Diversity of satDNA fraction in *Cestrum*, the Genus with the Largest Genomes within Solanaceae

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Abstract

Cestrum species present large genomes (~24 pg), a high occurrence of B chromosomes, and great diversity in heterochromatin bands. Despite this, there is maintenance of chromosome shape and karyotype symmetry. To deepen our knowledge on *Cestrum* genome composition, low coverage sequencing data of *C. strigilatum* and *C. elegans* were compared. Bioinformatics analyses showed retrotransposons comprising more than 70% of the repetitive fraction, followed by transposons (~18%). The four satDNA families that accumulated the most in the datasets were used as probes in FISH assays, and showed different distribution profiles along chromosomes. Most hybridization signals were located in the C-CMA/DAPI banding sites, including those related to AT-rich Cold-Sensitive Regions (CSRs) and heterochromatin. Although satellite probes hybridized in all tested species, a satDNA family named CsSat49 was highlighted as it predominates in centromeric regions. Data suggest that the satDNA fraction is still conserved in the genus, although there is variation in the number of FISH signals between karyotypes, as well as in the B chromosomes. This study brings an important advance in the knowledge on genome organization and heterochromatin composition in *Cestrum*, especially on the distribution and differentiation mechanisms of satellite fraction between species of a genus of Solanaceae with large genomes.

Introduction

Angiosperm species exhibit wide variation in the content of genomic DNA (DNA C-value), which can reach up to 2,400 times [1]. This variation may often be associated with polyploidy, as well as with accumulation or reduction events in the repetitive DNA fraction [2, 3]. Part of this repetitive fraction is represented by satellite DNA (satDNA) families, which predominate in the constitutive heterochromatin. Each satellite consists of monomers (units) tandemly repeated over hundreds to thousands of times, with differences in size and composition bases, depending on the species and clade in the phylogeny. SatDNA families can commonly be found as clusters in distal and proximal regions, and eventually in interstitial chromosome regions [4, 5]. Satellites can also be associated with other repetitive sequences, such as 3' UTR-derived tandem repeats derived from Gypsy/Tat/Ogre retrotransposons residing in the pericentromeres of *Lathyrus* and *Coffea* [6, 7], respectively, or with CENH3 centromeric histones, like in barley [8]. It is because of examples like these that proximal regions are considered hotspots of repetitive DNA families, although pericentromere residing sequences have diverged considerably in different plant clades [9]. In other plant groups, like *Medicago* [10] and *Arachis* [11], satellite repeats may also occur in interstitial and terminal regions.

Variability in number and distribution of repeat sequences along chromosomes may be associated with different rearrangements (insertions, recombinations, and amplification/reduction of sequences), as well as the nature of sequences [12]. *Cestrum* L. is an interesting plant group to test the dynamics of repeat elements, due to its abundance and diversity of heterochromatin bands located in proximal, interstitial, and terminal regions, respecting an equilocal and equidistant distribution profile [13, 14]. Species of *Cestrum* exhibit different heterochromatin families, such as C-Giemsa bands, CMA^+ (associated or not

with NORs), $CMA^+/DAPI^+$ and neutral CMA/DAPI bands, and $DAPI^+$ associated with Cold Sensitive Regions (CSRs), even when staining with fluorochromes is performed after treatment for band C, with acetic acid, Ba(OH)□, and 2× SSC [13, 15]. In general, there is a tendency for *Cestrum* species to exhibit large heterochromatic bands in intercalary and terminal regions of the short arms, while the minute bands occur preferentially in intercalary and proximal regions [14]. If we take into account at least three factors: i) satellite DNA families may be originated from different genome fractions, ii) rearrangements may influence the increase or decrease in monomer numbers, and iii) *Cestrum* species present the largest genomes among the Solanaceae (~20 pg), some questions can be raised. Do different repetitive families have the same chromosomal distribution profile in different *Cestrum* species? Do fluctuations in satDNA accumulation provoke significant variations between DNA C-values? Do different satellite families have the same chromosomal distribution profiles in different species? These questions are particularly interesting for *Cestrum*, because it is a recent genus, with approximately 30 Mya, and 150 species distributed in tropical and subtropical regions of the Americas [15], of which 23 species occur in Brazil [16].

The aim of this study was to obtain an overview understanding of the repetitive DNA families using partially sequenced genomes of *C. strigilatum* Ruiz & Pav. and *C. elegans* (Brongn.) Schltdl., and a deeper view of the diversity and distribution of satDNA families using bioinformatic and FISH tools, in South American species.

Material And Methods

Plant collection

Samples of seven *Cestrum* species (*C. bracteatum* Link & Otto {formerly named of *C. amictum*}, *C. corymbosum* Schltdl., *C. intermedium* Sendtn., *C. axillare* Vell. {formerly named of *C. laevigatum*}, *C. mariquitense* Kunth, *C. nocturnum* L., and *C. strigilatum*) were collected from natural environments of different South and Southeastern Brazilian regions (São Paulo and Paraná states) and cultivated in the greenhouse of the Laboratory of Cytogenetics and Plant Diversity, State University of Londrina, Paraná, Brazil (**Online resource 1**). The collection consisted of at least three individuals of each species, and the vouchers were kept in the FUEL herbarium, at the State University of Londrina.

DNA extraction, genome sequencing and assembling

High-molecular-weight DNA (with about 40Kb fragments) of *C. strigilatum* was isolated from young leaves using the Nuclear Isolation Buffer (NIB) method [17]. The sample was used for low-coverage sequencing by an Illumina HiSeq2000 system (Novogene Company). The input files containing 9,227,692 reads with a 150 bp length were assembled using the Repeat Explorer pipeline with default [18, 19] (https://repeatexplorer-elixir.cerit-sc.cz), corresponding to ~0.1× of coverage. Data of *C. elegans* genome sequencing was retrieved from the public database of NCBI (SRX1951472), corresponding to ~0.3× of coverage. Datasets were filtered by quality with 90% of bases equal to or above the cut-off value of 10. To expand searches for repetitive DNA sequences, input files containing 9,227,692 reads with a 150 bp

length (*C. strigilatum*) and 22,134,326 reads with a 197 bp length (*C. elegans*) were also assembled with the SPAdes v3.6.2 program using K-mers 31, 51, 71, in a local server.

DNA C-value estimates

Measurements of Nuclear DNA amounts were performed with young leaves using 1 mL of cold LB01 buffer plus 1 mg/mL propidium iodide [20]. Analyses were performed on a BD ACCURI C6 flow cytometer, in three independent estimations on different days. *Pisum sativum L. 'Ctirad'* (2C=9.09 pg), was used as standard [20]. Measurements of at least 30,000 nuclei were taken in each reading cycle (three times). The 2C values were calculated as sample peak mean / standard peak means × 2C DNA amount of standard (pg).

Repetitive fraction evaluation

Two approaches were used to evaluate the proportion of repetitive DNA families in these two datasets. Sequences were contrasted in the pipeline implemented on RepeatExplorer and also using local Blastn/x analyses against databases containing conserved domain sequences available on RepBase (http://www.girinst.org/censor/), GypsyDB (http://gydb.org/index.php/MainPage) and RexDB (http://repeatexplorer.org/), totaling 283,676 protein sequences. To evaluate the proportion of rDNA sequences, a second database composed of 1652 sequences from 35S and 5S sequences from various organisms obtained on NCBI (http://www.ncbi.nlm.nih.gov) was used, keeping as a criterion max_target_seqs 1 as parameter, and the others on default in a tabular output format.

To gain an overview of satellite DNA families on the large *Cestrum* genomes, sequences of *C. strigilatum* and *C. elegans* were subjected to the TAREAN (TAndem REpeat ANalyzer) tool, on RepeatExplorer [18, 19]. In order to expand the scope of the analysis, the output files produced with SPAdes assembler, sequences of both genomes, were used in the TRF script [21]. Output files were processed with the TRF-filter script designed by us, G-numeric-1.12.35, as well as with filtering commands based on bash scripts in the Linux environment. Data were compared to optimize the search for satellite, microsatellite, and transposable element sequences. Probable satellite DNA monomers were used in a comparison "all against all", and checked with the Dotter Version 4.44.1 [22]. The SSR frequency was estimated using SSRIT script (http://archive.gramene.org/db/markers/ssrtool), the SSRIT output file was filtered with SSR_Estimates.sh script (designed by us), and the motifs (from two to six nucleotides, nts) were tabulated and organized to compare the 20 most common motifs in the two genomes used in this work.

Cestrum elegans data were used for comparisons in the bioinformatics analyses, but this species does not occur in Brazil. Therefore, only *C. strigilatum* was used for sequencing and cytomolecular analysis, because, in addition to being a species easily found in several Brazilian biomes, it exhibits the highest heterochromatin amount between the studied species.

Probe labeling and FISH

PCR were performed using a mix composed of 2 mM $MgCl_2$, 0.4 µM of primers, dNTP containing dGTP (0.1 mM), dCTP (0.1 mM), dATP (0.1 mM), dTTP (0.07 mM), and Cy3-dUTP (0.03 mM), ~10 ng of DNA

template, 1.25 U of Taq polymerase and ultrapure water to complete 25 µL. The PCR conditions were 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 30 s at 60 ºC, and 1 min at 72°C, and then 10 min at 72°C. Amplicons were labelled with DIG and BIO, using the nick translation kit (Digoxigenin NT Labeling Kit and Biotin 16 NT Labeling Kit – Jena Bioscience). The oligos CsSat1 and CsSat72 were designed with 5 biotin modifications (ThermoFisher Scientific), CsSat74 and CsSat49 were labelled by PCR using digoxigenin-dUTP.

The retrotransposon probes were obtained by PCR using specific primers for a conserved stretch of reverse transcriptase of Sire/Copia and Athila/Tat/Gypsy elemments. A standard PCR [5 U µL–1 Taq polymerase (0.5 µL), 10× buffer (2.5 µL), 50 mm $MgCl_2$ (1.5 µL), 10 mm dNTP (1 µL), 5 mm primers (2 µL each), and H_2O up to a final volume of 25 µL] was used in the following conditions: 94 ºC for 2 min, 30 cycles of 94 ºC for 40 s, 59 ºC for 40 s and 72 ºC for 1 min, and a final extension of 72 ºC for 10 min. Reactions were tested using electrophoresis in an agarose gel at 3 V cm–1 and stained with ethidium bromide. Amplicons were used in a second PCR to produce probes, which were labeled using 0.2 mM dNTP, containing dGTP (25%), dCTP (25%), dATP (25%), dTTP (17.5%) and bio-dUTP (7.5%) or Cy3-dUTP (7.5%).

Fluorescence in situ hybridization (*FISH)*

For cytogenetic analysis, slides were prepared from root tips pretreated with 0.1% colchicine for 6 h, fixed in Farmer solution (ethanol/acetic acid 3:1, v:v) for at least 2 h, and stored at -20°C. Roots were also collected and directly fixed in order to obtain mitotic stages. To obtain Cold Sensitive Regions (CSR), root tips were pretreated in cold water (~0 ºC) for 26h and fixed in Farmer solution [23]. Samples were softened in 2% cellulase plus 20% pectinase (w:v), at 37°C and then dissected in a drop of 60% acetic acid, and subsequently squashed. Coverslips were removed in liquid nitrogen, and the preparations were used for in situ hybridization tests.

For FISH, the probes labeled with biotin and digoxigenin were added on the slides prepared with 30 µL of a denatured mix containing 100% formamide (15 µL), 50% polyethylene glycol (6 µL), 20× SSC (3 µL), 100 ng calf thymus DNA (1 µL), 10% SDS (1 µL), and 100 ng probe (~4 µL), reaching > 70% stringency. Both slide and mix were denaturated/hybridized at 95°C, 50°C, and 38°C for 10 min in a thermal cycler, and then at 37°C overnight in a humidified chamber. Post-hybridization washes were carried out in 2× SSC and 4× SSC/0.2% Tween 20. Probes were detected using avidin–fluorescein isothiocyanate (FITC) or anti-digoxigenin-rhodamine conjugate and the slides were mounted with 25 µL of a solution composed of glycerol (90%), DABCO (2.3%), 20 mM Tris-HCl, pH 8.0 (2%), 2.5 mM $MgCl_2$ (4%), and distilled water (1.7%), plus 1 µL of 2 µg/mL DAPI. Sequential hybridizations were performed on slides washed in baths of 4× SSC/0.2% Tween 20 (twice), 2× SSC, fixed in ethanol/acetic acid 3:1 (v:v), and dehydrated with absolute alcohol.

Image acquisition

The slides were analyzed in three replicates for at least 10 cells. All chromosome images were acquired using a Leica DM 4500B microscope, which is equipped with a DFC 300FX camera, and overlapped with blue for DAPI, greenish-yellow for FITC, and red for Cy3, using Leica IM50 4.0 software. The images were optimized for contrast and brightness using GIMP 2.8 Image Editor.

Results

DNA C-value variation and distribution of TEs in the sequenced genomes

The amount of DNA estimated in 2C ranged from 21.00 ± 0.25 pg in *Cestrum mariquitense* to 24.82 ± 0.31 pg in *C. axillare* (with a B chromosome), see Fig. 1 and **Online resource 1**. If we consider that all species have 2*n*=16 and symmetrical karyotypes, the variation found is of more than 3 pg, which can equate to almost two medium-sized chromosomes. *Cestrum axillare* and *C. mariquitense* have the smallest amounts of heterochromatic bands compared to other species [14].

The comparative analyses using datasets of low coverage genome sequencing of *C. strigilatum* (with 23.94±0.50 picograms) and *C. elegans* (with about 19.52 pg [24]) showed relative conservation of repetitive domains between the two species (**Online resource 2**). Class I elements (retrotransposons) were most abundant in *C. strigilatum* and *C. elegans*, with 79% and 78%, followed by those of Class II (transposons) with 17% and 18%, respectively (Fig. 2a). Among the retrotransposons, members of the Copia superfamily represented approximately 17% in both species, while those of Gypsy represented 45% in *C. strigilatum* and 48% in *C. elegans* (Fig. 2b). Regarding specific lineages, no significant differences were observed in either dataset. The most accumulated lineages were Ale-Retrofit, Ale-Sire and Tork (Copia) and Reina, Tekay, and the clade Athila/Tat (Gypsy). The non-LTR LINE sequences showed a difference of ~15% in *C. strigilatum* and ~9% in *C. elegans* (Fig. 2c). If we prospect the TE quantities in both datasets in relation to DNA content, which was ~20% higher in *C. strigilatum*, the most significant differences appeared in the non-LTR retrotransposons (LINE elements) fraction, which was also 30% higher in *C. strigilatum*.

The tests of FISH using retrotransposon probes showed a dispersed distribution profile for both Sire/Copia and Athila/Tat/Gypsy (**Online resource 6a-f**), which were the two most abundant elements in *C. strigilatum* and *C. elegans*. Other LRT-RT probes were tested, but without great success in hybridizations. It is possible that the scattered pattern of these repetitive DNAs makes it difficult to observe such signals by FISH.

Estimates of Simple Sequence Repeat (SSR) and satellite DNA (satDNA) sets

The overview of microsatellite composition using the SSRIT script showed a predominance of trinucleotides (3-nts), with 77.8% in *C. strigilatum* and 85% in *C. elegans*, dinucleotides (2-nts) exhibited

16.5% in *C. strigilatum* and 11.2% in *C. elegans*. Motifs with 4, 5, and 6-nts represented less than 6% of the SSR set (**Online resource 3**). The 10 main motifs were common to both genomes, and aga/tct, gaa/ctt, aag/ttc, aat/tta, at/ta, and tc/ag were the most accumulated. Even the percentage of each motif varied between genomic assemblies, there were no significant differences in the sum of the most representative SSR motifs.

The characterization of satDNA families using the *C. strigilatum* dataset was based on two main criteria: i) occurrence and numbers of monomers and ii) AT/GC content. Four satellite monomers, ranging from 20 bp up to 339 bp, were recognized by the TAREAN tool (Fig. 3**)**. The CsSat1 family with 20nt (nucleotides) exhibited 74% AT and 26% GC, with 73% match, 15% mismatch, and 12% indels, and this family was identified only by the RepeatExplorer/TAREAN, probably because this tool was able to recognize and organize short monomers, with numerous inversions and deletions (Fig. 4). The other AT-rich satDNA families were very different in size and base pair compositions. The CsSat74 family exhibits 78nt (57.7% AT and 42.3% GC, 72% match, 14% mismatch and 14% indels between monomers), while the CsSat49 family showed 339nt (55.5% AT and 44.5% GC, with 84% match, 10% mismatch and 6% indels between monomers). The only GC-rich identified family, CsSat72 with 31nt, exhibits 38.7% AT and 61.3% GC basis, with 68% match, 21% mismatch, and 11% indels between monomers (**Online resource 4**). These four sequences were also contrasted via Blastn against the genomes of *C. strigilatum* and *C. elegans* assembled by the SPAdes tool, and the results showed that both genomes share similar monomers, independent of each other (**Online resource 5**).

satDNA probes from C. strigilatum have hybridized in other Cestrum species

Probes of satDNA families obtained from *C. strigilatum* genome were hybridized in situ against chromosomes of *C. strigilatum* (our model), and *C. bracteatum*, *C. corymbosum*, *C. intermedium*, *C. axillare*, *C. mariquitense*. The FISH revealed different hybridization profiles, such as dots or large proximal, interstitial, and subterminal blocks, individually localized or co-localized signals. CsSat1 (20nt and AT-rich) was the most abundant in the *C. strigilatum* dataset, and hybridized in the cold sensitive regions (CSR) that are identified by $DAPI^+$ signals (Figs. 4a-b **and Online resource 4**). Six interstitial, two subterminal, and four terminal FISH signals were detected, besides four chromosomes without signals (Figs. 5a-b). The CsSat74 probe (78nt and AT-rich) also showed bright signals in all chromosomes, sometimes colocalized with CsSat1 and with other interstitial dots (Fig. 5c). The CsSat49 (339nt and 55.5% AT) probe produced strong hybridization signals in the pericentromeric region of all the chromosomes, besides weaker FISH signals distributed in some chromosome arms (Fig. 5d). At the end, the only probe with a higher concentration of GC bases (CsSat72 with 31nt and 61.3% GC) exhibited stronger signals collocated with AT-rich regions (CsSat1 and CsSat74), in addition to smaller FISH signals in pericentromeric, interstitial, and terminal regions (Fig. 5e). To better visualize the distribution of satDNA families in *C. strigilatum*, an idiogram was created (Fig. 5f).

Tests with the satDNA probes in the other five species showed that probes hybridized at the same chromosomal positions, however, for some probes the number of signals was lower. The CsSat1 probe showed signals in all species, varying in quantity and location, with interstitial and terminal signals. CsSat1 signals varied from interstitial in two pairs in *C. mariquitense* (Fig. 6a), interstitial in two pairs and terminal in one pair in *C. bracteatum* (Fig. 6g), and interstitial in four pairs, with a very weak signal on a chromosome, and terminal in one pair in *C. axillare*. This last species exhibited FISH signals in the terminal region of a B chromosome, in addition to two smaller dots (Fig. 6h). Part of the interstitial CsSat1 signals were co-localized with CsSat49 signals (Figs. 6a, g-i). The CsSat49 probe was conserved in the centromeric/pericentromeric region of all chromosomes of *C. mariquitense* (Fig. 6a), *C. corymbosum* (Fig. 6f), *C. bracteatum* (Fig. 6g), and *C. axillare* (Fig. 6i), except two chromosomes of *C. mariquitense* and *C. bracteatum* and four chromosomes of *C. axillare*, as previously mentioned. This probe exhibited an approximation of centromeric regions in the interphase nuclei (Figs. 6b-c, **and f**). In *C. intermedium*, the FISH using CsSat49 probe produced centromeric/pericentromeric signals in all the chromosomes, besides two subterminal signals in the B chromosome, adjacent to $DAPI^+$ bands (**Online resource 6g-j**). The CsSat72 probe showed small and scattered FISH signals in all chromosomes of *C. mariquitense*, and the CsSat74 probe appeared as strong signals in seven chromosomes and as small dots in two others (Figs. 6d-e). The CsSat74 probe exhibited FISH signals in half of chromosomes of *C. intermedium*, and two proximal signals in the B chromosome, adjacent to $DAPI^+$ bands (**Online resource 6k-n**).

satDNA families occupy equilocally related regions

To understand the distribution profile of satDNA families, in situ hybridizations were performed on root tips of *C. strigilatum* squashed without any pre-treatment. For this, double FISH were applied with different probe combinations. The CsSat1 (AT-rich) and CsSat49 (GC-rich) probes showed FISH signals occupying opposite nuclear regions (Fig. 7a), following the Rabl's orientation. The CsSat49 probe hybridized at proximal regions (associated with centromeres), predominating in the equatorial plate in metaphases (Fig. 7a). This location was also observed in the opposite centromeric regions in the anaphases. Otherwise, the FISH signals with CsSat1 exhibited equilocal and equidistant distribution in both metaphase and anaphase (Figs. 7c-d).

The double FISH with the CsSat72 and CsSat74 probes also showed signals on opposite sides of the nuclei (Fig. 7e), with the CsSat74 equilocal signals occupying the interstitial regions around the centromeres at metaphases (Figs. 7f-g), while the CsSat72 signals were colocalized with the $DAPI^+$ signals and CsSat1 (Figs. 7f-g). The equilocality of these FISH signals was also more evident in anaphases (Figs. 7h-i), which supports the "contamination" of heterologous chromatids.

Discussion

What do we know about the repetitive DNA families in Cestrum?

Cestrum species exhibit large genomes (ranging from 2C=21 to 24 pg) and karyotypes (2*n*=16) with very large chromosomes when compared to most Solanaceae. What particularly attracts our attention is the fact that species maintain similar and symmetrical karyotypes, despite the exaggerated increase in DNA content (~41 times) in relation to the other Solanaceae. In an estimate performed in the Plant DNA C-values Database (https://cvalues.science.kew.org), the average value of 1C for the family is 2.84 pg, the lowest value is 0.60 pg, and the highest value is 24.80 pg. The genera with the highest 1C values described to date are *Nicotiana* (1C reaching 6.40 pg), *Lycium* with 7.47 pg, and *Cyphomandra*, with values ranging between 6.80 and 24.80 pg. Although it is clear that species such as *C. strigilatum* and *C. axillare*, with high 2C values among the species tested, have more FISH signals with satDNA probes, *C. bracteatum*, also with high 2C, exhibited less FISH signals with the same probes. From the other species with an estimate nuclear DNA amount, i.e. *C. parqui* (1C=10.92 pg), *Cestrum* hybrid (1C=12.09 pg), and *C. elegans* (1C=9.76 pg) [25], the first two species exhibit large numbers of heterochromatin bands [14]. These data suggest that DNA C-value variation is not only due to the accumulation or loss of the satellite fraction, but also to the repetitive DNA of other natures.

The estimate of the repetitive fraction using low coverage sequencing data of *Cestrum elegan*s and *C. strigilatum* showed retrotransposons with LTR (LTR-RTs) as the most accumulated elements of repetitive fraction (~60%). Sequences were highly similar among these two genomes, and reports including FISH with LTR-RT probes always exhibited a scattered distribution along chromosomes [26, 27]. Accumulation of retrotransposons in *Cestrum* seems to follow the general trend of plants [28]. In maize (*Zea mays)*, retrotransposons represent more than 75% of the genome [29]. Comparing *Cestrum* with other Solanaceae, in some species of *Capsicum*, the accumulation of LTR-RTs varies from 70% in *C. baccatum* to 98% in *C. chinense* datasets [30], while this fraction represents ~61% in *Solanum lycopersicum* and ~40% in both *Petunia axillaris* and *Nicotiana tomentosisformis* [31]. Non-LTR elements, transposons, and Simple Sequence Repeats (SSRs) were less representative in the *C. elegans* and *C. strigilatum* datasets. As with the other repetitive families, those of rDNA also varied between these two datasets. Although variations in rDNA had already been reported [14, 32, 33], they don't seem to be enough to explain the large variations found in DNA content observed here. It would be wiser to attribute the differences in DNA C-value to fluctuations across the entire repetitive DNA set, including for example, the minisatellite 5’-A4-5CTGCT-3’ [24]. Finally, there are eight *Cestrum* species with B chromosomes that, although always exhibiting the same size in micrometers and morphology, are rich in different repetitive DNA sequences [26, 32, 33, 34]. These B chromosomes are approximately three times lower than the largest pair, and could represent a variation of ~0.5 pg in the total DNA amount.

satDNA sequences are conserved in Cestrum, but differ in quantity on chromosomes

The four main monomers of satDNA characterized by the RepeatExplorer/TAREAN pipelines and used as the probe for physically mapping by FISH, allowed us to identify almost all heterochromatin related bands. The AT-rich CsSat1 corresponded to the subterminal and interstitial DAPI$^+$ bands [14], that are hypomethylated in cold-sensitive heterochromatin regions [23]. This sequence was the only one related to changes in cytosine methylation levels after cold exposure, but other changes resulting from some chromatin stress, like changes in H3K9ac or H3K9me2 levels [35], were not tested here.

The AT-rich CsSat74 sequences appeared in colocalized CsSat1 sites, however, CsSat74 also appeared in four other chromosomes, independent of CsSat1, and without any relation to CSR sites. As alignment by Dotter support that these two sequences are different, it seems reasonable to assume that CsSat1 is associated with the formation of CSRs. There is no doubt that CSRs of *Cestrum* are DAPI$^+$ and hypomethylated on cytosine residues, and these data are similar to the CSRs/DAPI$^+$ bands reported in *Paris hainanensis* [36]. However, it is important to emphasize that other epigenetic changes may be related to the decondensed state of chromatin when cold treatment occurs. An example is the CSRs/GC-rich reported in *Viburnum* and *Sambucus* species, of the Caprifoliaceae family [37]. Only the pair 8 of *C. strigilatum* carries a CMA$^+$ band adjacent to the CSR, but this GC-rich region does not appear distended after cold treatment [23]. The only CMA$^+$/GC-rich satellite sequence tested here was CsSat72 and it did not associate with CSRs.

The last and most interesting probe was CsSat49, whose FISH signals showed colonization in the centromeric region of all *C. strigilatum* chromosomes. This region is likely equivalent to the bands previously revealed by C-Giemsa [13, 14]. This probe also produced signals in the centromeric regions of *C. bracteatum*, *C. corymbosum*, *C. intermedium*, *C. axillare*, and *C. mariquitense*. Although FISH results have shown a predominance of CsSat49 centromeric signals, we cannot consider this monomer as typical of centromeres, because there are also FISH signals in interstitial regions, close to the 5S rDNA region of *C. strigilatum* and *C. axillare*, and in subterminal regions of B chromosomes of *C. axillare* and *C. intermedium*. In plants, the proximal chromosomal region is enriched by different families of repetitive DNA. In fact, there is large diversity and accumulation of repeated sequences in the proximal chromosome regions, such as LTR retrotransposons [38, 39, 40] and satDNA [7, 41, 42]. It is important to mention that we used two different probes to locate centromeric retrotransposons in *Cestrum* (for conserved domains of reverse transcriptase and integrase), but we were not successful using the FISH, contrary to what we obtained in *Capsicum* [30]. Proximal repetitive sequences seem to play an important role in the dynamics of the centromeric region and, at least for CsSat49 repeats, this feature appears to be relatively conserved in *Cestrum*, a recent genus with approximately 30 Mya.

satDNA families are equilocally dispersed in Cestrum

FISH data showed that the four most representative satDNA families of *C. strigilatum* and *C. elegans* are relatively conserved in the genus, although great diversity in the number of FISH signals was evident when the karyotypes of six species were compared. Apparently, these satellites seem to occupy some preferential chromosome regions, i.e. subterminal, interstitial, or proximal, like the CsSat49 family that

was predominantly proximal. FISH performed on non-pretreated materials allowed us to visualize a clustering of the signals from the CsSat49 probe in opposite regions to the signals from the CsSat1 and CsSat74 probes, suggesting that satellite sequences may have been equilocally dispersed, following the Rabl configuration [43, 44]. Three models of organization of chromosomal regions in interphase nuclei have been considered. The first, named the rosette-like configuration, predicts that chromatin organizes from heterochromatic bodies, from which less condensed chromatin loops emanate [45]. The second, named the Rabl configuration, predicts that centromeric and telomeric regions occupy opposite nuclear poles, following the kinetics of anaphasic chromosomes, while in the third case, the so-called non-Rabl configuration, both centromeres and telomeres lose any orientation, appearing as dispersed throughout the nucleus [46]. Data obtained from crucifer species point out that nuclei with large genomes tend to present centromeres and telomeres arranged according to the Rabl-like configuration [47], and this may be what occurs in *Cestrum* species, which have very large genomes (from ~19 to 24pg). Although our in situ hybridization images were produced by the squashing technique, which can relatively interfere in the positioning of the chromosomal arms, the observation of dozens and dozens of cells gives us reliability to suggest that the equilocal dispersion, based on the Rabl configuration, may have been determinant for the distribution profile of satDNA families in *Cestrum*.

Final remarks

This study addresses a comparison between genome and chromosome data of species that have a large expansion in DNA C-values, when related to other species of the Solanaceae family. The mean 2C-value in *Cestrum* (~23 pg), that is about 8 times higher than the mean value in *Solanum*, i.e. ~2.8 pg (Plant DNA C-values Database, from https://cvalues.science.kew.org/search), together with similarities in karyotype symmetry, suggest that differentiation occurred due to changes in repetitive fraction and not by major chromosome rearrangements. The main satDNA families of *Cestrum* occur in all the studied species, but they are differentially accumulated, in some cases co-located in the same chromosome stretches (such as CSR and non-CSR). This study brings a great advance in the understanding of the satellite fractions, and their contribution to the organization of these large genomes. Nevertheless, even though we determined that there is a predominance of Copia/Ale, Gypsy/Athila/Tat, and non-LTR/LINE elements in the genomes of both *C. strigilatum* and *C. elegans*, there remains a gap in the knowledge on the physical distribution of transposable elements and their roles in the genome expansion in *Cestrum.*

Declarations

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate and publish All authors reviewed and approved the final version for publication.

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Figures

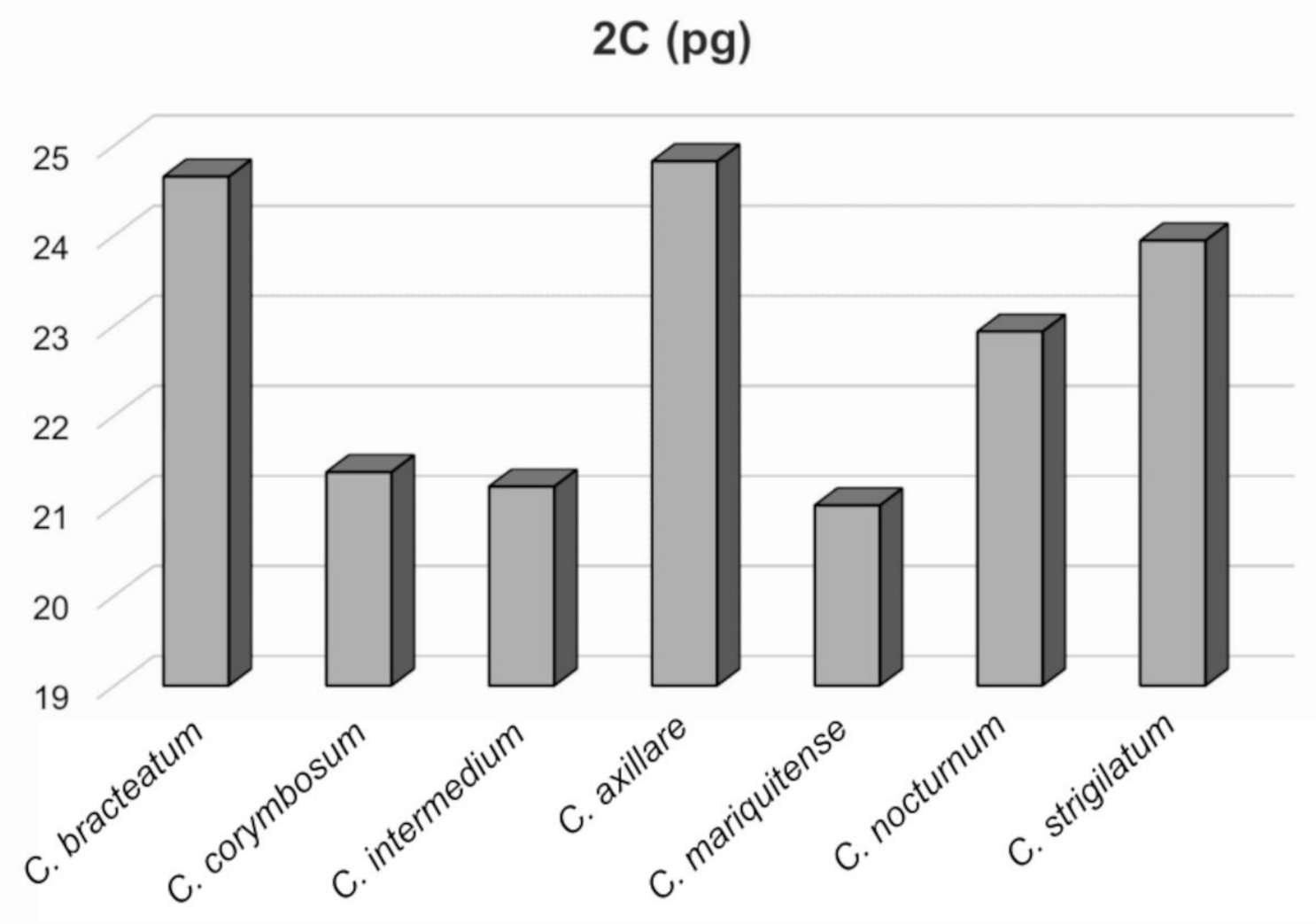

Figure 1

Comparison of amount of nuclear DNA between seven species of Cestrum. Note that the difference in 2C values between the largest and smallest genome is 15.4%, which represents about 3 µm

Figure 2

Occurrence of repetitive DNA families in two Cestrum genomes. (a) LTR-retrotransposon sequences were the most accumulated in C. strigilatum and C. elegans, followed by transposons and rDNA. (b) LTR-RTs Gypsy and Copia superfamilies presented similar predominance in the two datasets, mainly Ale-Copia and Athila/Tat-Gypsy. (c) Non-LTR retrotransposons differed between the two datasets, and LINE/SINE accumulated more in C. strigilatum than in C. elegans, followed by Sola transposons

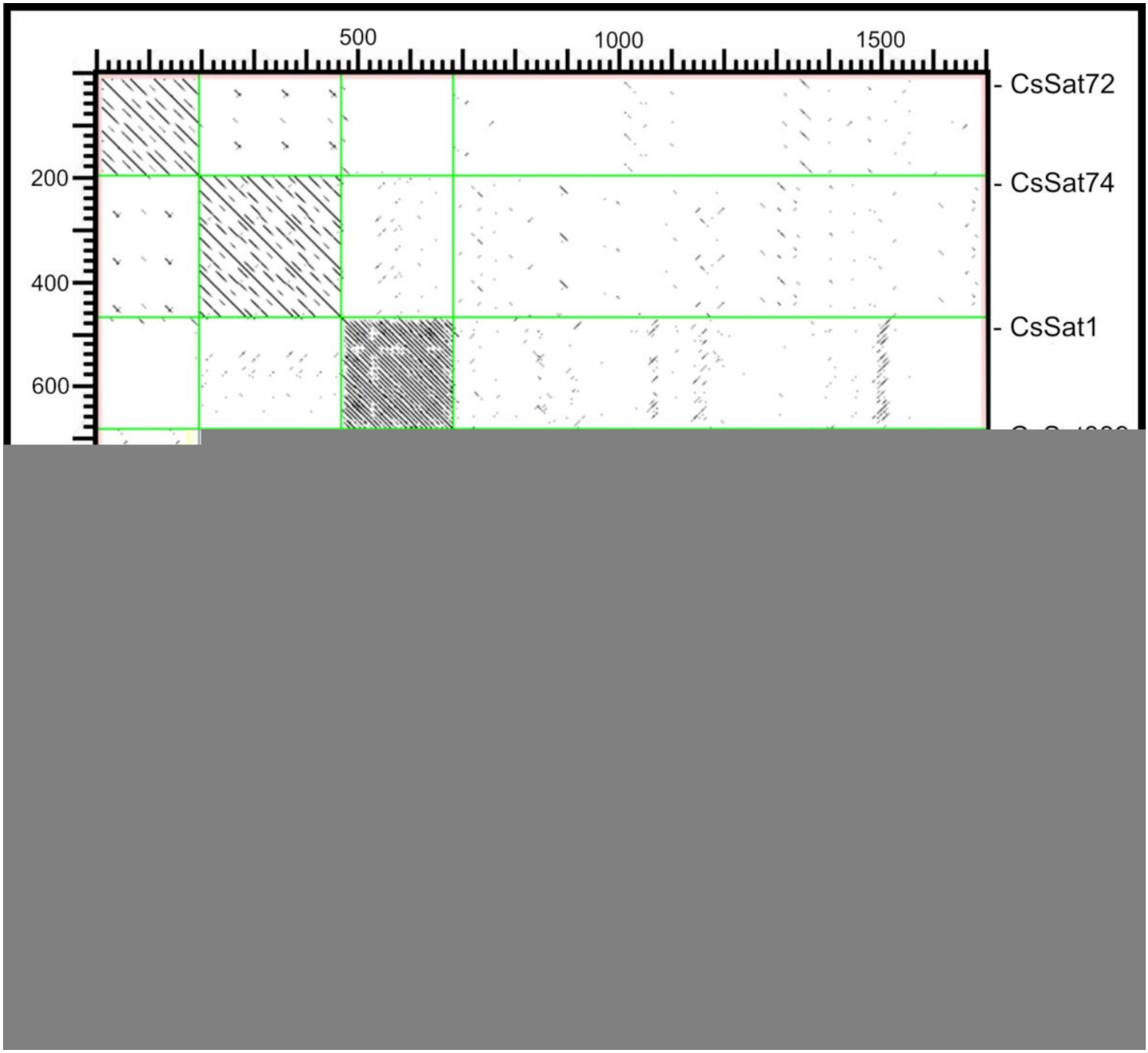

Figure 3

Dotplot of satDNA families identified in Cestrum strigilatum and C. elegans. For better visualization of the repeats relationship, contigs containing repeats in tandem were compared to each other on the plot. Note that all of them are independent. Analyses of contigs showed that CsSat72 and CsSat74 present numerous deletions, CsSat1 presented several small inversions and deletions, while CsSat49 seems to be more conserved

Figure 4

Organization and distribution of the AT-rich CsSat1 satellite. (a) prometaphase of Cestrum strigiltum, after cold treatment and DAPI staining. Note the non-condensed bands highlighted by the DAPI dye, which are named CSR (cold sensitive regions). (b) The same prometaphase hybridized with CsSat1 probe, showing the FISH signals in the CSR locations. (c) Dot plot of distinct contigs containing repeats of the CsSat1 family from C. strigilatum. Note that there are numerous deletions and inversions within each cluster. However, it is important to mention that repeats present from 10 nts, and the probe for FISH was designed with 2 repetitions, i.e. 20 nts

Figure 5

Distribution of the four selected satDNA families (CsSat1, CsSat74, CsSat49, and CsSat72) in C. strigilatum. Metaphase chromosomes were stained with DAPI (a). Satellite probes were detected with avidin-FITC or anti-dig–rhodamine conjugate, and pseudocolored after image capture. Note that CsSat1 (b), CsSat74 (c), and CsSat72 (d) FISH signals are colocalized in many regions, however, some specific signals were observed in some chromosomes. The CsSat49 probe (e) showed signals predominating in the centromeric regions of all chromosomes. (f) Idiogram representing the distribution of all satellites located by FISH. Note that most terminal and subterminal regions are colonized with different repeats, colocated in an equilocal arrangement, just like interstitial FISH signals. Scale bar 10μm

Figure 6

FISH using the four selected satDNA families (CsSat1, CsSat74, CsSat49, and CsSat72) from Cestrum strigilatum, against chromosomes of four other species (C. mariquitense, C. corymbosum, C. bracteatum and C. axillare). Chromosomes were DAPI stained. Probes were detected with avidin-FITC or with anti-dig–rhodamine conjugate, and pseudocolored after image capture. (a) FISH in C. mariquitense with CsSat1 (green) and CsSat49 (red). Observe a chromosome pair showing probe colocalization, in addition to amplification of the centromeric satellite to another subterminal region. Note also that the signals of CsSat1 (green) and CsSat49 (red) probes occupy opposite sides of the interphase nucleus (b). (c-e) Metaphase of C. mariquitense hybridized with two other satellites, (CsSat72 and CsSat74). The probe showed small FISH signals with CsSat72 probe (green), distributed throughout the interphase nucleus (c) and on most chromosomes and in proximal and interstitial regions (compare DAPI stained metaphase in (d) and the FISH in (e)). The CsSat74 probe (red) showed signals on one side of the nucleus (c) and on half of the chromosomes (e). Note more intense signals in four pairs in the terminal and subterminal regions, associated with a DAPI- region. (f) Metaphase of C. corymbosum hybridized with CsSat49. Observe FISH signals in all centromere regions and organizing the centromere ring at interphase. (g) Metaphase of C. bracteatum hybridized with CsSat49 (red) and CsSat1(green). Observe that all the centromere regions presented FISH signals with the CsSat49 probe, but there are less interstitial FISH signals with CsSat1 probes, when it is compared with C. strigilatum (see the Fig. 5). (h and i) Metaphase of C. axillare hybridized with CsSat49 and CsSat1. FISH with CsSat1 probe showed terminal and

subterminal signals, including the B-chromosome. The CsSat49 probe showed signals in the proximal region of all chromosomes, besides an additional signal in two pairs. Note that the B chromosome has FISH signals, co-located with CsSat49 at the terminal region. Scale bar 10μm

Figure 7

FISH with CsSat1, CsSat74, CsSat49, and CsSat72 probes in Cestrum strigilatum samples without any pretreatment, in order to obtain different phases of mitotic division. (a) Metaphase and interphase hybridized with CsSat1(green) and CsSat49(red) probes, showing an equilocal alignment of FISH signals. Note that the satellites occupy opposite regions in the interphase nucleus. (b-d) Anaphase hybridized with CsSat1(green) and CsSat49(red) probes, showing an equilocal arrangement of FISH signals. Observe that the CsSat49 probe occupies the proximal region, organizing a centromere ring, in opposition to the CsSat1 satellite, however both probes exhibit an equilocal alignment. (e-i) FISH with the CsSat72 (green) and CsSAt74 (red) probes, showing the opposite positioning of the satellites in the core interphase (e). Both probes showed interstitial signs, also with an equilocal alignment. This position is more evident in FISH in anaphase (h and i)

Supplementary Files

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- CestrumOnlineresource.docx