic chromosome number is x = 12, although x = 10, 11 numbers were also reported, especially for species of the genus *Chusquea* (Hilu 2004). The chromosome number observed for *G. chacoensis* and *G. angustifolia* (2n = 46) confirms previous counts for both species (Quarín 1977; Chen *et al.* 2003), while 2n = 44 is the first report for *C. tenella*. The chromosome numbers reported herein support the tetraploid karyotype in Neotropical woody bamboo species (Triplett *et al.* 2014; Guo *et al.* 2019).

In *Guadua*, 2n = 46 was also observed for *G. paraguayana* and *G. capitata* (Davidse & Pohl 1992; Gould & Soderstrom 1967), with the only deviation reported in *G. macclurei* (2n = 48; Davidse & Pohl 1992). For *Chusquea* species, the most common chromosome number reported is 2n = 40,



based on $x = 10$. Variation from the expected number was also reported to *C. oxylepsis* ($2n = 44$; Hunziker *et al.* 1989). Those variations from the expected chromosome numbers must be then related to dysploid events from an ancient tetraploid karyotype, since the basic chromosome number for the Bambuseae tribe ranges from $x = 10$ to 12 (Schubert & Lysak 2011; Clark *et al.* 2015).

Polyploidy is recognized as a major evolutionary force in plants and it seems to have played an important role in radiation and habitat exploitation of woody bamboos (Nirmala *et al.* 2014; Soltis *et al.* 2014). Genomic data suggest that polyploidy plays an important role regarding the origin of woody traits and regulation of flowering behavior within the group (Guo *et al.* 2019). In fact, woody bamboo diversity is a consequence of three independent allopolyploid events involving four monophyletic and ancestral subgenomes, revealing a complex pattern of reticulate evolution (Triplett *et al.* 2014; Guo *et al.* 2019).

Fundamentally, the cytogenetic assumption of a recently formed polyploid is the additive pattern of rDNA sites/heterochromatic blocks (Clarkson *et al.* 2005; Hasterok *et al.* 2006; Zhang *et al.* 2016). For instance, a hexaploid origin proposed for the Paleotropical woody bamboo lineage was corroborated by the presence of three pairs of 35S rDNA sites in *Bambusa oldhamii*, *B. gibboides*, and *Melocanna baccifera* (Zhou *et al.* 2017). Following this additive pattern in polyploids, two pairs of rDNA sites in tetraploids temperate and Neotropical woody bamboos would be expected (Triplett *et al.* 2014; Clark *et al.* 2015). However, our data revealed only one pair of rDNA sites and heterochromatic bands in three Neotropical woody bamboo species, suggesting a karyotype evolution shaped by diploidization events. The diploidization hypothesis is corroborated by analysis of temperate tetraploids (Arundinaria tribe; $2n = 48$) *Phyllostachys heterocycla*, *P. vivax*, *Indosasa gigantea*, and *Pleioblastus gramineus*, that indicate also only one pair of

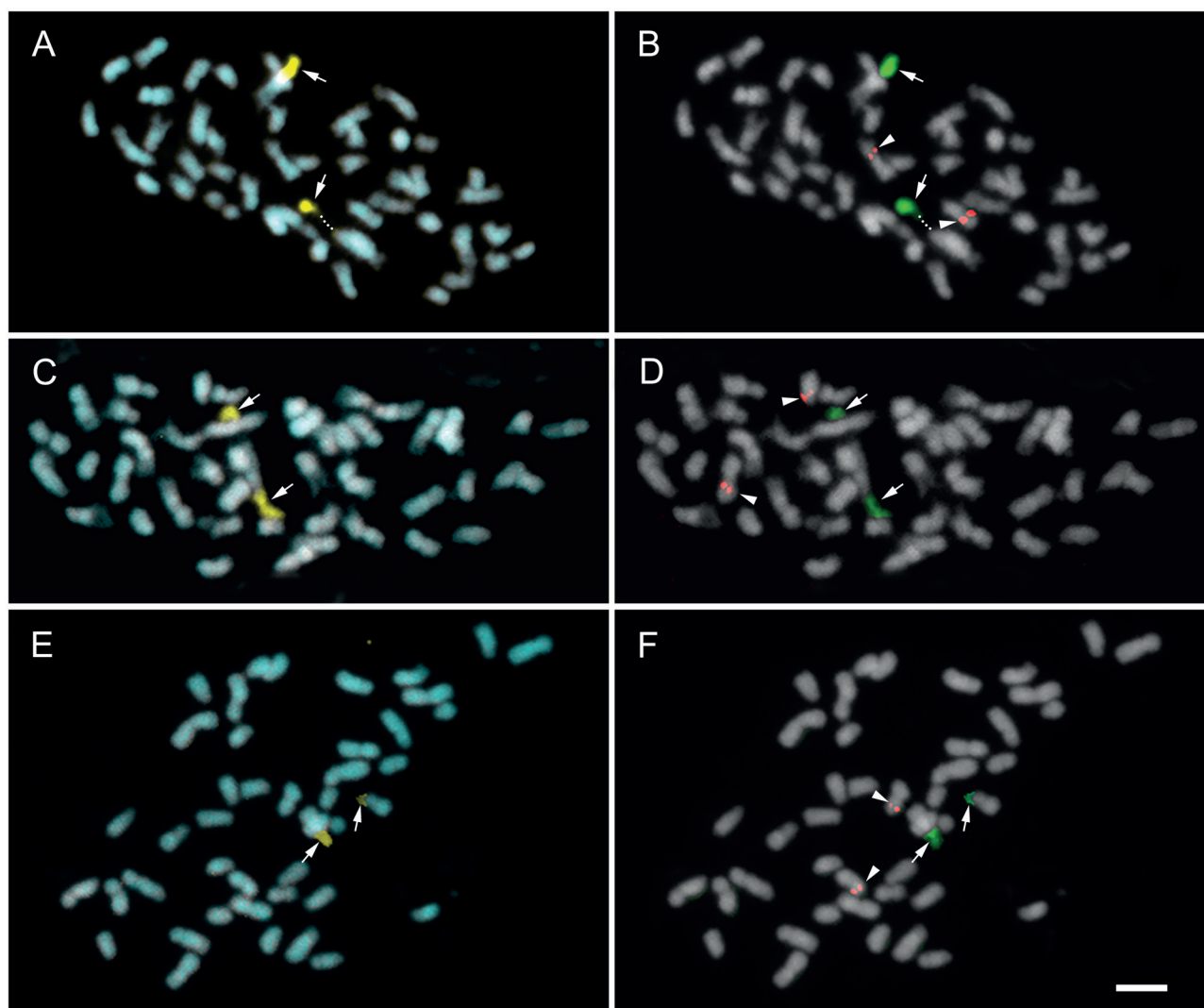


Figure 1. Distribution of heterochromatin and rDNA sites in *Guadua chacoensis* (A, B), *G. angustifolia* (C, D), and *Chusquea tenella* (E, F). Arrows in A, C, and E indicate CMA⁺/DAPI bands (yellow). In B, D, and F, arrows show 35S rDNA sites (green) and arrowheads show 5S rDNA sites (red). Bar = 5 μ m.

First cytomechanical characterization of three Neotropical woody bamboos (Bambusoideae, Poaceae) suggests ancient diploidized karyotypes

35S rDNA sites detected by FISH (Peng *et al.* 2013; Zhou *et al.* 2017).

Since phylogenetic relationships and polyploid origin within woody bamboo lineages are currently well established, the group could represent a model to understand the evolution of paleopolyploid karyotypes (Triplett *et al.* 2014; Guo *et al.* 2019). Therefore, given the Miocene origin (20 – 25 Mya) of the Neotropical woody bamboo lineage (Ruiz-Sanchez 2011; Guo *et al.* 2019), as well as the karyotype features and reduced number of heterochromatic bands and rDNA sites, it is reasonable to consider the three analyzed species as diploidized paleopolyploids (Clarkson *et al.* 2005; Berjano *et al.* 2009; Figueredo *et al.* 2016).

Both processes of genetic and cytological diploidization are frequently associated with the evolution of polyploid genomes, which has been widely recognized as an important evolutionary force across plant species (Ma & Gustafson 2005; Jiao *et al.* 2011). Genetic diploidization involves gene loss or rearrangement, while cytological diploidization leads to reduced ploidy level and/or chromosome number,

restoring the diploid-like meiotic behavior of the polyploid genome (Wolfe 2001; Hollister 2015; Dodsworth *et al.* 2016). Further karyotype and meiotic behavior analysis including a wide range of Neotropical woody bamboo species would be required to confirm the diploidized state of the group, which would be of great interest to resolve the complex genome evolution within the group.

Chromosome number and genome size variations have been widely suggested as an important character in grass evolution, mainly due to the effects of independent rounds of polyploidization (Bennetzen 2007; Devos 2010). Genome size estimations of woody bamboo species relied mainly on Paleotropical and temperate lineages, ranging from $2C = 1.67$ pg (*Bambusa affinis*) to $2C = 5.61$ pg (*Pseudosasa japonica*) (Gielis *et al.* 1997; Gui *et al.* 2007; Kumar *et al.* 2011; Jia *et al.* 2016; Zhou *et al.* 2017). The genome size of Neotropical woody bamboo had only been reported for *G. angustifolia* ($2C = 3.03$ - 3.98 pg; Gielis *et al.* 1997; Guo *et al.* 2019). Thus, the genome size data presented here are the first measurements for *G. chacoensis* and the genus *Chusquea*.

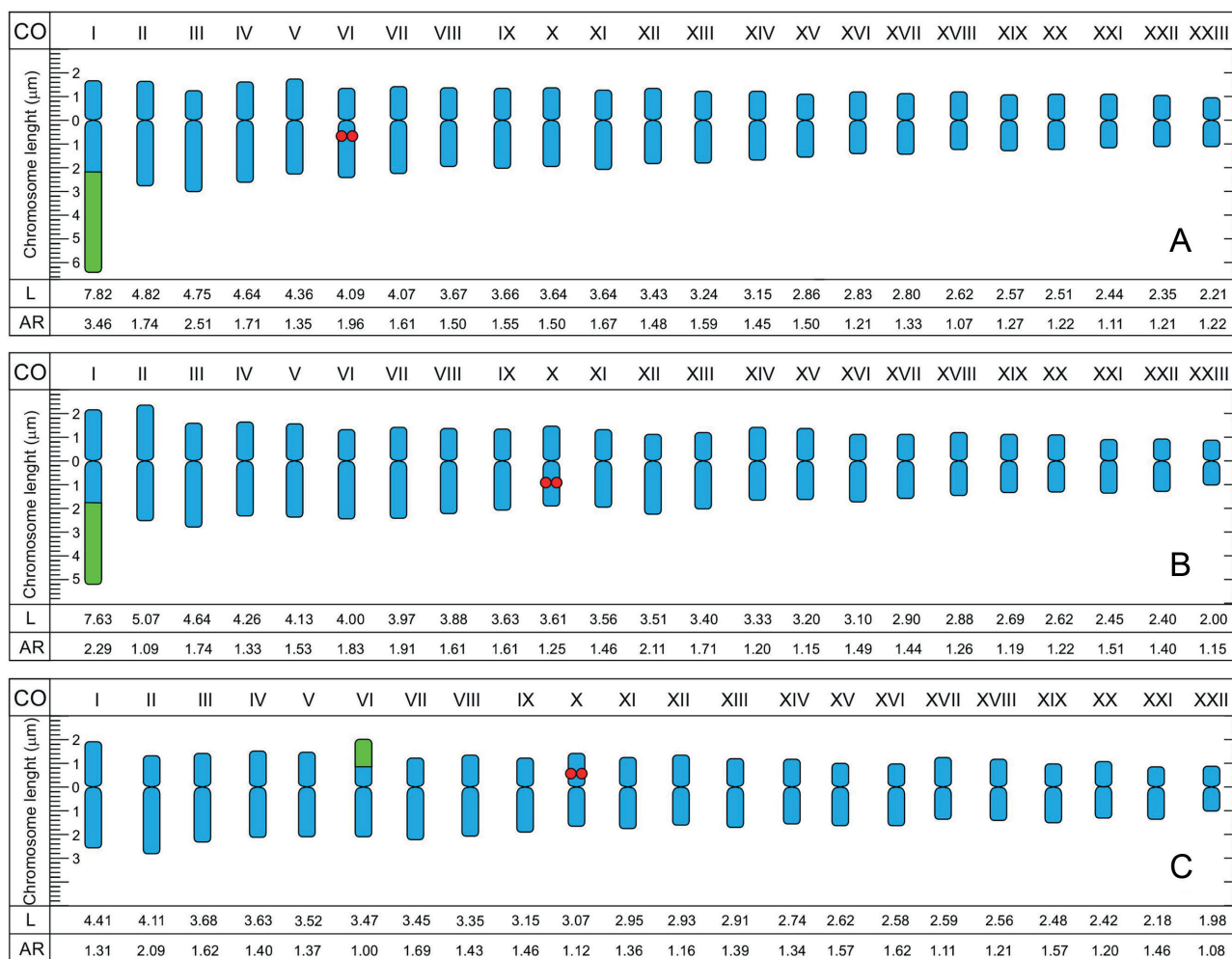


Figure 2. Idiograms of *Guadua chacoensis* (A), *G. angustifolia* (B), and *C. tenella* (C). Chromosomes are arranged by the position of centromere and by decreasing order of length. The 35S and 5S rDNA sites are represented by the green and red marks, respectively. CO: chromosome order; L: chromosome length; AR: arm ratio.

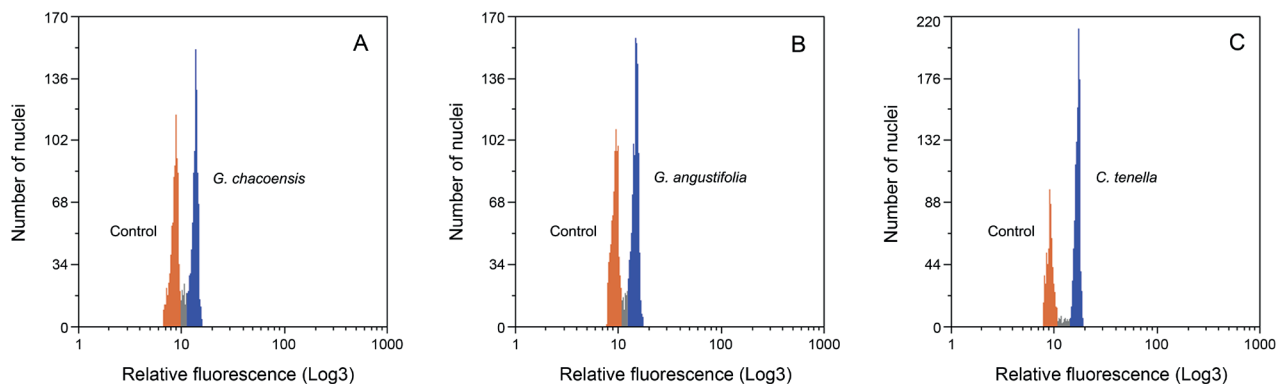


Figure 3. Histograms of relative fluorescence intensities obtained by FCM of nuclei suspension of *Glycine max* var. Polanka (internal standard; 2C = 2.5 pg) and **(A)** *G. chacoensis* (2C = 3.98 pg), **(B)** *G. angustifolia* (2C = 3.99 pg), and **(C)** *C. tenella* (2C = 4.77 pg).

Even though polyploidization events can directly affect karyotype features (e.g. chromosome number, genome size, etc.), these traits are shaped by independent evolutionary processes (Weiss-Schneeweiss & Schneeweiss 2013). *Chusquea tenella* ($2n = 44$) showed a larger genome size (2C = 4.77 pg) when compared to *G. chacoensis* and *G. angustifolia* (both with $2n = 46$ and 2C \approx 3.99 pg), corroborating the independent evolution of chromosome number/genome size (Zhou *et al.* 2017; Guo *et al.* 2019). On the other hand, both intrachromosomal and interchromosomal asymmetry indices revealed a symmetric karyotype in *C. tenella* and *Guadua* species (see Peruzzi & Eroglu 2013), suggesting that this is not a good cytogenetic parameter to differentiate the karyotype of these species.

Genome size and the total length of chromosomes (T) are often evolutionarily correlated (Schubert & Lysak 2011; Weiss-Schneeweiss & Schneeweiss 2013). The three bamboo species analyzed showed a very similar total chromosome length (T) despite differences in genome size. Differential chromatin condensation patterns can explain this apparent incongruity (see Feitoza *et al.* 2017). In general, early-condensed proximal and late-condensed terminal chromatin are related to smaller genomes (as observed here in *Guadua* species), and a uniformly condensed chromatin is usually found in species with larger genomes (as *C. tenella*).

The results herein presented the first chromosome count for *C. tenella*, as well as the first genome size estimation for *G. chacoensis* and the genus *Chusquea*, revealing the first insights into karyotype evolution within the Neotropical woody bamboo lineage. This finding highlights the complexity of genetic analysis within the group, regarding chromosome number (polyploidy), genome size, and karyotype features. Furthermore, considering the diploidization hypothesis must be crucial for cytogenetic/genomic studies.

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First cytomechanical characterization of three Neotropical woody bamboos (Bambusoideae, Poaceae) suggests ancient diploidized karyotypes

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