



## Karyotype differentiation of four *Cestrum* species (*Solanaceae*) based on the physical mapping of repetitive DNA

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### Abstract

We studied the karyotypes of four Brazilian *Cestrum* species (*C. amictum*, *C. intermedium*, *C. sendtnerianum* and *C. strigilatum*) using conventional Feulgen staining, C-Giemsa and C-CMA<sub>3</sub>/DAPI banding, induction of cold-sensitive regions (CSRs) and fluorescent *in situ* hybridization (FISH) with rDNA probes. We found that the karyotypes of all four species was  $2n = 2x = 16$ , with, except for the eighth acrocentric pair, a predominance of meta- and submetacentric chromosomes and various heterochromatin classes. Heterochromatic types previously unreported in *Cestrum* as neutral C-CMA<sub>3</sub><sup>0</sup>/DAPI<sup>0</sup> bands, CMA<sub>3</sub><sup>+</sup> bands not associated with NORs, and C-Giemsa/CSR/DAPI bands were found. The heterochromatic blocks varied in size, number, position and composition. The 45S rDNA probe preferentially located in the terminal and subterminal regions of some chromosomes, while 5S rDNA appeared close to the centromere of the long arm of pair 8. These results suggest that karyotype differentiation can occur mainly due to changes in repetitive DNA, with little modification in the general composition of the conventionally stained karyotype.

**Key words:** *Cestrum*, chromosome banding, CSRs, karyotype evolution, rDNA probes.

Received: August 24, 2004; Accepted: May 31, 2005.

### Introduction

Along with *Vestia* (Willd) and *Sessea* (Ruiz et Pav) the genus *Cestrum* (L.) is part of the tribe *Cestreae* (G. Don) of the *Cestroideae - Solanaceae*. The genus *Cestrum* contains about 250 species distributed in the tropical and subtropical regions of the Americas (D'Arcy, 1986) and includes herbs, shrubs and trees up to 12 m in height. Members of this genus are recognized by their actinomorphic flowers with tubular-infundibuliforme corolla, five stamens attached to the corolla tube up to the middle or higher, bilocular ovary with berry-like fruits, few compressed seeds and a straight embryo (Cronquist, 1988). The karyotype of *Cestrum* species contains  $2n = 2x = 16$  chromosomes of similar size and shape, most of which are meta- or submetacentric except for one subtelocentric pair (Berg and Greilhuber, 1992, 1993a,b). In general, *Solanaceae* species

such as those of the genera *Solanum* and *Capsicum* ( $2n = 2x = 24$ ) exhibit small chromosomes (1.2-2.4  $\mu\text{m}$  for *Solanum* and 3.5-4.0  $\mu\text{m}$  for *Capsicum*) and constancy in the distribution of meta- and submetacentric chromosomes (Bernardello and Anderson, 1990, Bernardello *et al.*, 1994, Moscone, 1993). Nevertheless, the chromosomes of the *Cestreae* are the largest in the family, reaching up to 14  $\mu\text{m}$  and containing 1.36 pg of DNA per chromosome (Berg and Greilhuber, 1992, 1993a,b, Sykorová *et al.*, 2003a).

Berg and Greilhuber (1992, 1993a,b) studied five species of *Cestrum* (*C. aurantiacum*, *C. elegans*, *C. fasciculatum*, *C. parqui*, and *C. strigilatum*) using several banding techniques, and found four different heterochromatic types: (i) large blocks unassociated with nucleolar organizing regions (NORs); (ii) numerous smaller blocks forming intercalary, proximal or distal dots; (iii) CMA<sub>3</sub><sup>+</sup> (chromomycin A<sub>3</sub>) bands associated with NORs; and (iv) DAPI<sup>+</sup> (4'-6-Diamidino-2-phenylindole) bands associated with cold-sensitive regions (CSRs). The CSRs were first described by Dyer (1963) and at present have been reported in 11 genera of monocotyledons but only three dicotyledons.

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Members of *Solanaceae* show large diversity in heterochromatin accumulation and dispersion. Species of - *Capsicum* exhibited heterochromatic blocks in all the centromeres and secondary constrictions, besides a variable number of large and small bands distributed along the chromosomes (Moscone *et al.*, 1993). Similarly, *Nicotiana* species show localized DNA repetitive segments (microsatellites, ribosomal DNAs and telomeric sequences), while other (virus-associated) segments are dispersed along the chromosomes (Yoong Lim *et al.*, 2000), and the literature suggests that *Cestrum* can also follow this model.

During the research reported in this paper we compared the karyotypes of four Brazilian *Cestrum* species (*C. amictum* (Schlecht.), *C. intermedium* (Sendtn.), *C. sendtnerianum* (Mart. & Sendtn.) and *C. strigilatum* (Ruiz & Pav.)) using Feulgen staining and chromosomal markers produced by C-Giemsa and C-CMA<sub>3</sub>/DAPI banding, CSRs and fluorescence *in situ* hybridization (FISH) with 45S and 5S rDNA probes. The results are discussed considering the possible mechanisms of dispersion and distribution of heterochromatic segments and of rDNA cistrons in the karyotypes of the four species.

## Materials and Methods

Seeds of the four *Cestrum* species cited above (eight populations) were obtained from different localities and cultivated in tubes in the seedling nursery of the Laboratory for Biodiversity and Restoration of Ecosystems (LABRE), Biology Science Centre, State University of Londrina, Paraná, Brazil. Vouchers were deposited at the FUEL herbarium (Table 1).

Slides were prepared of root tips pre-treated with 0.05% colchicine, fixed in Carnoy solution (ethanol/acetic acid 3:1, v:v), for up to 24 h and stored at -20 °C. Conventional staining was carried out by the Feulgen method. The cover slips were removed after freezing slides in liquid nitrogen and the slides permanently mounted in Entellan. To obtain CSRs, roots were collected and pre-treated in Bristol nutritive solution (Bold, 1949) for 24 h at 0 °C and fixed in Carnoy solution. Roots were hydrolyzed in 1 M HCl, dissected in a drop of 45% (v/v) aqueous acetic acid and

squashed. The cover slips were removed after freezing in liquid nitrogen and the preparations stained with 2% (w/v) Giemsa and the cells photographed using Kodak Imagemink HQ ASA 25 film.

For chromosomal banding, roots were digested in an enzyme solution composed of 4% cellulase and 40% pectinase (both w/v) at 37 °C and dissected in a drop of 45% acetic acid. After removal of the cover slips, the slides were aged for three days, and then incubated in 45% acetic acid, 5% barium hydroxide and 2xSSC, pH 7.0 (Schwarzacher *et al.*, 1980, with modifications). The materials were stained in two different ways, either with 2% Giemsa or with the fluorochromes 0.5 mg/mL CMA<sub>3</sub> for 1.5 h and 2 µg/mL DAPI for 30 min. Slides stained with Giemsa were mounted in Entellan and those stained with the fluorochromes mounted with a medium composed of glycerol/McIlvaine buffer (pH 7.0) 1:1 plus 2.5 mM MgCl<sub>2</sub>. The cells were photographed using Kodak Imagemink HQ ASA 25 and T-max ASA 100 film.

Fluorescent *in situ* hybridization (FISH) was performed according to the procedures described by Heslop-Harrison *et al.* (1991) and Cuadrado and Jouve (1994), with minor modifications. Slides were prepared as described for banding. The wheat probes pTa71 containing the 45S rDNA sequence (Gerlach and Bedbrook 1979) and pTa794 containing the 5S rDNA sequence (Gerlach and Dyer 1980) were both labeled with biotin-14-dATP by nick translation and used for FISH. Each slide was treated with 30 µL of hybridization mixture containing 100-150 ng of labeled probe (4 µL), 50% formamide (15 µL), 50% polyethylene glycol (6 µL), 20xSSC (3 µL), 100 ng of calf thymus DNA (1 µL), and 10% SDS (1 µL). The material was denatured at 90 °C for 10 min, and hybridization was performed overnight at 37 °C in a humidified chamber. Post-hybridization washes were carried out in 2xSSC, 20% formamide in 0.1xSSC, 0.1xSSC and 4xSSC/0.2% Tween 20, all at 42 °C. The probes were detected with avidin-FITC conjugated and the post-detection baths were conducted in 4xSSC/0.2% Tween 20 at room temperature. Slides were mounted in 26 µL of a solution composed of antifade (12.5 µL) and 50% glycerol in McIlvaine buffer (pH 7) with 2.5 mM

**Table 1** - Chromosome measurements and morphology of the *Cestrum* species and populations analyzed. CL = Length of large and small chromosome pairs; THL = Total length of haploid set.

<i>Cestrum</i> species	Natural location in Brazil	CL (µm)	THL	Metacentric	Submetacentric	Acrocentric	FUEL
<i>C. amictum</i> Schlecht.	Conquista farm, Rolândia, Paraná (PR)	6.9 to 9.8	70.2	1. 2. 3. 4. 6 and 7	5	8	30386
<i>C. intermedium</i> Sendtn.	Mata dos Godoy State park, Londrina, PR	7.3 to 11.8	77.3	1. 2. 3. 5 and 7	4 and 6	8	31197
	Mata Doralice reserve, Ipirorã, PR						30255
	Rubi valley, Londrina, PR						31398
<i>C. sendtnerianum</i> Mart.	Hermógenes de Freitas Leitão Filho ecological park, Campinas, SP	6.4 to 8.7	63.4	1. 2. 3 and 4	5. 6 and 7	8	33213
<i>C. strigilatum</i> Ruiz & Pav	São Jerônimo native Brazilian reserve, São Jerônimo da Serra, PR	7.2 to 8.6	63.3	1. 3. 4. 6 and 7	2. 5 and 8	-	31953
	Sapopema, PR						30984
	Mata Doralice reserve, Ipirorã, PR						29682

MgCl<sub>2</sub> (12.5 µL), plus 1 µL of 50 µg/mL propidium iodide. Photographs were taken using Kodak Proimage color ASA 100 film.

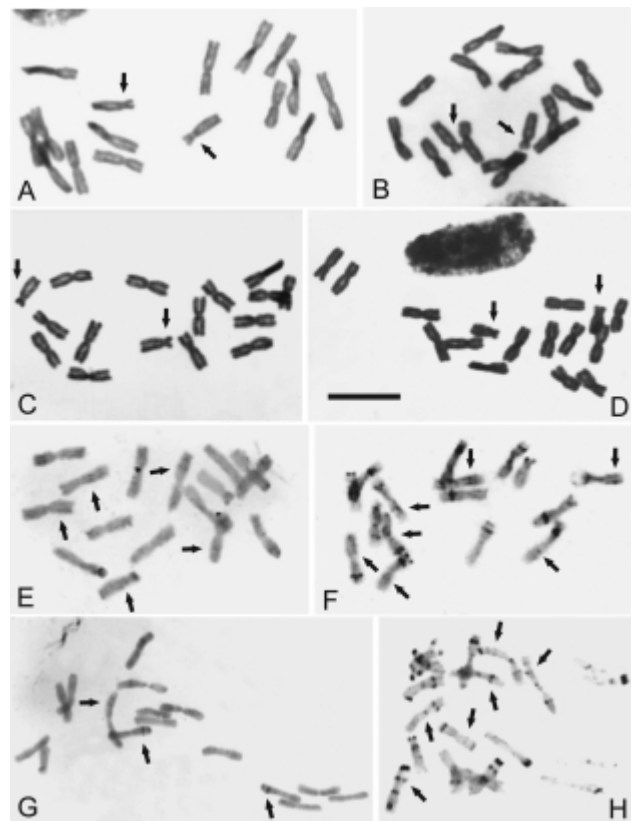
In general, 15 specimens were analyzed for each of the eight populations and idiograms constructed based on the analyses of about 10 metaphases and/or pre-metaphases obtained from each treatment. The chromosomes were classified based on the nomenclature proposed by Guerra (1986).

## Results

All four *Cestrum* species studied had a diploid chromosome number of  $2n = 2x = 16$ , with chromosomes varying gradually in size from about 6.4 µm to 11.8 µm (Table 1), although *C. intermedium* exhibited slightly larger chromosomes. The predominant chromosome forms were meta- and submetacentric, but pair 8 of *C. strigilatum* was submetacentric while in the other species this pair was acrocentric (Table 1). Satellites were rarely seen, and the interphase nuclei were always reticulate. Karyotypical differences among the populations were not detected, even when the chromosomes were studied using several techniques (conventional staining, banding and FISH).

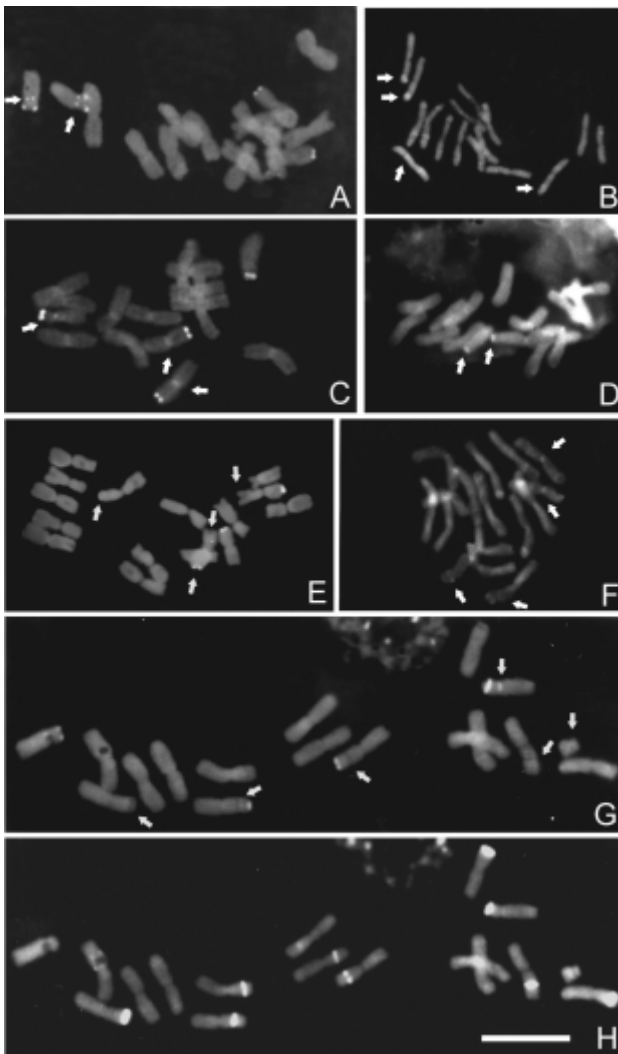
We found that *C. amictum* exhibited six metacentric pairs, one submetacentric and one acrocentric (Figure 1A, Table 1). C-Giemsa banding revealed heterochromatic blocks in all the chromosomes, with the intercalary bands being smaller and tenuous (or inconstant) in some preparations while the terminal bands were larger and more evident. The tenuous bands were distributed equidistantly on the long arms of almost all pairs except for pairs 2 and 8. Large C-Giemsa blocks were visualized mainly in the terminal/subterminal regions of the short arms of the minor chromosome pairs (Figures 1E and 5A) but fluorochrome banding showed large C-CMA<sub>3</sub><sup>+</sup> blocks associated with NORs in the terminal regions of the short arms of pairs 6 and 8, (Figures 2A and 5A). The C-DAPI<sup>+</sup> bands were tenuous and slightly fluorescent, located in the terminal/subterminal regions of the short arms of pairs 6 and 7, and on the long arms of pairs 4 and 6 (Figures 2B and 5A). Neutral bands (C-CMA<sub>3</sub><sup>0</sup>/DAPI<sup>0</sup>) appeared in the terminal regions of the short arm of pair 3 and proximal on the long arm of pair 8 (Figures 2A, 2B and 5A). Cold-sensitive regions (CSRs) were visualized in the intercalary region of the short arm of chromosome pair 4 of *C. amictum* (Figures 3A and 5A). Terminal signals on the short arms of pairs 6 and 8 were detected with FISH with the 45S rDNA probe, while FISH with the 5S rDNA probe gave a proximal signal on the long arm of pair 8 (Figures 4A, 4B and 5A).

The karyotype of *C. intermedium* was composed of five metacentric pairs, two submetacentrics and one acrocentric pair (Figure 1B and Table 1). After C-Giemsa banding, heterochromatic regions were visible in all the chromosomes (Figures 1F and 5B). Large C-Giemsa bands



**Figure 1** - (A-D) Feulgen-stained metaphases of *Cestrum amictum* (A), *C. intermedium* (B), *C. sendmerianum* (C) and *C. strigilatum* (D). Arrows indicate the acrocentric pair. (E-H) Giemsa C-banded metaphases of *Cestrum amictum* (E), *C. intermedium* (F), *C. sendmerianum* (G) and *C. strigilatum* (H). Arrows indicate some interstitial and inconspicuous C-bands. Bar represents 10 µm.

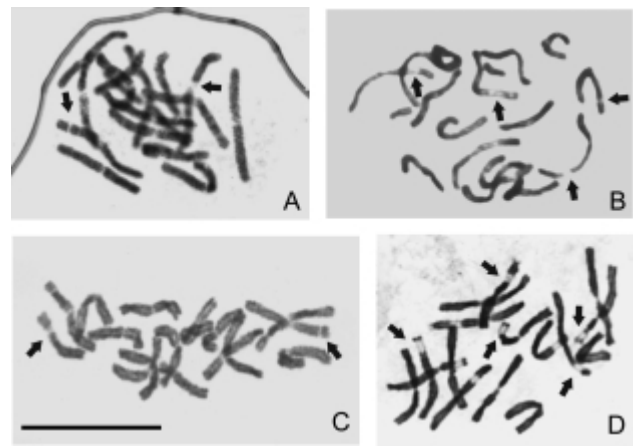
were found in the intercalary/terminal positions of the long arms of pairs 4 and 5, in the terminal/subterminal regions of the short arm of pair 7 and 8 (both associated with NORs) and in the terminal regions of the short arms of pairs 2 and 3. More tenuous bands were detected at intercalary/terminal positions on the long arms of pairs 3 and 6, as well as in the intercalary part of the short arm of pair 6. Weak and inconstant Pericentromeric C-Giemsa bands occurred in pairs 1, 2, 5, 6 and 7 (Figure 5B). Fluorochrome banding revealed C-CMA<sub>3</sub><sup>+</sup> NOR-associated blocks adjacent to C-DAPI<sup>+</sup> bands in the terminal and subterminal regions of the short arms of pairs 7 and 8 plus a tenuous neutral C-CMA<sub>3</sub><sup>0</sup>/DAPI<sup>0</sup> band in the pericentromeric region of pair 8. Weak DAPI<sup>+</sup> staining was also found in the pericentromeric region of pair 7 (Figures 2C, 2D and 5B). In some preparations cold-treatment (CSR) produced evidence of intercalary sites on the long arms of pairs 4 and 5 and C-Giemsa banding indicated that these sites were associated with large heterochromatic blocks (Figures 3B and 5B). Fluorescent *in situ* hybridization with the 45S rDNA probe revealed hybridization sites in the terminal region of the short arm of pair 7 and the subterminal region of pair 8 in addition to a weaker C-CMA<sub>3</sub> negative terminal site on



**Figure 2** - Chromosome banding with fluorochromes in *Cestrum* species. (A) C-CMA<sub>3</sub> banding in *C. amictum*, arrows indicate the minor and proximal band in the pair 8. (B) C-DAPI banding in *C. amictum*, arrows indicate minor and interstitial bands. (C) C-CMA<sub>3</sub> banding in *C. intermedium*, arrows indicate inconstant centromeric-pericentromeric bands and minor and proximal band in the pair 8. (D) C-DAPI banding in *C. intermedium*, arrows indicate major terminal bands. (E) C-CMA<sub>3</sub> banding in *C. sendtnerianum*, arrows indicate minor and terminal bands. (F) C-DAPI banding in *C. sendtnerianum*, arrows indicate some proximal, terminal and interstitial bands. (G) C-CMA<sub>3</sub> banding in *C. strigilatum*, arrows indicate interstitial, proximal (pair 8) bands and a B-chromosome. (H) C-DAPI banding in *C. strigilatum*, arrows indicate minor interstitial bands and a B-chromosome. Bar represents 10  $\mu$ m.

the long arm of pair 3. Hybridization sites with the 5S rDNA probe were found near the centromere of the long arm of pair 8 (Figures 4D and 5B).

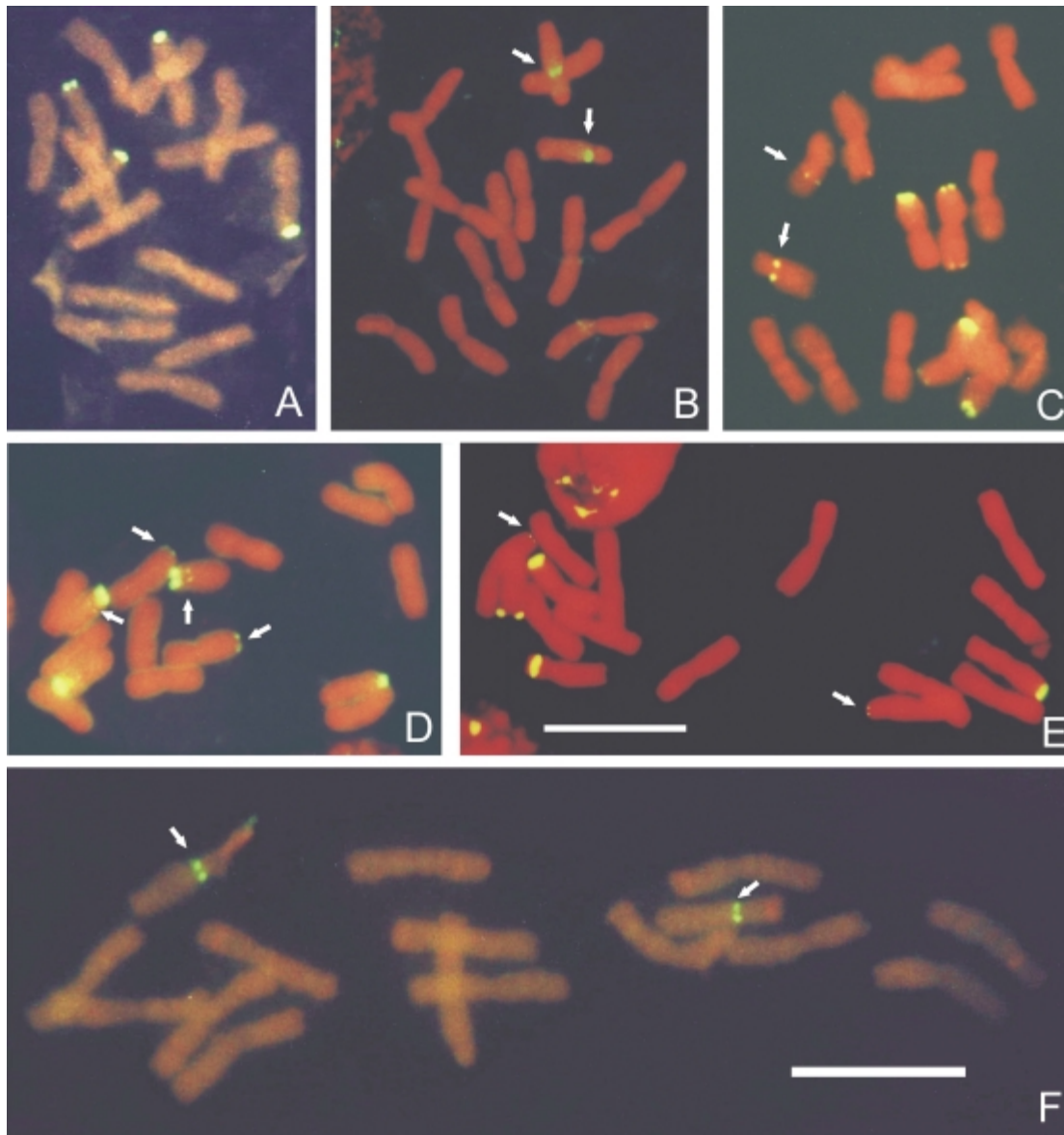
The karyotype of *C. sendtnerianum* consisted of four metacentric pairs, three submetacentrics and one acrocentric pair (Figure 1C and Table 1). After C-Giemsa banding, large blocks were observable in the terminal region of the short arm of pair 1 and in the proximal region of the short arm of pair 8. Large NOR-associated terminal blocks were revealed by C-CMA<sub>3</sub> banding in both arms of pair 3



**Figure 3** - Cold sensitive regions (CSRs) in *Cestrum*. (A) *C. amictum*, (B) *C. intermedium*, (C) *C. sendtnerianum* and (D) *C. strigilatum*. Arrows indicate terminal and interstitial CSRs. Bar represents 10  $\mu$ m.

and on the short arm of pair 6. Some of these blocks also occurred in the intercalary regions of the short arms of pairs 1, 2, 3 and 4, on the long arm of pair 6, in the proximal region of the long arm of chromosome 7 and in both subterminal regions of pair 8. Equidistantly distributed tenuous and inconstant staining was found in the pericentromeric regions (mainly of the long arms) and intercalary/distal position of the long arms of all the chromosomes (Figure 1G). Fluorochrome banding showed NOR-associated terminal C-CMA<sub>3</sub><sup>+</sup> bands on both arms of pair 3 and the short arm of pair 6, with another intercalary band (unassociated with NORs) also occurred on the short arm of pair 6. Tenuous C-DAPI<sup>+</sup> bands were found at the subterminal regions of the short arms of pairs 3 and 7. One neutral (C-CMA<sub>3</sub><sup>0</sup>/DAPI<sup>0</sup>) band occurred in the pericentromeric region of pair 8 (Figures 2E, 2F and 5C). A single CSR was found at the intercalary position on the short arm of pair 7, coinciding with a C-Giemsa/DAPI positive band (Figures 3C and 5C). Fluorescent *in situ* hybridization with the 45S rDNA probe revealed terminal signals in both arms of pair 3, plus a terminal site on the short arm of pair 6. In these three cases, the hybridization sites coincided with C-Giemsa and C/CMA<sub>3</sub> positive bands. A CMA<sub>3</sub>-negative 45S rDNA hybridization site occurred in the terminal region of the long arm of pair 4. Hybridization with the 5S rDNA probe showed a proximal signal on the long arm of pair 8 (Figures 4C and 5C).

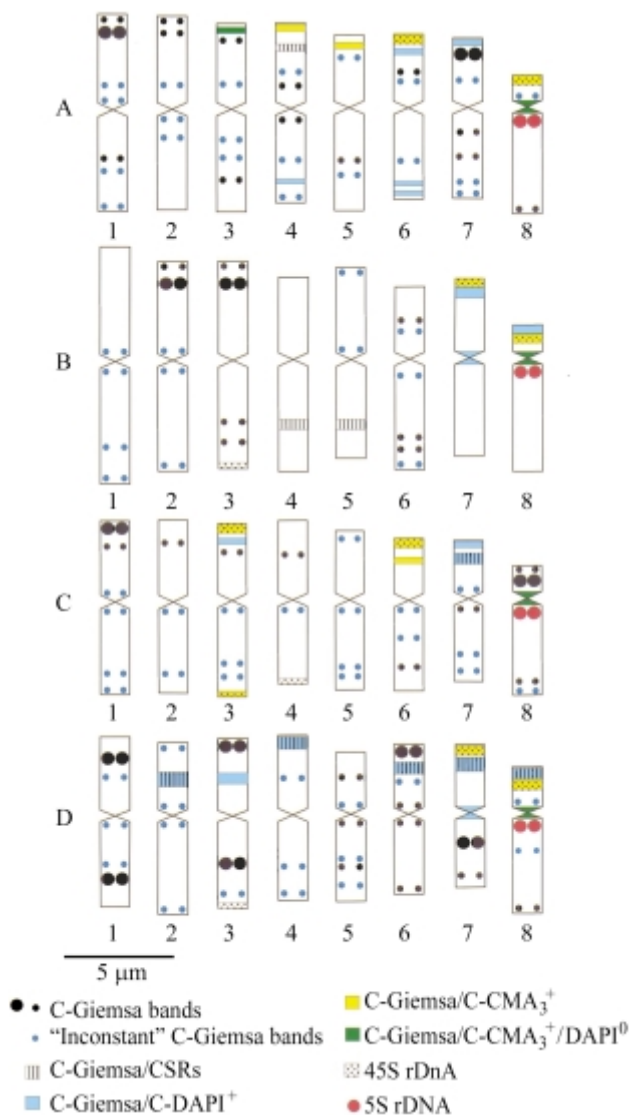
The karyotype of *C. strigilatum* is composed of five metacentric and three submetacentric pairs (Figure 1D and Table 1). After C-Giemsa banding, large intercalary blocks were observed on both arms of pair 1, on the long arms of pairs 3 and 7 and in the subterminal regions of the short arms of pairs 3 and 6. Smaller blocks were visualized in the intercalary regions of both arms of pair 5, in the pericentromeric regions of pairs 5 and 6 and in the subterminal regions of pairs 6, 7 and 8. Except for pair 7, all the pairs showed equidistantly distributed tenuous or inconstant



**Figure 4** - Fluorescent *in situ* hybridization (FISH) with the pTa71 (45S rDNA) and pTa794 (5S rDNA) clones in *Cestrum* species. (A) Metaphase hybridized with 45S rDNA probe in *C. amictum*. (B) Metaphase hybridized with 5S rDNA probe in *C. amictum*, arrows indicate out proximal signals. (C) Metaphase of *C. sendtnerianum* hybridized at the same time with 45S and 5S rDNA probes, arrows indicate terminal and tenuous 45S rDNA signals (note a chromosome with two hybridization signals) and proximal 5S rDNA hybridization signals. (D) Metaphase of *C. intermedium* hybridized at the same time with 45S and 5S rDNA probes, arrows indicate terminal and tenuous 45S rDNA signals and proximal 5S rDNA hybridization signals. (E) Metaphase of *C. strigilatum* hybridized with 45S rDNA probe, arrows indicate a terminal and tenuous hybridization signal. (F) Metaphase of *C. strigilatum* hybridized with 5S rDNA probe, arrows indicate proximal hybridization signals.

bands in their intercalary, proximal and distal regions (Figures 1H and 5D). Distal C-CMA<sub>3</sub><sup>+</sup> bands, coinciding with NORs and C-Giemsa bands, were found on the short arms of pairs 7 and 8 (Figure 2G) and C-Giemsa associated C-DAPI<sup>+</sup> bands were intercalary on the short arms of pairs 2, 3, 6 and 7, subterminal in pairs 4 and 8 and the pericentromeric in pair 7 (Figure 2H). A single neutral band was detected in the proximal region of the long arm of pair 8 (Figures 2G, 2H and 5D). Cold sensitive regions were consistently observed in association with the C-DAPI<sup>+</sup> bands of pairs 4, 6, 7 and 8, although the block observed on

the short arm of pair 2 did not constantly exhibit a CSR and the C-DAPI<sup>+</sup> block in pair 3 was not induced by the cold (Figures 3D and 5D). We found that *in situ* hybridization with the 45S rDNA probe gave a very similar distribution to that found in *C. intermedium*. There was a terminal signal on the short arm of pair 7 and subterminal in pair 8 and also a weaker C-CMA<sub>3</sub> negative terminal site on the long arm of pair 3. Fluorescent *in situ* hybridization with the 5S rDNA probe located a site next to the centromere on the long arm of pair 8 (Figures 4E, 4F and 5D).



**Figure 5** - Idiograms and chromosomal physical mapping of *Cestrum amictum* (A), *C. intermedium* (B), *C. sendtnerianum* (C) and *C. strigilatum* (D). The 'inconstant' bands correspond to small C-Giemsa<sup>+</sup> bands that were not detected in all the preparation.

## Discussion

The family *Solanaceae* shows karyotypical uniformity within the same genus mainly with relation to chromosome numbers and shapes. Illustrative examples are the species of the genera *Solanum*, with  $2n = 24$  (Bernardello and Anderson 1990, Bernardello *et al.* 1994), *Lycium* (Stiefkens and Bernardello 2002) and *Capsicum* (Moscone 1993). However, variation in the chromosome base number, with  $x = 14$  and  $17$ , has also been reported for *Hyoscyamus* (Masoud *et al.* 1999). Species of *Cestrum* exhibit uniformity in chromosome number ( $2n = 2x = 16$ ) and type, including our new chromosome numbers for *C. amictum* and *C. sendtnerianum*. In general, the *Cestrum* karyotypes present meta- to submetacentric chromosome

morphology for pairs 1 to 7 and subtelocentric for pair 8, as previously reported by Berg and Greilhuber (1992, 1993a,b). The *Cestrum* karyotypes accumulate numerical alterations related only to the occurrence of B chromosomes (Sykorová *et al.* 2003b, Fregonezi *et al.* 2004), except for a few structural changes probably associated with amplification and loss of repetitive DNA (Greilhuber 1992, 1993a,b).

The four *Cestrum* species studied by us showed heterochromatic blocks dispersed throughout all the chromosomes. The heterochromatic blocks varied in size, number, position and composition, this variation being most apparent when comparing the 14 large blocks present in 7 chromosome pairs of *C. strigilatum* with the 9 to 15 medium or large-sized blocks of *C. intermedium*, *C. sendtnerianum* and *C. amictum*. The same profile appeared when comparing the distribution of the smaller blocks and the inconstant dots within and between the species, these dots corresponding to very small C-Giemsa<sup>+</sup> bands that were not detected in all the preparations and were probably due to small variations in the banding and/or fixation conditions. The larger and smaller heterochromatic blocks preferentially occupied the intercalary and subterminal regions equidistantly. Five or more dots were found in some cases (*e.g.* *C. amictum* pairs 1, 2, 3, 4 and 7, *C. intermedium* pair 6 and *C. strigilatum* pair 5) while in the other chromosomes of the four species there were at most three or four dots. The preferential localization of particular heterochromatic segments in terminal and subterminal regions was also very evident for the C-CMA<sub>3</sub><sup>+</sup>, C-CMA<sub>3</sub><sup>+</sup>/NOR, C-DAPI<sup>+</sup> and C-DAPI<sup>+</sup>/CSRs bands, except for a few, most often intercalary, bands found in *C. amictum* (pair 4) and *C. intermedium* (pairs 6, 4 and 5), *C. sendtnerianum* (pairs 6 and 7) and *C. strigilatum* (pairs 2, 3, 6 and 7). Similar variation has been reported by Berg and Greilhuber (1992, 1993a,b) in other five *Cestrum* species. According to Guerra (2000), quantitative and qualitative variation in heterochromatin could be common for different populations and species. Moscone *et al.* (1993) reported that the amount of heterochromatin in *Capsicum* varied by up to a factor of 10, with about 4% of the total length of *Capsicum chacoense* chromosomes being occupied by C-bands and up to 33% of *Capsicum campylopodium* chromosomes containing mainly centromeric C-bands along with a variable number of large and small distal bands and a few intercalary bands. Thus there are at least two possible heterochromatin dispersion modes in *Cestrum* species, non-equidistantly dispersion (Schweizer and Ehrendorfer 1976) in which isolated bands could have appeared from initial amplification sites and equidistant dispersion (Schweizer and Loidl 1987) in which a heterochromatic region could disperse repetitive segments to nearby heterologous chromosomal arms positioned at the same distance from the centromere in the 'chromosomal field' (Lima-de-Faria 1976).

Berg and Greilhuber (1992, 1993a,b) found five different types of heterochromatin in *Cestrum*: (i) C-Giemsa<sup>+</sup> bands, (ii) intercalary, proximal or distal C-Giemsa<sup>+</sup> dots, (iii) CMA<sub>3</sub><sup>+</sup> bands associated with NORs, (iv) CMA<sub>3</sub><sup>+</sup> bands not associated with NORs, and (v) DAPI<sup>+</sup> bands associated with cold-sensitive regions (CSRs). Despite the different procedures employed by us our results confirm the existence of the same heterochromatic types plus two new types, *i.e.* C-CMA<sub>3</sub><sup>0</sup>/DAPI<sup>0</sup> neutral bands (see also Fregonezi *et al.* 2004) and C-Giemsa/CSR/DAPI negative bands.

We found that the number and distribution of the CSRs varied greatly between the *Cestrum* species studied, *C. strigilatum* showing a large number of CSRs while the other species showed few such sites. Variation in the size and number of CSRs has already been described in five other *Cestrum* species by Berg and Greilhuber (1992, 1993a, b), these authors having found a high number of CSRs in a different *C. strigilatum* population and a low number in *C. parqui*, even though the latter species has been considered the species with the larger amount of heterochromatin. In our study qualitative and quantitative variations were evident in both the heterochromatic segments and the CSRs, a fact to be expected because these regions consist of highly repetitive DNA segments. The occurrence of CSRs in terminal positions in *C. aurantiacum* (Berg and Greilhuber 1993b) and intercalary and subterminal positions in the four species studied by us also indicates that CSRs can be equidistantly dispersed, except for *C. amictum* and *C. sendtnerianum* because CSR segments appear in only one chromosome pair. In respect to AT and GC base composition, the CSRs were CMA<sup>+</sup>/DAPI<sup>+</sup> in *C. strigilatum* and *C. sendtnerianum* but negative for both fluorochromes in *C. amictum* and *C. intermedium*. In the species studied by Berg and Greilhuber (1992, 1993a,b), CSRs were always CMA<sup>+</sup>/DAPI<sup>+</sup>. Cold-sensitive CMA<sub>3</sub><sup>+</sup>/DAPI<sup>0</sup> regions have been found in the genera *Sambucus* and *Viburnum*, Caprifoliaceae (Benko-Iseppon and Morawetz 1993). All these data suggest the existence of four CSR classes in plant genomes: (i) C-Giemsa<sup>+</sup>/DAPI<sup>+</sup>, (ii) C-Giemsa<sup>+</sup>/CMA<sup>+</sup>, (iii) C-Giemsa<sup>+</sup>/CMA<sup>0</sup>/DAPI<sup>0</sup> and (iv) C-Giemsa<sup>+</sup> without fluorescence. However, the constitution of the CSR segments does not necessarily depend on the proportion of AT or GC bases but perhaps on the type of associated proteins or the different levels of methylation among the heterochromatic segments.

Conventional staining did not allow us to visualize secondary constrictions in any *Cestrum* chromosomes, difficulty in observing satellites or secondary constrictions having previously been described in *Cestrum* species by Berg and Greilhuber (1992, 1993a,b) using silver nitrate (AgNO<sub>3</sub>) banding. In their study of a number of *Cestrum* species Berg and Greilhuber (1992, 1993a,b) found NOR-bearing areas in the terminal region of metacentric pair 1 and subtelocentric pair 8 of all the studied species ex-

cept for *Cestrum parqui*, the NOR region of which was not in pair 8 but in two other metacentric pairs. We found that FISH with the 45S and 5S rDNA probes was useful in localizing rRNA genes in *Cestrum*. The occurrence of 45S rDNA hybridization sites in the terminal regions of *Cestrum* chromosomes follows a common distribution pattern in the large majority of plant species (see Lima-de-Faria 1976). This profile has also been found in *Capsicum* (Moscone *et al.* 1995) and *Nicotiana* (Yoong Lim *et al.* 2000). In our study, *C. amictum* was the only species with four 45S rDNA sites, supporting the initial proposal by Berg and Greilhuber (1992, 1993a,b). The other species showed more hybridization sites, three in *C. intermedium* and *C. strigilatum* and four in *C. sendtnerianum*. Hybridization also detected major and minor sites in *C. strigilatum*, *C. sendtnerianum* and *C. intermedium*, which could reflect intraspecific differences in the number of repeats. Although FISH is not a completely quantitative technique, the intensity of the signal can be related to the copy number of similar structures (Leicht and Heslop-Harrison 1992). We found that variation appeared not only in the number of rDNA sites but also in the organization of these segments, as seen in the occurrence of 45S rDNA segments not associated with C-CMA<sub>3</sub><sup>+</sup> heterochromatin. We also found that *in situ* hybridization with the 5S rDNA probe revealed a single proximal site on the long arm of pair 8 in the four species, also associated with a proximal Giemsa<sup>+</sup>/CMA<sup>+</sup>/DAPI<sup>+</sup> band. In other genera such as *Nicotiana* (Yoong Lim *et al.* 2000, Kitamura *et al.* 2001) and *Lycopersicon* (Lapitan *et al.* 1991, Xu and Earle 1996) 5S rDNA sites are also located preferentially in the pericentromeric region of the long and short arms. The available information points to the conclusion that, at least for *Cestrum* species, the location of 45S and 5S rDNA sequences can be conserved in the chromosome field, in the terminal and subterminal region of some chromosomes (45S) and next to the centromere on the long arm of pair 8 (5S).

All the *Cestrum* species so far studied exhibited a uniform karyotype. We found that certain heterochromatic segments showed preferential localization (*e.g.* C-CMA<sub>3</sub><sup>+</sup>/NOR blocks located terminally) or appeared in the same chromosome pairs in all species (*e.g.* C-Giemsa<sup>+</sup>/CMA<sup>0</sup>/DAPI<sup>0</sup> pericentromeric bands and 5S rDNA sites in pair 8). However, the majority of heterochromatic segments showed variation in size and distribution among the species studied. This suggests that differentiation among *Cestrum* species occurred mainly through alterations in the organization of repetitive DNA segments, with few modifications in karyotype composition as observed by conventional staining.

## Acknowledgments

The authors thank the Brazilian agencies Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Pró-Reitoria de Pesquisa e Pós-graduação

(ProPPG-UEL) for financial support, Edson Mendes Francisco and Karina L. V. Ramalho de Sá for help with botanical material and Dr. Albert Leyva for help with the English.

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Associate Editor: Marcelo Guerra