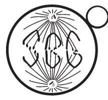
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Research Article

Distribution of a *Ty3/gypsy*-like retroelement on the A and B-chromosomes of *Cestrum strigilatum* Ruiz & Pav. and *Cestrum intermedium* Sendtn. (Solanaceae)

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Abstract

Retroelements are a diversified fraction of eukaryotic genomes, with the *Ty1/copia* and *Ty3/gypsy* groups being very common in a large number of plant genomes. We isolated an internal segment of the *Ty3/gypsy* retroelement of *Cestrum strigilatum* (Solanaceae) using PCR amplification with degenerate primers for a conserved region of reverse transcriptase. The isolated segment (p*Cs*12) was sequenced and showed similarity with *Ty3/gypsy* retroelements of monocotyledons and dicotyledons. This segment was used as probe in chromosomes of *C. strigilatum* and *Cestrum intermedium*. Diffuse hybridization signals were observed along the chromosomes and more accentuated terminal signals in some chromosome pairs, always associated with nucleolus organizer regions (NORs). The physical relationship between the hybridization sites of p*Cs*12 and p*Ta*71 ribosomal probes was assessed after sequential fluorescence *in situ* hybridization (FISH). Hybridization signals were also detected in the B chromosomes of these species, indicating an entail among the chromosomes of A complement and B-chromosomes.

Key words: Cestrum strigilatum, *Cestrum intermedium*, karyotype organization, retroelements, Solanaceae, *Ty3-gypsy*.

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Introduction

Retroelements comprise a common group of repetitive DNA elements which are found dispersed in a number of plant genomes. Such elements form distinct subgroups and can occupy a great portion of the genome. Maize (*Zea mays* L.) is a very illustrative genus, since the genomes of some species can consist of 50-85% of retroelements (Brandes *et al.*, 1997, Kumar and Bennetzen, 1999). The *Ty3/gypsy* and *Ty1/copia* retrotransposons are typical groups with long terminal repeat (LTR), abundant in the genomes of gymnosperms and angiosperms (Van Sluys *et al.*, 2001). These two retrotransposons differ in the gene positioning of reverse transcriptase, integrase and RNase-H, in the *Pol* sequence (Kumar and Bennetzen, 1999). The *Ty1/copia* elements occur dispersed along the chromosomes, and can vary in repeat number according to the genome (Heslop-Harrison *et al.*, 1997). Although the *Ty3/gypsy* are less studied than *Ty1/copia*, they have been shown also to occur dispersed in the genome (L'Homme *et al.*, 2000). In some species of *Helianthus* (Asteraceae) and some monocotyledons *Ty3/gypsy*-like retroelements exhibit a dispersed pattern, but they can appear concentrated in all centromeric regions (Santini *et al.*, 2002, Kumar and Bennetzen, 1999). Additionally, Belyayev *et al.* (2001) found *Ty3/gypsy* to be associated with secondary constrictions and with subterminal and intercalary heterochromatin in both *Hordeum spontaneum* and *Aegilops speltoides*.

The *Ty3/gypsy*-like and other retrotransposons have been characterized in some Solanaceae representatives, such as *Solanum tuberosum* and *Lycopersicum esculetum* (Van Sluys *et al.*, 2001), but their physical localization has not been explored.

The genus *Cestrum* contains sub-tree and tree species, native to tropical and subtropical America (D'Arcy, 1979). The species possess karyotypes composed of 2n = 16, with a predominance of meta- and submetacentric chromosomes (Berg and Greilhuber, 1992, 1993a,1993b). The *Cestrum* group is an interesting plant group because it possesses genomes with different heterochromatin families, such as C-Giemsa bands, GC-rich bands associated

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with nucleolar organizing regions (NORs), GC-rich bands not NOR-associated, AT-rich bands associated with cold sensitive regions (CSRs), C-Giemsa/CSR/AT$^-$ bands and C-GC/AT neutral bands (see Berg and Greilhuber, 1992, 1993a, 1993b, Fregonezi *et al.*, 2004, Fregonezi *et al.*, 2006).

The objectives of this study were isolate and characterize sequences of the *Ty*3/*gypsy* group of the *Cestrum strigilatum* genome, and to locate physically these elements in *C. strigilatum* and *Cestrum intermedium* chromosomes because both species present B-chromosomes. The chromosome localization of *Ty*3/*gypsy*-like retroelements could help to understand the role and the dynamics of repetitive DNA in the genome and karyotype of *Cestrum*, as well as their relationships with the B-chromosomes of both species.

Materials and Methods

Seeds of *C. strigilatum* were collected at São Jerônimo da Serra (23°43'37" S, 50°43'07" W) and those of *C. intermedium* at Londrina (23°27'21" S, 51°13 48" W), both locations being in the southern Brazilian state of Paraná. Samples were cultivated at the Laboratory of Biodiversity and Ecosystem Restoration (LABRE) at the Universidade Estadual de Londrina, Paraná, Brazil. Vouchers were deposited at the FUEL herbarium. Cytogenetic analysis was carried out with young root tips that were pretreated with 0.05% (w/v) aqueous colchicine (Sigma), fixed in 3:1(v:v) ethanol:acetic acid for 12 h to 24 h and stored at -20 °C until use. The samples were softened in a solution containing 4% (w/v) cellulase and 40% (w/v) pectinase (all of Sigma) at 37 °C for 1 h and then squashed in a drop of 45% acetic acid. The coverslips were subsequently removed in liquid nitrogen. Slides were used immediately after their preparation or then kept at -20 °C.

Genomic DNA of *C. strigilatum* was extracted from young leaves using the cetyltrimethyl ammonium bromide (CTAB) method described by Rogers and Bendich (1988). The GyRT1 (5' MRNATGTGYGTNGAYTAYMG) and GyRT4 (5' RCAYTTNSWNARYTTNGCR) degenerate primers (Friesen *et al.* 2001), giving a 450 bp product, were used for the PCR reactions. The band containing the 450 bp fragment was purified with the Concert Rapid Gel Extraction System Kit (Gibco) and then re-amplified with the GyRT3 (5' YKNWSNGGNTAYCAYCARAT) and GyRT4 primers, as described above, to obtain a 320 bp product. The last fragment was cloned in *E. coli* with the TOPO TA Cloning for Sequencing Kit (Invitrogen Life Technologies, USA) and several clones were sequenced using the DYEnamic ET dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) on MEGABACE 1000 in the forward and reverse directions, according to the manufacturer's instructions. The sequence analysis and contig construction were performed with PhredPhrapConsed software (http://bozeman.mbt.washington.edu/consed/consed.html). The consensus sequence was compared with GeneBank sequences using the BLAST(X) Software tools. (http://www.ncbi.nlm.nih.gov).

The p*Cs*12 plasmid containing a segment of the *Ty*3/*gypsy* retroelement and the p*Ta*71 plasmid containing 18S-5.8S-26S rDNA (Gerlach and Bedbrook, 1979) were used as probes. FISH was performed according to Heslop-Harrison *et al.* (1991), with modifications. The 45S rDNA and *Ty*3/*gypsy* probes were labeled with biotin-14-dATP by nick translation (Invitrogen Bionick Kit). The slides were pretreated with RNase (100 µg/mL) at 37 °C for 1 h, washed in 2xSSC, post-fixed in 4% paraformaldehyde (w/v), and washed again in 2xSSC at room temperature. Samples were dehydrated in a graded ethanol series and air-dried. Slides were treated with 30 µL of a hybridization mixture composed of 100 ng of labeled *Ty*3/*gypsy* probe, 50% (v/v) formamide, 50% polyethylene glycol (v/v), 20xSSC, 100 ng of fragmented *E. coli* DNA, and 10% (v/v) SDS. The mixture was denatured at 70 °C and chilled on ice prior to use. Both chromosomes and hybridization mixture were denatured/renatured using a thermal cycler at 90 °C for 10 min, 50 °C for 10 min and 38 °C for 10 min. The hybridization was performed at 37 °C overnight in a humidified chamber. The post-hybridization washes for the p*Cs*12 probe were in 6xSSC for 20 min at room temperature, 6xSSC at 37 °C for 3 min, and 4xSSC/ 2% Tween 20 at room temperature for 5 min (about 40% of stringency to point out to dispersive pattern of *Ty*3/*gypsy*). Biotin-labeled probe was detected with avidin-fluorescein isothiocyanate (avidin-FITC) conjugate (Sigma). Chromosomes were analyzed without counterstaining, and photographed with Kodak Proimage 100 ISO film. Afterwards, slides were washed with 2xSSC and 4xSSC containing 0.2% Tween 20 (v/v) at room temperature for 30 min each, dehydrated in a graded ethanol series and air-dried. Slides were hybridized again with a 45S rDNA probe, as described above, except for the blocking DNA that was calf thymus DNA (Invitrogen). Washes were performed in 2xSSC, 0.1xSSC containing 20% formamide, then in 0.1xSSC and finally in 2xSSC at 42 °C for 5 min each (about 80% of the stringency was due to the 45S rDNA size and location pattern). The hybridization sites were detected again with avidin-FITC conjugate and the chromosomes counterstained with 2.5 µg mL^{-1} propidium iodide and photographed.

For characterization and differentiation of *C. intermedium* and *C. strigilatum* B-chromosomes, samples were submitted to C-Giemsa and C- CMA_3/DAPI banding. Root tips were digested at 37 °C in a solution composed of 4% (w/v) cellulase and 40% (w/v) pectinase and the chromosome spread was made in a drop of 45% (v/v) acetic acid. Coverslipped slides were frozen in liquid nitrogen and then the cover slips removed. Slides were aged for three days at room temperature, treated with 45% (v/v) acetic acid, 5% (w/v) barium hydroxide, and 2xSSC (pH 7) and then stained with 2% (w/v) Giemsa (Schwarzacher *et al.*,

1980). Alternatively, the fixed slides were aged for three more days at room temperature and sequentially stained with 0.5 mg mL^{-1} chromomycin A_3 (CMA_3) GC-specific fluorochrome for 1.5 h and 2 μg mL^{-1} 4'-6-diamidino-2-phenylindole (DAPI) AT-specific fluorochrome for 30 min (Vanzela *et al.*, 2002). Samples stained with Giemsa were mounted with Entellan (Merck), but those stained with the fluorochromes were mounted in a medium composed of glycerol/McIlvaine buffer (pH 7) containing 2.5 mM $MgCl_2$. The cells were photographed with Imagelink HQ ASA 25 or T-max ASA 100 film (Kodak). Analyses were based on five well-spread metaphases for each treatment.

Results

The reaction using the GyRT1/GyRT4 primers generated several fragments of different sizes, including a stronger band of about 450 bp (Figure 1A). This fragment was isolated, purified and used as in Friesen *et al.* (2001) and then re-isolated, purified and used as a template for a new reaction with the GyRT3 (internal) and GyRT4 primers. This new PCR reaction produced a band of about 320 bp (Figure 1B), which was isolated, purified and cloned. Clones were sequenced and one corresponding to the *Ty*3/*gypsy* elements (p*Cs*12) included in the NCBI GeneBank Sequence Viewer.htm (gi:53801553, accession number AY744673). The sequence showed similarity with several related reverse transcriptase fragments (Figure 2A). The comparisons showed that the p*Cs*12 insert was very similar to sequences of *Ty*3/*gypsy*-like retroelements found in monocotyledons and dicotyledons. The strongest similarity was with a retroelement of the monocotyledon *Oryza sativa* (rice) (gi: 37533358, e-value 2e-29) followed by the *Solanum demissum* (gi: 47824972, e-value 2e-29) and *Elaeis guineensis* (gi:22859203, e-value 2e-28) and the monocotyledon *Ananas comosus* (gi:2995405, e-value 2e-28), and was also similar to the monocotyledon *Lolium multiflorum* (gi:13559324, e-value 2e-25). Figure 2B shows the more significant alignment obtained with BLAST(x).

The FISH analysis using the p*Cs*12 probe revealed diffuse signals along the chromosomes of *C. strigilatum*, but brighter signals were visualized at the terminal positions of a metacentric pair and on the short arm of the eighth chromosome pair. Besides the signals visualized on the A complement chromosomes, two more accentuated signals were detected at intercalary positions on the short and long arms of a B chromosome (Figure 3A and 3I). A negative band was detected in the intercalary region of the short arm of a *C. strigilatum* chromosome pair showing the absence of hybridization with p*Cs*12 probe (see arrow heads in Figure 3A). The absence hybridization signals on these chromosomal segments indicated that the low stringency used (about 40%) did not interfered nor did it overestimate the dispersive pattern of occurrence of *Ty*3/*gypsy* elements (p*Cs*12). In *C. intermedium* FISH also revealed dispersed signals along the chromosomes, very similar to that observed in the A complement of *C. strigilatum*, and also the small hybridization signals at the terminal positions of a metacentric pair and on the short arm of the eighth chromosome pair as well (Figure 3B). The two *C. intermedium*

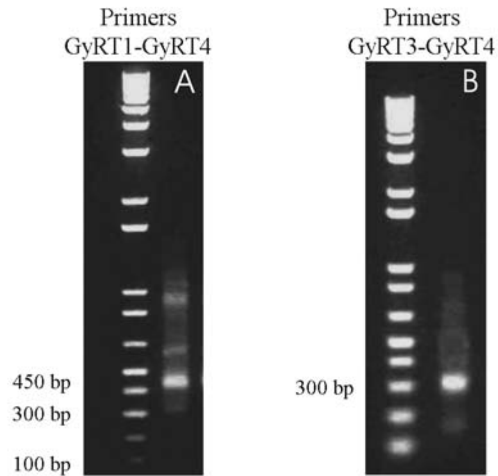

Figure 1 - A) Results of the PCR reaction using the GyRT1/GyRT4 primers. The 450 bp band was removed, purified and used in a semi-nested PCR reaction. B) Results of semi-nested PCR reaction using the GyRT3/GyRT4 primers. The 320 bp band was purified, cloned and several clones sequenced. The p*Cs*12 probe was labeled and used for fluorescence *in situ* hybridization (FISH).

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GCATTTTCTTAGTTTGGCGACGAGTTTGTGCTCCTTGAGTGTCTGTAAGG
TGATCCTGCGGTGTTCCACACGGTCTCCCCTACTTTTTGAGTACAACAGT
ATATCATCAATAAGTACAATAATCAAGGTGTCCAAATAAGGCCTGAACAC
TCGATTCATTAAATCCATGAAAGCTGCAGGGGCATTTGTTAGCTCGAAAG
GCGATTACCAAAAATTCATAGTGCCCGTATCTAGTCCTGAACACTGTTTT
TAGGAAGTTCCCCCCCTAATCGTCATTTGATGATAACCTCTGCA
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Figure 2a - Results of sequencing of the p*Cs*12 probe obtained by PCR from *Cestrum strigilatum.*

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A)  gi:53801553  01 GNFQKTVFRTRYGHYEFLVMPFELTNAPAAFMDLMNRVFRPYLDTFIIVFIDDILLYSKSRGEHEEHLSITLQTLKEHKLF 81
B)  gi:47824972  01 ADIPKTAFRTRYGHYELLVMSFGLTNAPAAFMDLMTRVFRPYLDSFVIVFIDDILIYSRSRGDHEQHLRVVLQTLRDQRLY 81
C)  gi:2995405   01 EDVSKTAFRTRYGHYEFAVMPFGLTNAPTAFMDLMNRVFKPYLDRFVVVFIDDILVYSRSDADHEEHLRIVLQVLREKELY 81
D)  gi:22859203  01 EDVPKTAFRTRYGHYEFLVMPFGLTNAPAAFMDMMNRIFKPYLDQFIVVFIDNILVYSKNIEEHERHLRIVFQTLRKEKLF 81
E)  gi:37533358  01 SDIPKTAFRARYGHYEYLVMPFGLTNAPAAFMDLMNRIFKPYLDQFVVVFIDDILIYSKTKEDHANHLRIVLQTLRDHKLF 81
F)  gi:13559324  01 EDVPKTAFVSRYGHHEYLVVPFGLTNAPAIFMNLMNKIFMPCLDKFVIVFIDDILIYSKDKAEHAEHLRIVLQTLREHQLY 81
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Figure 2b - ClustalW alignment comparing the amino acid sequence of the *Cestrum strigilatum* retroelement with the most similar sequences recovered from databanks. A) *C. strigilatum*, B) *Solanum demissum*, C) *Ananas comosus*, D) *Elaeis guineensis,* E) *Oryza sativa*, F) *Lolium multiflorum*.

B-chromosomes also showed hybridization signals on both arms, but both were larger and more evident than the signals obtained on the *C. strigilatum* B-chromosome (Figure 3N). The accentuated p*Cs*12 hybridization signals observed in B-chromosomes also reinforce the fact that the 40% stringency did not overestimate the dispersive occurrence of *Ty*3-*gypsy* on the A-chromosomes of both species. The slides hybridized with p*Cs*12 probe were washed and again hybridized with the 45S rDNA probe. In both species, bright terminal rDNA signals were visualized on the same positions as the p*Cs*12 probe signals (Figure 3C and 3D). However, in *C. intermedium* the bright intensity was not as strong as on the terminal region of the A-chromosomes. It is important to point out that, unfortunately, not all the eight 45S rDNA sites were detected after the new hybridization, probably due to technical problems. However, based on the physical map proposed by Fregonezi *et al.* (2006), it is safe to say that at least one representative of each chromosome pair containing a NOR was detected by the p*Ta*71 probe. The chromosome region observed as a negative segment after the hybridization with the p*Cs*12 probe (see arrows at Figures 3A and 3B) appeared stained with propidium iodide (Figures 3C and 3D). This further shows that the stringency and hybridization technique was correct. The Figure 3E to 3I (Bs of *C. strigilatum*) and 3J to 3N (B-chromosomes of *C. intermedium*) show a comparison of B-chro-

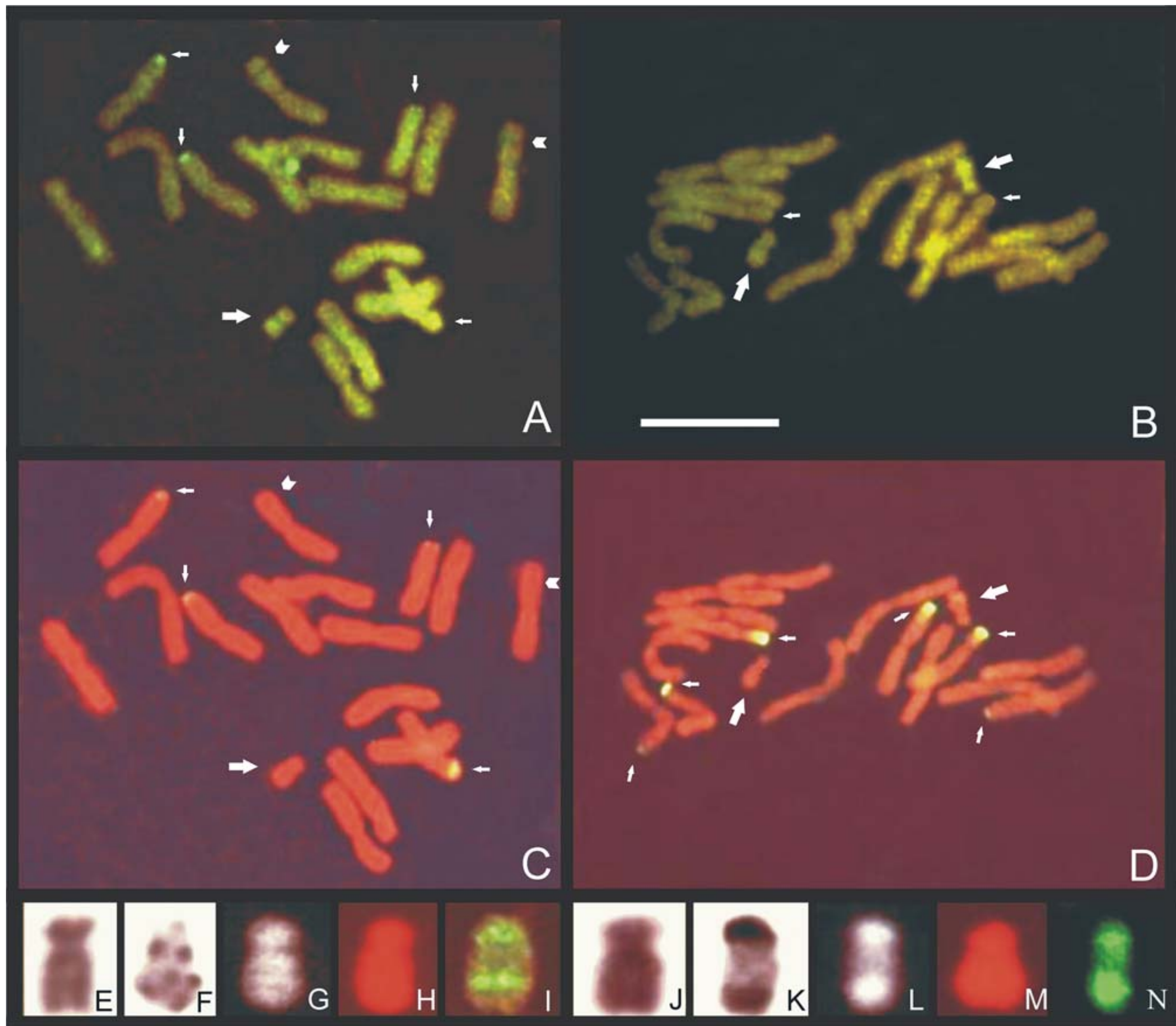

Figure 3 - A) Fluorescence *in situ* hybridization (FISH) with the p*Cs*12 probe in *Cestrum strigilatum*. B) FISH with p*Cs*12 probe in *Cestrum intermedium*. Small arrows point out terminal sites and the large arrow point out B-chromosomes. C) FISH with p*Ta*71 probe in *C. strigilatum*. D) FISH with p*Ta*71 probe in *C. intermedium*. Small arrows point out the terminal 45S rDNA sites and the large arrow point out B-chromosomes. The arrow heads show that the chromosome region observable as a negative segment after the hybridization with the p*Cs*12 probe (3A) appears perfectly stained with propidium iodide (3B). E-I) B-chromosomes of *C. strigilatum*, treated for conventional Giemsa, Giemsa C-banding, DAPI, propidium iodide and FISH with p*Cs*12 probe, respectively. J-N) B-chromosomes of *C. intermedium,* treated for conventional Giemsa, Giemsa C-banding, DAPI, propidium iodide and FISH with the p*Cs*12 probe, respectively. Bar, 10 μm.

mosomes analyzed by several techniques, that is, conventional Giemsa staining (3E and J), C-Giemsa (3F and 3K), C-DAPI (3G and 3L), propidium iodide (3H and 3M), and FISH with p*Cs*12 (3I and 3N). These techniques showed that B-chromosomes are very similar when stained with Giemsa and propidium iodide, but exhibit differences evident when treated with C-Giemsa and C-DAPI banding and FISH with the p*Cs*12 probe.

Discussion

The retroelement families are characterized by heterogeneity in many of their sequences, mainly due to the propagation mechanism through reverse transcriptase, which produces base substitutions in the copy processes (Van Sluys *et al.*, 2001). The *Ty*1/*copia* and *Ty*3/*gypsy* retroelement groups usually occur in multiple families in related organisms, but different retroelement families can exist within the genome of an organism. Marín and Llórens (2000) found nine different *Ty*3/*gypsy* forms in *Arabidopsis thaliana* through the comparison of conserved sequences. It was also suggested by these authors that the differences among these forms might have originated prior to the separation of monocotyledons and dicotyledons. The occurrence of different retrotransposon forms could be considered a general feature of the modern angiosperms. The sequence found in *Cestrum* was highly similar to the retroelements of three monocotyledons (*O. sativa, A. comosum* and *L. multiflorum*) and two dicotyledons (*S. demissum* and *E. guineensis*) and if these data were analyzed from only the point of view of the *Ty*3/*gypsy* retroelements, the possible ancestral genome of the dicotyledons could contain *Ty*3/*gypsy* retroelements similar to the segment cloned in p*Cs*12.

In regard to the physical position of the retroelements, these can occur dispersed along the chromosomes, or concentrated in specific regions, according to the genomic organization of the species (Heslop-Harrison *et al.*, 1997). In our study, *C. strigilatum* and *C. intermedium* showed a diffuse distribution of the p*Cs*12 probe, indicating that the *Ty*3-*gypsy* retroelements also occur distributed along the chromosomes. Besides this general dispersed tendency, both species exhibited a concentration of these elements in some terminal regions, associated with NORs, which are also associated with GC-rich heterochromatin (Fregonezi *et al.*, 2004). Retroelements have previously been reported to be associated with the NORs of some Triticeae (Belyayev *et al.*, 2001), with the heterochromatin of *Allium cepa* (onion) (Pearce *et al.*, 1996) and *A. thaliana* (Brandes *et al.*, 1997), and with centromeric regions in several monocotyledons (Kumar and Bennetzen, 1999) and dicotyledons (Santini *et al.*, 2002). Retrotransposons have also been reported to be associated with the whole genome as segments dispersed along the A- and D-chromosomes of *Avena sativa* (oat) (Linares *et al.*, 2001). However, the association of the retroelements with specific DNA segments is not obligatory. Schmidt *et al.* (1995) demonstrated that the BNR1 and Tbv retrotransposon families of *Beta vulgaris* (sugar beet) are heterogeneous and highly amplified on the genome but are largely excluded from regions rich in 18S-5.8S-25S rRNA genes.

Hybridization signals showed that the B-chromosomes of both *C. strigilatum* and *C. intermedium* possess *Ty*3-*gypsy* elements but with visible differences in the number, position, and size of the repeats. This is in agreement with the suggestion of Camacho *et al.* (2000) for B-chromosomes. According to Jones and Houben (2003) B-chromosomes are described as heterochromatic in about half of plants that carry them. In general, the repetitive DNA families of Bs are similar to that of their A chromosomes. Species of *Cestrum* contain more than one family of repetitive DNA, which seems indicate different dynamics from that observed in the A-chromosomes for C-banding and C-DAPI segments (Fregonezi *et al.*, 2004) and *Ty*3-*gypsy* elements. Repetitive DNA families occupying a large portion of B-chromosomes have already been described in several plants species, such as *Brachycome dichromosomatica* (Houben *et al.*, 2001), *Medicago* (Hossain and Bauchan, 1999) and *Zea mays* (Stark *et al.*, 1996). The presence of *Ty*3-*gypsy* retroelements in the B-chromosomes of *C. strigilatum* and *C. intermedium* indicates that these repetitive segments may contribute to the formation and stabilization of B-chromosomes in these species, and that this might have occurred through the amplification of existing segments in the chromosomes of the normal complement.

It is important to point out that the probe used by us was obtained with degenerate primers designed for the conserved portion of the reverse transcriptase of the *gypsy* elements, and that the reverse transcriptase genes of all retrotransposons are related by their monophyletic origin (Hansen and Heslop-Harrison, 2004). As different retroelements can also have followed different evolutionary pathways it is not uncommon to find associations among different types of retrotransposons and different DNA families, as those found by us among the *Ty*3/*gypsy* retroelements, NOR sites and B-chromosomes of *C. strigilatum* and *C. intermedium*.

Acknowledgments

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