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Research Article

Nuclear DNA content and karyotype of Rosewood (*Aniba rosaeodora*)

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Abstract

Rosewood (*Aniba rosaeodora* Ducke, Lauraceae) is ecologically and economically important to the Amazon region. As a consequence of its economic importance, rosewood populations have been decimated in the Amazon forest. Species of nine genera of the Lauraceae family have characterized karyotypes with n = x = 12 chromosomes in the gametophytic phase but the genus *Aniba* is one of the least studied Lauraceae genera with a previously undescribed genome. We used cytogenetic techniques to determine that the *A. rosaeodora* karyotype contained 12 pairs (2n = 24) of relatively small submetacentric chromosomes with lengths ranging from 1.34 to 2.25 μm and a nucleolar organizer region (NOR) in the short arm of chromosome 7. Flow cytometry gave 2C = 2.32 pg of DNA, equivalent to approximately 2.24 x 10^9 base pairs.

Key words: rosewood, *Aniba rosaeodora*, Lauraceae, karyotype, flow cytometry.

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Introduction

The Amazon forest is mega-biodiverse but its ecosystems are still poorly known (Bawa and Seidler, 1998). Rosewood (*Aniba rosaeodora* Ducke, Lauraceae) is an Amazonian tree that produces an essential oil which is in great demand both in Brazil and internationally. Rosewood oil contains large amounts of linalool, a compound widely used by the cosmetics industry (Vainstein *et al.*, 2001) but which may also have therapeutic properties as an anesthetic (Ghelardini *et al*., 1999) and an antimicrobial agent (Rosa *et al*., 2003; Inouye *et al*., 2001) that may lead to the development of new products. Due to commercial demand, rosewood populations have decimated in its center of origin in the Amazon forest but a small number of individual trees continue to exist in the Brazilian states of Amazonas and Pará (Rosa *et al*., 1997).

The Lauraceae family contains 52 genera and about 2500 described species (Ribeiro *et al*., 1999) distributed in the tropics and subtropics, the greatest concentration of species being in the Neotropics and Southeast Asia. The chromosome number has been quantified for many species of the nine Lauraceae genera, mostly from the northern hemisphere. The characteristic gametophytic chromosome number is n = 12 but some polyploids also occur (Goldblatt and Johnson, 2000). The genera *Adenodaphne* (Carr and McPherson, 1986), *Lindera* (Wu, 1995), *Machilus* (Sandhu and Mann, 1988), *Neolitsea* (Chatha and Bir, 1987), *Persea* (Chen, 1993), *Phoebe* (Sandhu and Mann, 1988) and *Sassafras* (Huang *et al*., 1989) all possess n = 12 but the genus *Litsea* (Huang *et al*., 1988) contains five species with n = 12 and one (*L. glutinous*) with n = 24, while the genus *Lauros* has species with 2n = 12 times X, where X varies from 3 to 6 (Todua, 1987).

The only Lauraceae species to have had its nuclear DNA content quantified by flow cytometry is *Persea americana*, which has 2C = 1.86 pg (Arumuganathan and Earle, 1991), although Bennett and Leitch (2003) used other methods to establish that *Persea indica* is 2C = 3.30 and *Cinnamomum camphora* C = 1.80.

The genus *Aniba* is one of the least known members of the Lauraceae family and in spite of the great scientific, ecological and economic importance of *Aniba rosaeodora* its genome size, genetic structure and chromosome number and morphology have not yet been characterized. In the work described in this paper we quantified the amount of nuclear DNA in *A. rosaeodora* and characterized its karyotype

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Material and Methods

Plant material

Amazonian rosewood (*Aniba rosaeodora* Ducke, Lauraceae) seeds were collected from two wild populations in reserves in the Brazilian state of Amazonas, one being the Reserva Florestal Adolpho Ducke belonging to the Brazilian National Institute for Amazonian Research (Instituto Nacional de Pesquisas da Amazônia (INPA) Manaus, Amazonas, Brazil) and the other the Silves Reserve (Associação Vida Verde (AVIVE) da Amazônia, Silves, Amazonas, Brazil). The seeds were placed in germination chambers for 15 days at 30 °C and cultivated in the INPA forest seedling greenhouse in Manaus (3°8' S, 59°52' W).

Flow Cytometry

The method used was described by Dolezel and Göhde (1995), with minor modifications. Young and vigorous seedling leaves were washed, placed in recipients containing distilled water and maintained at 4 °C. Leaf fragments (2 cm^2) were macerated in 1 mL of Otto-I lysis buffer (0.1 M citric acid monohydrate plus 0.5% (v/v) Tween 20) and the suspension filtered through 40 μm pore-size membranes and transferred to clean tubes which were centrifuged at 250 g. After centrifugation the pellet containing nuclei was collected and homogenized in 100 μL of Otto-I buffer which was separated in two aliquots, one being stained with 15 μM 4',6'-Diamidino-2-phenylindole (DAPI) solution in Otto-II buffer (0.4 mM $Na_2HPO_4.12H_2O$) for 15 min in the dark and the other with 75 μM propidium iodide (PI) plus 50 μg/mL Rnase in Otto-II buffer for 30 min. The analysis of the nuclei suspension was performed using a Partec PAS II/III Flow Cytometer (Partec Gmbh, Munster, Germany). For analysis of DAPI stained nuclei we used a high pressure mercury lamp (HBO-100 W) with KG 1, BG 38 and GG 435 filters while for propidium iodide stained nuclei we used a 480 nm argon ion laser (20 mW) with TK 560 and RG 610 filters. For internal controls we used *Raphanus* sativus cv Saxa (2C = 1.11 pg) kindly provided by Dr Jaroslav Dolezel (Institute of Experimental Botany, Tchec Republic). Three young leaves from each of three plants and approximately 10 thousand nuclei per plant sample were analyzed using the FlowMax® Partec software. Samples with coefficients of variation above 3% were not used in these analyses. The results presented in pg were transformed into base pairs as described by Bennett and Smith (1976).

Cytogenetic preparations

Thirty roots of rosewood seedlings were treated with 5 mM Oryzalin solution for 3 h at 30 °C, washed in distilled water for 15 min and fixed in 3:1 methanol:acetic acid at -20 °C. After 24 h the roots were washed and macerated in 1:10 Flaxzym:distilled water at 35 °C for 90 min. The root tips were washed in distilled water for 20 min, fixed (3x) in 3:1 methanol:acetic acid and stored at -20 °C. The cytogenetic preparations were proceeded by cellular dissociation of the apical meristem (Carvalho and Saraiva, 1997) and then left to dry at room temperature before staining with 5% Giemsa solution in phosphate buffer (pH 6.8) for 5 min, washing twice in distilled water and drying at 50 °C.

Image analysis

Twenty images of good quality metaphases per root meristem were captured using a charge-coupled device (CCD) video-camera connected to an Olympus™ BX60 microscope equipped with a 100x immersion lens. Morphological analyses of the chromosomes were performed using the Image SXM software (Rasband, 1997) running on a Macintosh™ G4 computer. The arms of each chromosome were measured in pixel units and converted to a micrometer scale. The centromeric index was determined according to the criteria for morphologic classification of chromosomes (Guerra, 1986).

Results and Discussion

Flow cytometry

The flow cytometry analysis of the nuclei suspension generated histograms with peaks corresponding to the average of the G1/G0 nuclei DNA content. The *R. sativus* cv Saxa peak was calibrated to channel 100 (internal standard with 2C = 1.11 pg DNA). The results from the three *A. rosaeodora* DAPI stained nuclei samples showed G1/G0 peaks in channels 212, 213 and 214, corresponding to a mean value of 2C = 2.36 pg DNA (Figure 1a). The equivalent samples stained with PI generated G1/G0 *A. rosaeodora* nuclei peaks in channels 207, 209 and 211, corresponding to a mean value of 2C = 2.32 pg DNA (Figure 1b). The two populations of rosewood showed overlaying peaks, that is, no differences in DNA content. The second DNA content measurement (2C = 2.32 pg) was calculated to be equivalent to 2.24 x 10^9 base pairs.

Cytogenetics

Chromosome preparations and image analysis allowed us to obtain metaphases with appropriate cytogenetic quality. After incubation in a solution (5 mM) of the herbicide Oryzalin for 3 h at 30 °C, the morphology of the chromosomes showed standard C-metaphase condensation, although in some cells the nucleolus remained attached to the secondary constriction (Figure 2). These data were used for morphologic characterization and karyogram assembly (Figure 3). The morphology of the rosewood chromosomes was characterized according to arm length (in micrometers) and classified as pairs of homologues (Table 1). This species contains relatively small submetacentric chromosomes (2n = 24) with lengths varying from 1.34 to 2.25 μm. The criteria for chromosome classification (Guerra, 1986) was used in this analysis and all chromo-

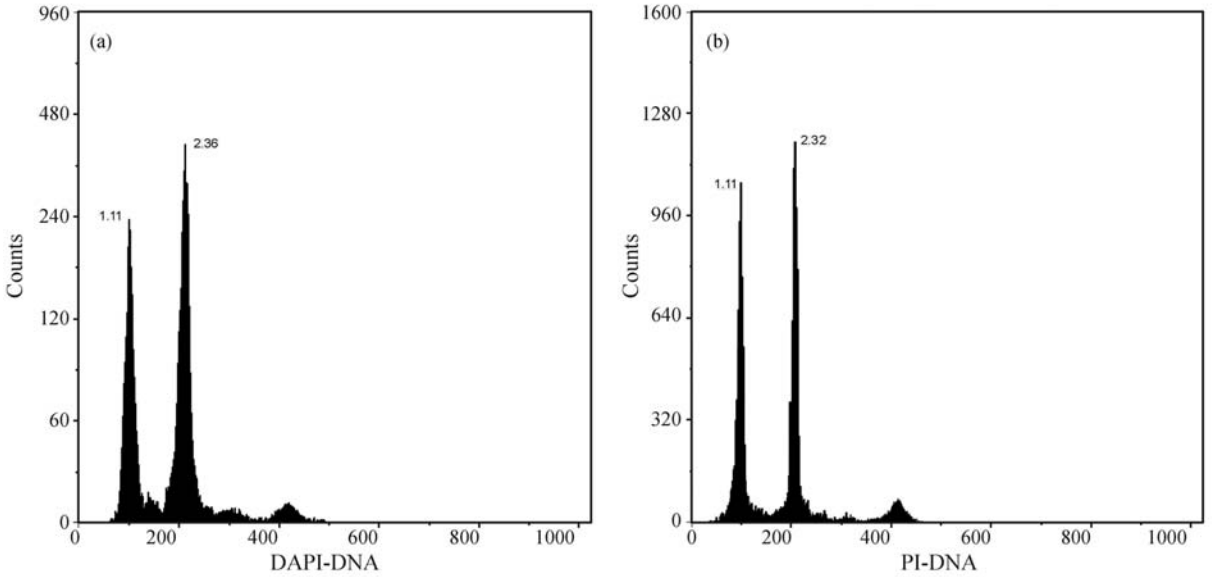

Figure 1 - DNA-histogram showing G1/G0 peaks resulting from simultaneous processing of nuclear suspensions of young leaves tissue of: (a) *Raphanus sativus* cv Saxa (internal standard: 2C = 1.11 pg) and *Aniba rosaeodora* (2C = 2.36 pg) stained with DAPI; and (b) *Raphanus sativus* cv Saxa and *Aniba rosaeodora* (2C = 2.32 pg) stained with propidium iodide.

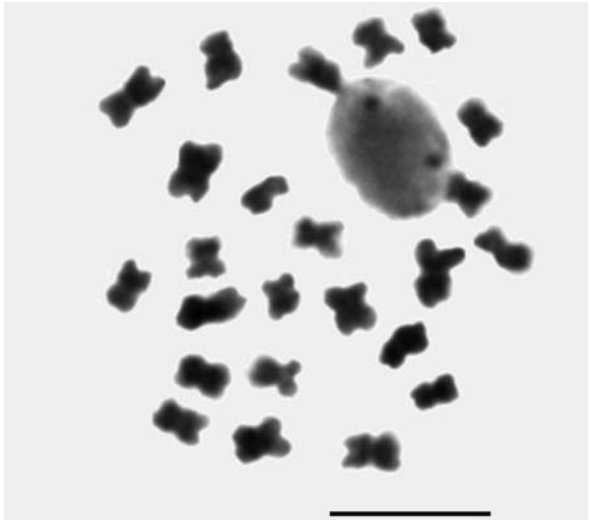

Figure 2 - Metaphase chromosomes (2n = 24) obtained from rosewood root tip cells pre-treated with 5 mM the herbicide oryzalin and Giemsa stained. The nucleolar organizing region can be seen in the short arm of the chromosome pair with secondary constrictions and satellites associated with the nucleolus. Note that after oryzalin treatment the chromosomes are condensed as in standard C-metaphase morphology and the nucleolus remains attached to the secondary constriction. Bar = 5 μm.

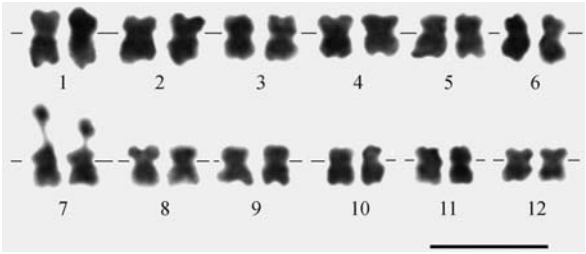

Figure 3 - Karyogram (2n = 24) of the Figure 2 chromosome set showing 12 submetacentric chromosome pairs, including chromosome pair 7 showing the secondary nucleolus organizing region (NOR) constriction) are shown. The gap in the short arm of pair 7 was produced by the stretching of stalk of the satellite. The nucleolus was digitally removed from the original image. Bar = 5 μm.

somes were classified according to the decreasing size criteria (1 to 12). The nucleolar organizing region (NOR) was identified by the presence of a secondary constriction in the short arm of chromosome 7 (Figure 2). No differences in karyotype were observed between the two populations.

The presence of a nucleolus attached to the condensed chromosomes (Figure 2) is not uncommon after herbicide treatment in slide preparations, similar results having also been observed in *Capsicum* sp and *Bixa orellana* (data not shown).

The 2C value of rosewood showed a nuclear DNA content of 2.36 pg using DAPI or 2.32 pg using PI as fluorochromes to stain the nuclei suspension. Although these nuclear DNA contents were similar, the 2C value of 2.32 pg is more representative because PI staining has no bias for AT or GC-rich sequences within genomes (Dolezel *et al*., 1998; Shapiro, 2003)

The *A. rosaeodora* haploid group corresponds to the basic number of chromosomes (x = 12) characteristic of most Lauraceae species, as verified by Goldblatt and Johnson (2000) in nine other genera of this family. Although the characteristic chromosome number is conserved, the nuclear DNA content varies considerably among the members

Table 1 - Analysis and classification of the chromosome morphology of *Aniba rosaeodora* (2n = 24).

Chrom	Fl	Arm		r	cr	Cc*	Rl
		short	long				
1	2.25	0.89	1.36	1.53	39.6	SM	11.09
2	1.88	0.71	1.17	1.65	37.8	SM	9.27
3	1.80	0.69	1.11	1.61	38.3	SM	8.87
4	1.79	0.64	1.15	1.80	35.8	SM	8.82
5	1.76	0.61	1.15	1.89	34.7	SM	8.67
6	1.75	0.57	1.18	2.07	32.6	SM	8.62
7	1.62	0.46	1.16	2.52	28.4	SM	7.98
8	1.55	0.50	1.05	2.10	32.3	SM	7.64
9	1.55	0.50	1.05	2.10	32.3	SM	7.64
10	1.51	0.49	1.02	2.08	32.5	SM	7.44
11	1.49	0.50	0.99	1.98	33.6	SM	7.34
12	1.34	0.48	0.86	1.79	35.8	SM	6.60

*SM = submetacentric. Chrom: Chromosome. Fl: Full length (μm). r: Ratio between arms. cr: Centromeric rate. Cc: Chromosome class. rl: Relative length (%).

of the Lauraceae and the results are within the previously reported range.

Collectively these data describe the basic characteristics of the genome organization of *A. rosaeodora* with the quantification of the nuclear DNA content and the number and morphology of the chromosomes.

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