Classical and Molecular Cytogenetic Tools to Resolve the *Bixa* Karyotypes

Pedro Marcos de Almeida, Carlos Roberto Carvalho* and Wellington Ronildo Clarindo

Laboratory of Cytogenetics and Cytometry, Departamento de Biologia Geral,
Universidade Federal de Viçosa, 36570-000, Viçosa-MG, Brazil

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Summary The cytogenetic studies performed in *Bixa orellana* have reported $2n=14$ or $2n=16$ chromosomes with little karyotypic information. In this context, an improved cytogenetic protocol using enzymatic maceration of meristematic cellular walls and digital image analysis was applied to *B. orellana* and *B. arborea*. In *B. arborea*, the high-resolution technique, Ag-NOR and C banding methods, and FISH using a probe of 45S rDNA genes, were also performed. Prometaphasic and standard C-metaphasic chromosomes presented well-defined primary and secondary constrictions facilitated the pairing of homologues and assembly of the karyogram for the 2 species. These species showed similar karyotypes with $2n=14$ chromosomes, being composed of 5 metacentric pairs (1, 2, 3, 4 and 6) and 2 submetacentric pairs (5 and 7). The chromosome 1 is approximately 2 times as long as the others and possesses the secondary constriction adjacent to the centromere, which suggests that Robertsonian translocation between 2 smaller chromosomes may have occurred during the karyotype evolution of *Bixa*. Additionally, the active NOR, the NOR-adjacent heterochromatic region and 18S, 5.8S and 26S rDNA genes were identified in the secondary constriction of chromosome 1.

Key words Ag-NOR banding, *Bixa*, C-banding, FISH, High-resolution technique, Robertsonian translocation.

The family Bixaceae presents only one genus, *Bixa* L., with 6 tropical species. *Bixa orellana* is a bush that synthesizes, in the seed coat, economically important natural dye pigments, such as the carotenoids bixin and norbixin (Scotter *et al.* 1998, Paiva Neto *et al.* 2003). Despite being classified into the same genus, *Bixa arborea* exhibits arboreal bearing and does not produce bixin and norbixin. Nevertheless, this species is interesting for the wood industry and used in reforestation projects (Lorenzi 1998).

Distinct authors have reported the chromosomic number only of *B. orellana* as $2n=2x=16$ (Simmonds 1954, Krishnan and Ayyangar 1987) or $2n=2x=14$ (Mukherjee 1975, Morawetz 1986, Krishnan and Ayyangar 1987). J.F.R.P. Carvalho (2000. Thesis, Univ. Fed. of Viçosa) not only confirmed the number of $2n=2x=14$, but also showed the nucleolar organization region (NOR) active in chromosome 1. Therefore, considering the worldwide food and industrial interest for the natural pigments of *Bixa* (J.F.R.P. Carvalho 2000. Thesis, Univ. Fed. of Viçosa), greater cytogenetic knowledge of this plant is required in order to aid in breeding programs.

As demonstrated by other authors, the classical and molecular cytogenetic tools have been successfully applied for obtaining information from high quality plant chromosomes. Using cellular wall enzymatic maceration and dissociation of root-tips (Almeida and Carvalho 2004, Contim *et al.* 2005), and image capture by CCD cameras with digital image analysis softwares, chromosome im-

* Corresponding author, e-mail: ccarvalh@ufv.br

ages permitted accurate cytogenetical characterization and measurements (Carvalho and Saraiva 1993, 1997, Guzzo *et al.* 2000, Bauchan and Hossain 2001, Neumann *et al.* 2002).

The present study was conducted in order to provide good quality cytogenetical preparations for application of the high-resolution methodology, C and Ag-NOR banding techniques and for localization of the 45S rDNA by FISH in *B. arborea*. In addition, some karyotype comparisons and chromosome evolution aspects were shown for *B. orellana* and *B. arborea*.

Materials and methods

Plant material

B. orellana seeds were obtained at Universidade Federal de Viçosa (UFV-MG, Brazil) and *B. arborea* seeds were supplied by the Reserva Florestal de Linhares (ES, Brazil). The analyses were carried out at the Laboratory of Cytogenetic and Cytometry, General Biology Department, UFV, Brazil.

Pre-treatment and fixation

Seeds of *Bixa* were germinated in a Petri dish at 30°C for 5 d. Root-tips were treated with 3 µM Trifluralin (Nortox[®]) and 100 µl dimethyl sulfoxide (Sigma[®]) solution for 18 h at 4°C, subsequently washed with distilled water for 20 min, fixed in fresh methanol:acetic acid (Merck[®]) solution (3:1), with 3 solution changes, and then stored at -20°C. For high-resolution chromosome preparations, the roots of *B. arborea* were pre-treated, during 18 h at 4°C, with a solution containing 0.1 mg ml⁻¹ of ethidium bromide (Sigma[®]) and 0.1 mg ml⁻¹ of colchicine (Sigma[®]), as described by Carvalho and Saraiva (1997).

Slide preparations

Root-tips were washed and enzymatically macerated with a 1:14 Flaxzyme (Novo FermentTM): distilled water solution, for 1 h 45 min at 34°C. Next, roots were washed for 20 min in distilled water and fixed again, then stored at -20°C. The slides were prepared as described by Carvalho and Saraiva (1993, 1997).

Giemsa conventional staining

Some slides were immediately stained with a 5% Giemsa (Merck[®]) solution in phosphate buffer (pH 6.8) for 5 min, washed twice in distilled water, air-dried and placed on a hot-plate (50°C).

Ag-NOR banding

The Ag-NOR banding technique was applied as described by Goodpasture and Bloom (1975), with minor modifications for plant chromosomes. Slides were aged for 3–5 d, stained with 4 to 5 drops of 50% AgNO₃ (Merck[®]) solution and incubated in a humid chamber at 30°C for 18–20 h, in the dark. The coverslips were removed and the slides were washed in distilled water for 2 min.

C banding

C banding technique was based on the protocol described by Carvalho and Saraiva (1993). Two-week-old slides were treated in 5% Ba(OH)₂·8H₂O (Ridel-Ecibra[®]) solution for 15 s, at 56°C, with continuous agitation. Next, the preparations were successively washed, for 5 s, in 70% ethanol (Merck[®]) and methanol:acetic acid (8:1) solutions, air-dried, placed on a hot-plate (50°C) and stained with 5% Giemsa (Merck[®]) solution for 8 min.

FISH

FISH was performed according to the methods described by Contim *et al.* (2003). rDNA probes (pDm 238) containing the 45S rDNA gene of *Drosophila melanogaster* were labeled with biotin by nick translation, according to manual of the BIONICK kit (GIBCO®). Slides with well-spread chromosomes of *B. arborea* were incubated with $100 \mu\text{g ml}^{-1}$ RNase in $2\times\text{SSC}$ (150 mM NaCl, 15 mM $\text{Na}_3\text{-citrate}$, pH 7.0) for 1 h in a humid chamber at 37°C . Next, the slides were treated with 0.005% pepsin in 10 mM HCl at 37°C for 10 min, washed with the same buffer, then with PBS (130 mM NaCl, 7 mM Na_2HPO_4 , 3 mM NaH_2PO_4 , pH 7.0). Afterwards, the slides were sequentially dehydrated in 70%, 80% and 100% ethanol (3 min each) at room temperature, and finally air-dried. Probe amounts of $5 \mu\text{l}$ ($1 \mu\text{l}$ of probe diluted in $4 \mu\text{l}$ of hybridization mixture) were added to each hybridization area and the slide was covered with a 20×20 mm coverslip and incubated for 3 min at 80°C and overnight at 37°C . After incubation, the slides were maintained for 2 min in each of 2 baths of 50% formamide and in PBS at 37°C . Signal detection was performed on slides treated with $5 \mu\text{g ml}^{-1}$ anti-biotin (VECTOR: SP 3000) and $10 \mu\text{g ml}^{-1}$ anti-goat IgG-FITC (VECTOR: FI 5000) for 1 h at room temperature. The preparations were counterstained with $100 \mu\text{l}$ propidium iodide ($1 \mu\text{g ml}^{-1}$), washed in phosphate-buffered saline (PBS) for 1 min and mounted with $15 \mu\text{l}$ Vectashield.

Image Analysis

Images of the chromosomes were captured with a CoolSNAP-Pro *cf* (Roper Scientific®) video camera of 12 bits, assembled on an Olympus™ BX-60 fluorescence microscope with a $100\times$ objective lens and a WB filter. The frame was digitized using the Kit Cool-SNAP Pro® and Image Pro-Plus® 4.5 analysis system (Media Cybernetics®). Image analysis was performed on a Power Macintosh G4® computer, using the freely available (<http://reg.ssci.liv.ac.uk>) Image SXM software (Barrett 2002). This is a spin-off of the public domain image analysis application NIH Image, which was developed by Rasband (1998).

Results

The pre-treatment, fixation and slide preparations provided excellent prometaphases and standard C-metaphases exhibiting few overlapped chromosomes and well-defined primary and secondary constrictions (SC), which facilitated the pairing of homologues and assembly of the karyograms for *B. orellana* and *B. arborea* (Fig. 1a). The morphometry of the *Bixa* chromosomes was characterized according to the arm length (in micrometers) criteria described by Levan *et al.* (1964) and revised by Guerra (1986).

Cytogenetic analysis of the 2 species showed $2n=2x=14$, being 5 metacentric (1, 2, 3, 4 and 6) and 2 submetacentric chromosome pairs (5 and 7), and the presence of one SC adjacent to the centromere of the chromosome 1 (Fig. 1a). Furthermore, the 14 chromosomes of *Bixa* presented lengths varying from 3.27 to $1.40 \mu\text{m}$ in *B. arborea* (Fig. 1a) and 3.82 to $1.24 \mu\text{m}$ in *B. orellana* metaphases (Fig. 1a).

The arm length ratios, the presence of SC adjacent to the centromere of chromosome 1 and the morphometric data suggest that these species are cytogenetically similar. The karyograms of *Bixa* also showed that the total length of chromosome 1 is, approximately, 2 times as large as the other chromosomes (Fig. 1a).

The high-resolution technique allowed the observation of a stalk stirred portion, on SC of the chromosome 1, associated with nucleoli (Fig. 1b, d). This region of *B. arborea* was confirmed to be an active NOR, by Ag-NOR banding (Fig. 1c), and C banding revealed heavily stained bands in the NOR-adjacent heterochromatic region (Fig. 1a). FISH analysis identified 2 sites for the 45S rDNA in *B. arborea*. This technique evidenced 2 strong fluorescence signals localized in the active NOR

Fig. 1. Chromosomes obtained from root apical meristems of *B. arborea* or *B. orellana* treated with 3 μ M trifluralin and 100 μ l dimethyl sulfoxide solution for 18 h at 4°C. a) the C banding karyograms of *B. arborea* (above) and *B. orellana* (below) showing positive bands in the pericentromeric and flanking NOR-adjacent regions. The *Bixa* karyograms possess metacentric (1 to 4 and 6) and submetacentric (5 and 7) chromosomes and the SC adjacent to the centromere of chromosome 1. Note that the chromosome 1 possesses a total length approximately two times as large as the other chromosomes. b) *B. arborea* chromosomes like prophase obtained after application of high-resolution technique in root apical meristems. Chromosome 1 (left) emphasizing the stalk stirred SC and chromosome 1 (right) associated to the nucleoli. c) The Ag-NOR karyogram of *B. arborea* showing the active NOR as one dark band at the SC position of chromosome 1. d) Prophasic chromosome 1 pairs nucleoli-associated. Bar=5 μ m.

Fig. 2. *B. arborea* chromosomes after FISH technique showing 45S rDNA sites. a) *B. arborea* metaphasic chromosomes showing intense fluorescence bands of the 45S rDNA sites on medial chromosome 1. b) prometaphasic chromosome 1 (left) exhibiting two fluorescent spots of 45S rDNA sites near centromeric region and a profile 3D density plot of the chromosome 1 (right) to highlight the rDNA sites fluorescence signals. Bar=5 μ m.

(Fig. 2a). The rDNA sites were illustrated by a density profile 3D plot of the chromosome 1 to highlight the fluorescence signals (Fig. 2b).

Discussion

Microtubule inhibiting agent trifluralin was efficient in the accumulation of prometaphasic and metaphasic cells with subtle range on chromosome condensation. We considered this aspect as important for screening minor differences between chromosomes of the same class and with seemingly similar lengths (Fig. 1a).

Cell dissociation of macerated root apical meristems, associated with air-dried slide preparations, was suitable to provide clear and preserved chromosomes, evidencing the main morphological characteristics that facilitated the assembly of the *Bixa* karyograms (Fig. 1a). These techniques allowed the obtention of chromosomes well-spread in the same focus plane, without cytoplasmic background, overlaps or structural deformations. Identical results were obtained in *Zea mays* (Carvalho and Saraiva 1993, 1997), *Capsicum annuum* (Almeida and Carvalho 2004) and *Aniba rosaeodora* (Contim *et al.* 2005).

The CCD video camera was an efficient tool for obtaining good quality images of chromosomes due to its 12 bits information for each pixel, representing 1 of 4,096 possible gray levels with 0.12 $\mu\text{m}/\text{pixel}$. Besides, the digital image analysis system contributed to discriminate between subtle measurements of chromosomes. Ohmido *et al.* (1998) and Guzzo *et al.* (2000) mentioned that these digital equipments are essential for capture and digitization of faint fluorescent signals.

In accordance with Murkherjee (1975), Morawetz (1986) and Carvalho *et al.* (2005) the *B. orellana* chromosomal number is $2n=2x=14$ (Fig. 1a). However, some authors found $2n=2x=16$ chromosomes for this species (Simmonds 1954, Krishnan and Ayyangar 1987). Morawetz (1986) related that the chromosome 1 can break in 2 fragments during slide preparations, because of the presence of SC adjacent to the centromere. This fact may have been inducing the carrying out of incorrect measurements.

Although *B. orellana* and *B. arborea* have shown a few distinct botanical characteristics, such as those concerning their bearing, color of petals and production of carotenoids (Baliane 1984, Lorenzi 1998), the arm length ratios, the presence of SC adjacent to the centromere of chromosome 1 and the morphometric data (Fig. 1a) indicate that these species are cytogenetically similar.

The chromosome 1 of both species exhibited a total length approximately 2 times as large as the other chromosomes (Fig. 1a). In that same chromosome, the stalk stirred portion on the SC of the *B. arborea*, associated to the nucleoli, was evidenced by the high-resolution technique (Fig. 1b) and the SC is clearly adjacent to the centromere (Fig. 1b). This result suggests that the Robertsonian translocation between 2 smaller chromosomes may have occurred during the evolution of *Bixa*. Robertsonian interchange is characterized by centromeric fusion (Jones 1998, Zhang *et al.* 2001, Oakey and Beechey 2002, Switonski *et al.* 2003) between 2 acrocentric (Gupta and Gupta 1991) or telocentric (Zhang *et al.* 2001) non-homologous chromosomes, resulting in one metacentric chromosome (Gupta and Gupta 1991, Jones 1998, Zhang *et al.* 2001, Oakey and Beechey 2002). Considering that many species present the SC in the distal portion of the short arm (Leitch and Heslop-Harrison 1992, Hanson *et al.* 1996, Liu *et al.* 1997) and the morphometric data of the chromosome 1, a break involving the long arm of 1 chromosome and the short arm of the other probably led to the formation of the metacentric chromosome 1 with the SC adjacent to the centromere, during the genomic evolution of these species.

Considering that, during mitosis, active NORs possess transcription factors of rDNA genes, that impregnate with silver (Moraes-Cecilio *et al.* 2000, Besendorfer *et al.* 2002, Brasileiro-Vidal *et al.* 2003, Neves *et al.* 2005), the presence of a single active NOR was confirmed at the portion of chromosome 1 adjacent to the centromere, in *B. arborea* (Fig. 1c), indicating that this region contains the chromosomal domain around which nucleoli are organized at the end of mitosis, when rDNA transcription is initiated. Identical results were observed in *B. orellana* by Carvalho (2000).

Additional results obtained from the SC of *B. arborea* after C banding presented heavily

stained bands at the NOR-adjacent heterochromatic region. Moreover, this technique also evidenced the pericentromeric heterochromatin in all chromosomes (Fig. 1a). Almeida and Carvalho (2004), after subjecting *Zea mays* and *Capsicum annum* chromosomes to the fluorescent banding of heterochromatin associated to the SC with the use of acridine orange (Hsc-FA banding), suggested that this NOR-adjacent heterochromatic region consists of β -heterochromatin. In addition to acridine orange, the fluorochrome chromomycin A3 has also been used to identify NOR-adjacent heterochromatin (Sato 1988, Melo and Guerra 2003, Marcon *et al.* 2005) in plants, indicating that this region is a GC-rich chromosomal portion.

The FISH of 45S rDNA resulted in fluorescence signals that evidenced the 18S, 5.8S and 26S rDNA genes, located in the active NOR of *B. arborea* (Fig. 2a). These genes occur as a large number of tandem repeats of single transcription units (45s rDNA). These sequences are predominantly located in the NOR of eukaryotic chromosomes (Ansari *et al.* 1999). Moreover, the digital image analysis system allowed the assembly of a density profile 3D plot of the chromosome 1 that highlighted the fluorescence signals (Fig. 2b). FISH proved valuable for both fundamental and applied aspects of studies of genome evolution and plant breeding (Osuji *et al.* 1998). Besides, this technique is a powerful tool for regional mapping of DNA sequences on chromosomes in interphase and metaphase (Ansari *et al.* 1999, Weiss and Maluszynska 2001).

This work represents an advance on the cytogenetics of *Bixa*, concerning the morphometry of the *B. orellana* and *B. arborea* chromosomes, the mitotic comparison of the 2 species and evidences of occurrence of Robertsonian translocation during the genome evolution of these species. In addition, the high quality of the chromosomes permitted the application of the techniques that identified the active NOR and characterized the NOR-adjacent heterochromatic region.

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