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Research Article

A karyotypic study of three southern Brazilian Asteraceae species using fluorescence *in situ* hybridization with a 45S rDNA probe and C-CMA$_3$ banding

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Abstract

The Asteraceae, one of the largest families of flowering plants, contains about 1,100 genera and 20,000 species, and is well known for its extensive karyotypic variation. In this study, conventional Feulgen staining, C-CMA$_3$ banding, and fluorescence *in situ* hybridization with a 45S rDNA probe were used to determine the chromosome number and the number and physical position of GC-rich heterochromatin and 45S rDNA sites in three Asteraceae weed species (*Crepis japonica, Galinsoga parviflora* and *Chaptalia nutans*). The three species exhibited karyotype differences in the chromosome number and shape, as a commom feature of Asteraceae. However, the 45S rDNA sites always occurred on the short chromosomal arms, associated with GC-heterochromatin. Althought of these differences, it suggests that commom features of plant karyotype are maintained.

Key words: CMA$_3$, FISH, heterochromatin, polyploidy, 45S rDNA.

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Introduction

The Asteraceae is a large family of flowering plants that contains about 1,100 genera and 20,000 herbs, shrubs and, to a lesser extent, trees. The plants in this family are characterized by having reduced flowers that are organized into an involucrate pseudanthium in the form of a head or capitulum. This family is well known for its extensive variation in karyotype. Inter- and intraspecifc chromosomal variations have been reported in some Brazilian genera, such as in *Mikania* (Ruas and Ruas, 1987; Ruas and Aguiar-Perecin, 1997; Maffei *et al.*, 1999). Other examples of karyotypic variation in Asteraceae include the occurrence of cytodemes in *Brachycome dichromosomatica*, with 2n = 4, 8, 10, 12 and 16 (Watanabe *et al.*, 1999a), as well as variations in the number and distribution of 45S rDNA sites and the occurrence of B-chromosomes (Houben *et al.*, 1999).

The search for chromosomal markers in wild and cultivated species of Asteraceae has contributed to our understanding of the karyotypic organization and has provided useful information for taxonomic applications. Of the DNA segments used for physical chromosome mapping, rDNA (45S and 5S) has been the most widely employed because it provides excellent chromosomal landmarks for investigating karyotypic evolution in diploid and polyploid species. According to Jiang and Gill (1994), the copy number of rDNA sites can vary among populations of common wheat, although the location of most of the loci is conserved in different diploid and polyploid wheat species. Our understanding of karyotypic features, including abnormalities and variations, can be improved by cytogenetic methods such as CMA$_3$/DAPI banding and fluorescence *in situ* hybridization (FISH).

The cytogenetic information provided by a combination of chromosome banding and FISH can be useful for comparing species within a genus, as well as species of different genera. In this study, three annual Asteraceae weeds (*Crepis japonica* (L.) Benth., *Galinsoga parviflora* Cav. and *Chaptalia nutans* (L.) Pol.), which are very common, small plants found in grasslands and disturbed areas in southern Brazil where they colonize degraded or newly open habitats, were studied. The aim of this study was to determine the chromosome number and to establish the number and physical location of 45S rDNA sites and

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C-CMA_3 bands in these groups. The findings are discussed with regard to the number and location of rDNA sites and their relationship with C-CMA_3 bands, in both diploid and polyploid specimens.

Material and Methods

Plant material and conventional analysis

Five samples of each species were collected (*Crepis japonica* and *Galinsoga parviflora* from Londrina in the State of Paraná - PR) and *Chaptalia nutans* from Londrina-PR and Laguna, in the State of Santa Catarina). The specimens were cultivated in a greenhouse and voucher material was deposited in the FUEL herbarium. Chromosomal preparations were obtained from root tips pretreated with 2 mM 8-hydroxyquinoline for 24 h followed by fixation in ethanol:acetic acid (3:1, v/v) for 12 h and storage at -20 °C. The Feulgen method was used for conventional analysis. Chromosomal recounts were done in at least five complete metaphases.

C-CMA_3 banding

The chromosomal preparations were processed for C-banding according to Schwarzacher *et al.* (1980), with modifications (see Vanzela *et al.*, 2002). The samples were softened in a solution containing 4% cellulase and 40% pectinase at 37 °C for 1 h and then squashed in a drop of 45% acetic acid. The coverslips were subsequently removed in liquid nitrogen. After three days, the samples were hydrolyzed in 45% acetic acid at 60 °C for 10 min followed by incubation in 5% $Ba(OH)_2$ at 25 °C for 10 min and in 2xSSC, pH 7.0, at 60 °C for 80 min. After a further three days, the samples were stained for 1.5 h with a drop of solution containing 0.5 mg of CMA_3/mL diluted in McIlvaine buffer (pH 7.0) and distilled water (1:1, v/v) containing 2.5 mM $MgCl_2$. The slides were mounted in a solution of glycerol:McIlvaine buffer (1:1, v/v) containing 2.5 mM $MgCl_2$.

Fluorescence *in situ* hybridization (FISH)

The root tips were softened in a solution containing 4% cellulase and 40% pectinase and slides were prepared as described for C-CMA_3 banding. FISH was done according to Heslop-Harrison *et al.* (1991) and Cuadrado and Jouve (1994), with modifications. The p*Ta*71 probe containing 18S-5.8S-26S rDNA (Gerlach and Bedbrook, 1979) was labeled with biotin-14-dATP by nick translation (Bionick Gibco 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 (10 min each at room temperature). The slides were dehydrated in a graded (70%-100%) ethanol series and air-dried. Each slide was treated with 30 µL of labeling reagent containing 100 ng of labeled probe, 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 probe was denatured at 70 °C and chilled on ice prior to use. Chromosomes were denatured/hybridized using an MJ Research thermal cycler at 90 °C for 10 min, 50 °C for 10 min, 38 °C for 10 min, and 37 °C overnight in a humidified chamber. Following hybridization, the slides were washed at 80% stringency in 2xSSC, 0.1xSSC/20% formamide, 0.1xSSC, 2xSSC, and 4xSSC/0.2% Tween 20 at 42 °C for 5 min each. Signals were detected with avidin-FITC (Sigma) and the chromosomes were counterstained with propidium iodide (2.5 µg/mL).

To assess the relationship between C-CMA_3 blocks and 45S rDNA sites, *C. nutans* with 2n = 50 were processed sequentially for FISH and C-CMA_3 banding. Photographs were taken using Kodak Imagelink HQ ISO 25 film for conventional staining, T-Max ISO 100 film for C-CMA_3 banding and Proimage ISO 100 film for FISH.

Results and Discussion

Variations in the chromosomal number have been reported for many groups of Astereaceae, including diploidy as in *Mikania* (Maffei *et al.*, 1999) and polyploidy as in *Helianthus* (Vanzela *et al.*, 2002); other groups, such as *Stevia*, show numerical stability (Frederico *et al.*, 1996).

As shown here, *Crepis japonica* (L.) Benth. had 2n = 16 chromosomes which were metacentric and submetacentric and of similar size (~1.9-2.2 µm); satellites were seen in only one submetacentric pair (Figure 1A). FISH revealed only two signals for 45S rDNA in interphase nuclei, and these were observed on almost the entire short arm of one submetacentric pair (Figure 1C). CMA_3^+ NOR-associated heterochromatin has been observed on the short arm of a submetacentric pair in three *Crepis foetida* subspecies (2n = 10), in addition to smaller pericentromeric and intercalary dots revealed by Giemsa-C banding (Dimitrova *et al.*, 1999). Guerra (1982) and Noguchi and Ohno (1989) described large C-bands on the short arm of submetacentric pairs in *C. vesicaria* and *C. capillaris*, respectively, in which this large heterochromatic block was associated with NOR, at least in *C. capillaris*. The observations agree with the FISH results obtained here, and suggest a constancy in the localization of the NOR on the submetacentric short arm in these species.

Galinsoga parviflora Pav. had 2n = 16 with small meta- and submetacentric chromosomes, the smallest pair being of the acrocentric type. Chromosomal lengths varied from 1.4 µm to 1.9 µm. According to Strother and Panero (1994), x = 8 is the probable basic number for *Galinsoga*. The largest submetacentric pair had a pericentromeric CMA_3^+ block that occupied most of the short arm. Additional C-CMA_3^+ bands appeared as interstitial dots on the other two small chromosomes (Figure 1F). FISH with a 45S rDNA probe revealed two signals in interphase nuclei and a

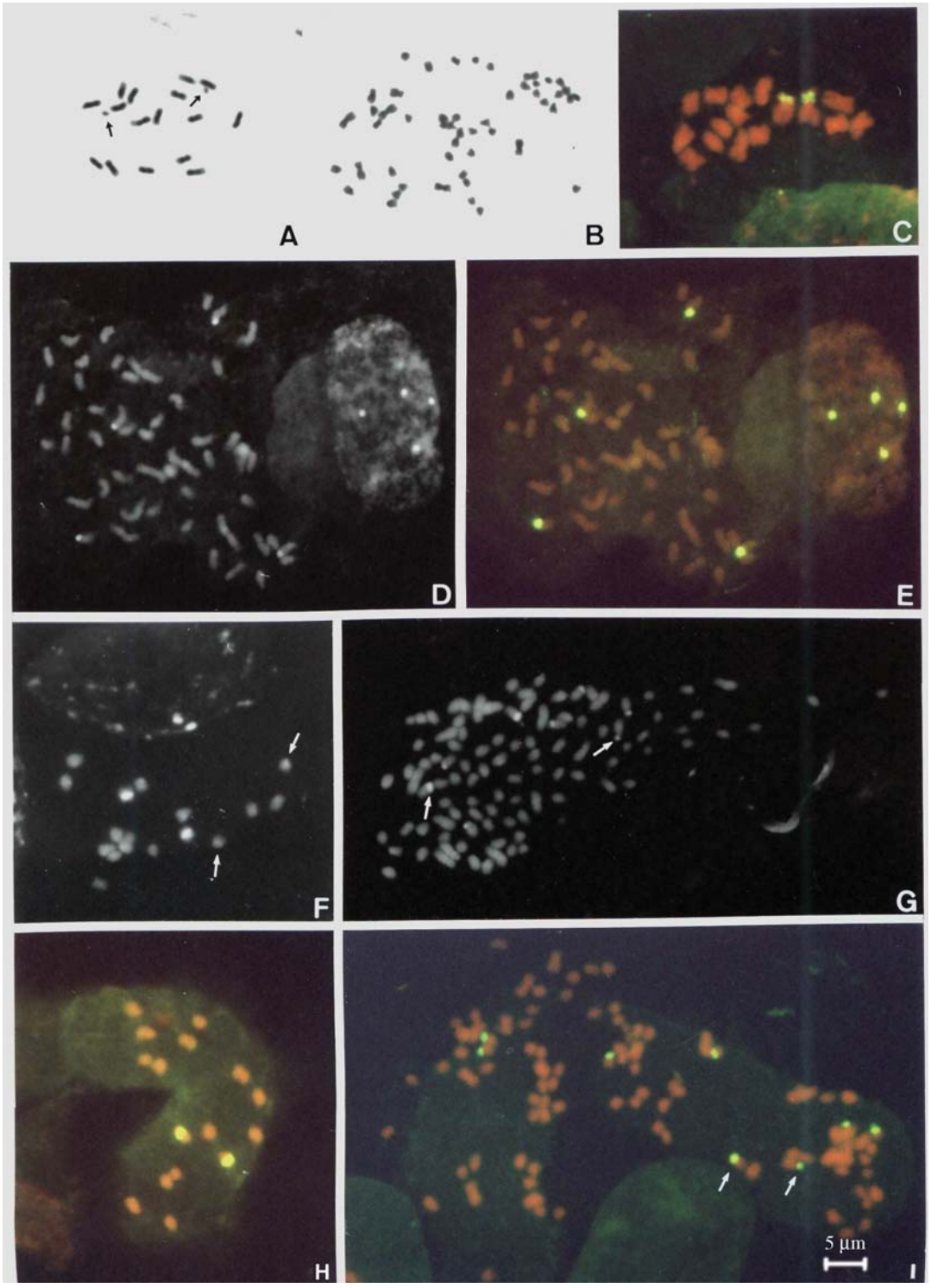

Figure 1 - (A) Feulgen-stained metaphase of *Crepis japonica* (2n = 16). Arrows indicate the satellites. (**B**) Feulgen-stained metaphase of *Chaptalia nutans* (2n = 50). (**C**) FISH with a 45S rDNA probe in *Crepis japonica*. Chromosomes appear larger than with conventional staining because of the hybridization process. (**D**) Premetaphase of *Chaptalia nutans* (2n = 50) showing C/CMA$_3$ banding after FISH. (**E**) FISH with a 45S rDNA probe in *Chaptalia nutans* (2n = 50). (**F**) *Galinsoga parviflora* showing C/CMA$_3$ banding. Arrows indicate minor intercalary CMA$_3$ blocks. (**G**) Metaphase of *Chaptalia nutans* (2n = 100) showing C/CMA$_3$ banding. Note the variation in size among the terminal NOR-associated C/CMA$_3$ signals (arrows). (**H**) FISH with a 45S rDNA probe in *Galinsoga parviflora*. (**I**) FISH with a 45S rDNA probe in *Chaptalia nutans* (2n = 100). Arrows indicate terminal hybridization signals that vary in brightness and size. Bar = 5 µm.

large hybridization site in the major submetacentric pair (Figure 1H), probably at the same position as the CMA_3^+ block.

Chaptalia nutans (L.) Pol. showed polyploidy in two cytotypes, 2n = 50 collected in Londrina-PR and 2n = 100 from Laguna-SC (the conventional staining is not shown here). These chromosomal numbers agree with previous counts of n = 25 and 2n = 50 for this species (Teppner and Tropper 1984). The chromosomal number for *C. graminifolia* and *C. pilloselloides* has been reported to be 2n = *ca.* 48 (Wulff *et al.*, 1996) and 2n = *ca.* 50 for *C. arechavaletai* (Waisman *et al.*, 1984). The Laguna cytotypes studied here had karyotypes with duplicated chromosomal numbers, *i.e.*, both were asymmetrical and consisted of large meta-submetacentric types (~2.5 μm in size) and several small acrocentric pairs ~1.3 μm long (Figure 1B). There were no major differences in chromosomal size and type. Neither cytotype showed differences in plant morphology. The C-CMA_3-banding of the 2n = 50 and 100 cytotypes revealed four and eight terminal C-CMA_3^+ blocks, respectively (Figure 1D and G). In the 2n = 50 cytotype, FISH with rDNA 45S revealed four hybridization signals of the same size (Figure 1E), whereas in the 2n = 100 cytotype one pair of chromosomes gave a much stronger hybridization signal than the other (Figure 1I). This size difference was also observed in these segments after C-CMA_3 banding (Figure 1G).

The results described here support the idea that the C-CMA_3 banding method is useful for examining the correlation between terminal GC-rich segments and 45S rDNA sites, as demonstrated by sequential FISH and C-CMA_3 banding in the *C. nutans* cytotype with 2n = 50 (Figures 1D, E). In addition, C-CMA_3 banding was effective in revealing fine bands (see arrows in Figure 1F) that were not visualized when stained directly with CMA_3. Vanzela *et al.* (2002) used this same procedure in some *Helianthus* species and found terminal GC-rich segments associated with 45S rDNA, as well as fine GC-rich bands that were not visualized after CMA_3 and Giemsa C banding. GC-heterochromatin associated with 45S rDNA sites is a common karyotypic feature that has been reported for many plant species (see Guerra, 2000a). The occurrence of interstitial C-CMA_3 blocks in a smaller chromosomal pair of *G. parviflora* (Figure 1F) suggested that, at least in this species, not all GC-rich heterochromatin was associated with rDNA sites.

The three species showed a conserved location for the 45S rDNA sites on the short chromosomal arms, *i.e.*, a terminal position in *C. nutans* and proximal-intercalary positions in *C. japonica* and *G. parviflora*. Clearer differences were observed in the size of the hybridization sites among the three species and within *C. nutans* with 2n = 100. The chromosomes bearing 45S rDNA were either submetacentric or acrocentric. Although FISH is a qualitative rather than a quantitative technique, an association between the size of the hybridization signal and the number of rDNA cistrons can be demonstrated (see Zurita *et al.,* 1997). Thus, the most brightly stained 45S rDNA sites likely represented cistrons with more repetitions than those with less intense signals.

Polyploidy is very common in plants and occurs in 70% of angiosperms (Wendel, 2000). According to Greilhuber and Ehrendorfer (1988), when polyploids are established as a population, they can become reproductively isolated and morphologically distinct from their parent species. Some *Vernonia* species collected in South America have a high level of ploidy and numerous interspecific karyotypic differences, especially in karyotype form, total chromosomal length and degree of asymmetry (Dematteis and Fernández, 2000). The two cytotypes of *C. nutans* studied here (2n = 50 and 100) were morphologically indistinct and certainly polyploids since the basic chromosome numbers proposed for angiosperms are x = 4, 5 and 6 (Guerra, 2000b). However, there was no evidence indicating that they were auto- or allopolyploids, despite the larger number of C-CMA_3 blocks and 45S rDNA sites.

Cytotypes originating from polyploidy have also been reported for other Brazilian Asteraceae, including *Mikania micrantha* (Ruas and Ruas, 1987) and *Vernonia polyphylla* (Ruas *et al.*, 1991). In some cases, the karyotypes of Asteraceae, such as those belonging to the Gnaphalieae group (Watanabe *et al.,* 1999b), can vary considerably in number, from 2n = 6 in *Podolepis* to 2n = 120 in *Craspedia*, and in symmetry, from unimodal in *Podotheca* to bimodal in *Schoenia*. Thus, the karyotype differences observed among *Crepis japonica, Galinsoga parviflora* and *Chaptalia nutans* appear to be common in the Asteraceae family. In contrast, the constancy of the rDNA position on the short arm of submetacentric pairs, as well as the association of this chromosomal segment with CMA_3^+ heterochromatin, are common features of most plant karyotypes.

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