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Cytogenetic Characterization of *Aloysia virgata* Ruiz and Pavan (Verbenaceae)

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ABSTRACT

Since previous cytogenetic reports of Aloysia *have only described the meiotic behavior and chromosomal number of some species, the aim of this work was to provide detailed cytogenetic description of* Aloysia virgata *that would contribute to the understanding of the taxonomical organization of the Verbenaceae.* Aloysia virgata *had a karyotype with 2n = 36 metacentric chromosomes, all with similar size. The large amount of heterochromatin seen after Giemsa staining was confirmed by C-banding. Four nucleolar organizer regions (NORs) were detected with an rDNA 45S probe in two homologous pairs and two sites of 5S rDNA located on one chromosomal pair were detected by fluorescence in situ hybridization. The interphase nucleus was classified as semi-reticulate. Meiotic analysis showed a normal chromosomal behavior, with 18 bivalents in some parts of prophase I and in metaphase I. The number of chromosomes, NORs and 5S rDNA segments did not exclude a possible polyploid origin.*

Key Words: Chromosome, Cytogenetic characterization, FISH, *Aloysia*, Verbenaceae

INTRODUCTION

The genus *Aloysia* was described by Ortega and Patau in 1807 and contained about 30 species, widely distributed in America, from the southern USA and Mexico to northern Patagonia (Botta, 1979, 1980; Troncoso, 1980). The taxonomical limits of the genus are not well defined and *Aloysia* species have frequently been included in other genera that are morphologically similar to *Aloysia*, e.g. *Lippia*. This has resulted in some confusion in identifying *Aloysia* species (Sanders, 1987, 2001; Salimena, 2000, 2002).

There have been few cytogenetic studies of the family Verbenaceae. The small size of the chromosomes and the reduced size of the flowers make cytogenetic analyses difficult (Kummar and Dutt, 1989). Cytogenetic studies have been reported for only five of the 30 well-known species: *A. grattisima* (Gill. and Hook.) and *A. lycioides* Cham. with 2n = 54 (Corazza-Nunes et al., 1995; Andrada et al., 1998), *A. ligustrina* (Gill. and Hook.) and *A. polystachya* (Gris.) with 2n = 36 (Covas and Schnack, 1946 *apud* Corazza-Nunes et al., 1995; Andrada et al., 1998) and *A. scorodonioides* HBK with 2n = 72 (Diers, 1961 apud Corazza-Nunes 1995).

Since cytogenetic studies are an important tool for understanding the phylogenetic relationships and systematics of plants and since previous cytogenetic reports of *Aloysia* have only described the meiotic chromosomal behavior and

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chromosome number for a few species, the aim of this work was to provide a detailed cytogenetic analysis that would contribute to the understanding of the cytogenetics of the Verbenaceae and of the taxonomical organization of this group.

MATERIAL AND METHODS

Plant material

Five hundred seeds of *A. virgata* were collected from at least 10 trees in the Reserva Municipal Santa Genebra (220 49´45"S, 47006´33"W), Campinas, SP, Brazil. Only one voucher specimen (accession nº 39.308) was deposited in the CESJ Herbarium at the Universidade Federal de Juiz de Fora (UFJF).

Sample and slide preparation

The seeds collected from different trees were placed in Petri dishes and incubated in a germinator on a 12 h photoperiod at 25 ºC until the root emission. The root stips were pre-treated with 12 µM oryzalin at room temperature for 5 h and then washed and fixed with fresh, cold methanol:acetic acid solution (3:1, v/v) for at least 24 h. Enzymatic maceration was done with a Pectinex (Novo Nordisk™): 2 mM sodium citrate mixture (1:10, v/v), pH 3.5, at 34 ºC for 1 h. The slides were prepared by the air drying technique (Carvalho, 1995).

For meiotic analysis, immature inflorescences were fixed in the fresh, cold methanol:acetic acid solution (3:1, v/v). Meiotic preparations were obtained using air drying technique with enzymatic maceration. Twelve flowers and about 40 anthers were used. After fixation, the anthers were transferred to an adapted tube with a polyester screen at the bottom. The material was washed with the distilled water for 10 min and the adapted tube with anthers was then immersed in a concentrated enzymatic solution at 34º for 20 min, after which the anthers were washed with the distilled water for 10 min. The adapted tube was then placed in the microtube (0.5 mL) containing distilled water in which the anthers were broken into fragments with a special needle to collect the pollen mother cells (PMCs). The cell suspension was centrifuged four times at 4000 rpm (three min each). After the fourth centrifugation, fresh, cold fixative (methanol:acid acetic, 3:1, v/v) was added to complete the microtube volume. After 20 minutes, the cells were resuspended and about six drops of the suspension were used for slide preparation. After drying on a hot plate, the slides were submerged in 45% acetic acid for 10 s and then dried again and stained with 10% Giemsa solution (pH 6.8) for 90 s.

Interphase nuclei

The morphology of nearly 1000 interphase nuclei was analyzed after Giemsa staining and the nuclei were classified according to Guerra (1987a, b).

C–Banding

Constitutive heterochromatin was detected as described by Schwarzacher et al. (1980), with slight modifications. The slides were incubated in 0.2 N HCl at room temperature for 75 s, treated with 5% barium hydroxide at room temperature for 8 min and finally incubated with 2 x SSC at 60 ºC for 80 min. The staining was done with Giemsa solution for 15 min.

Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization was done according to Heslop-Harrison et al. (1991), with a few modifications. The probes p*Ta*71 containing 18S-5.8S-26S rDNA and p*Ta*794 containing 5S rDNA sequences, both isolated from wheat (Gerlach and Bedbrook, 1979; Gerlach and Dyer, 1980) were used. The probes were labeled with biotin by nick translation. The preparations were incubated with RNAse (100 µg/mL) at 37 ºC for 1 h and then post-fixed in 4% (w/v) paraformaldehyde, dehydrated in a 70-100% graded ethanol series and air dried. The hybridization mixture (30 µl/slide) consisted of 100% formamide (15 µl), 50% dextran sulphate (6 µl), 20 x SSC (3 µl), denatured blocking DNA (100 ng/slide), 10% SDS (1 µl) and probe (100 – 200 ng/slide). The mixture was denatured at 70 ºC in a moist chamber and quickly chilled on ice. Chromosome denaturation was done at 90 ºC for 10 min, 48 ºC for 10 min, 38 ºC for 5 min and 37 ºC for 5 min using a PTC 100 thermal cycler (MJ Research). Hybridization was done at 37 ºC in a moist chamber overnight. Post-hybridization washes were done in 20 x SSC for 45S rDNA and in 6 x SSC for 5S rDNA, always with constant shaking. The hybridization sites were detected with an avidin-FITC conjugate. The chromosomes were counterstained with propidium iodide solution (2.5 mg/mL) and the slides were mounted in VectaShield antifade (Vector Laboratories).

Meiosis

About 100 cells in each stage of meiosis were analyzed and identified using meiotic images of *Hordeum*, *Artemisia* and *Zea mays*, as described by Singh (1993) and Fukui and Nakayama (1996).

RESULTS

Twenty metaphases were analyzed to determine the chromosomal number. There were 2n = 36 metacentric chromosomes, all with similar size (Fig. 1a).

Blocks of chromatin that stained strongly with Giemsa were seen in mitotic chromosomes (Fig. 1a). C-Banding (Fig. 1b) coincided with these blocks, indicating a large amount of heterochromatin. The interphase nucleus was classified as semi-reticulate, according to Guerra (1987a) (Fig. 1c).

Since the localization of active NORs by Ag-NOR technique (Howell and Black, 1980) resulted in non-specific staining, FISH was used, which revealed four rDNA 45S sites in two homologous pairs (Fig. 1d). Two signals regions of 5S rDNA were also seen in one homologous pair (Fig. 1e).

Cells at different meiotic phases were observed in middle pachytene (Fig. 2a), middle diplotene (Fig. 2b), diakinesis (Fig. 2c), anaphase I (Fig. 2d), telophase I (Fig. 2e) and final telophase II (almost tetrad) (Fig. 2f). No signs of meiotic irregularities were observed. Eighteen bivalents were seen during diakinesis (Fig. 2c).

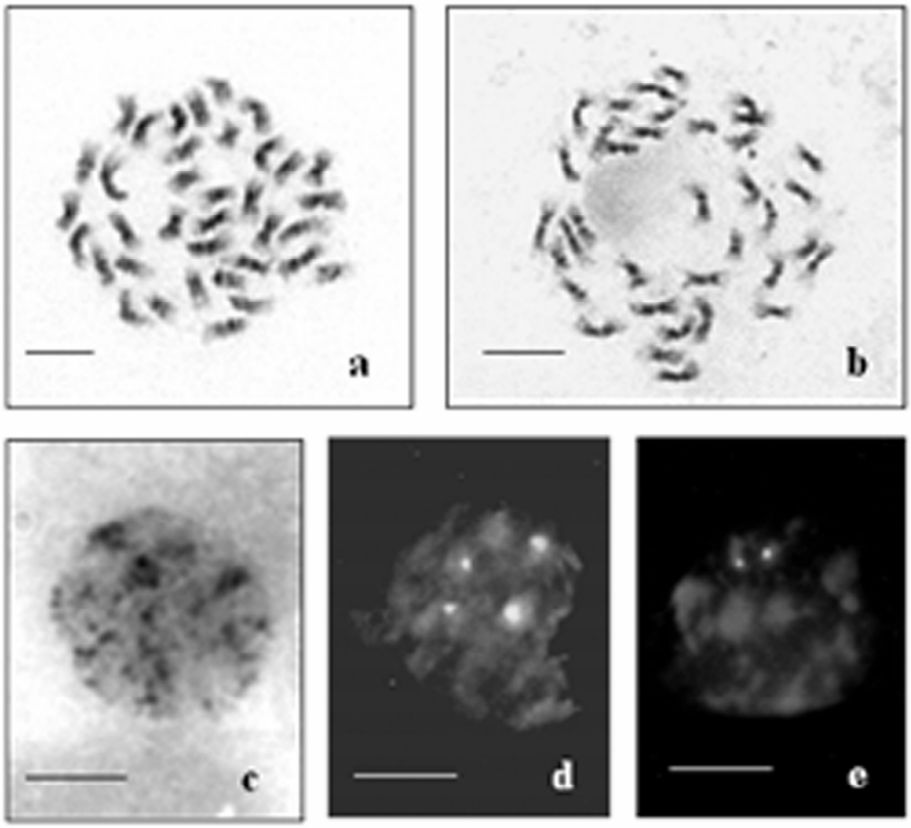

Figure 1 - Mitotic cells: metaphase (a) stained with Giemsa 10%. Metaphase after C-banding (b). Interphase nuclei (c) stained with Giemsa 10%. Interphase showing four NORs (d) and two segments of 5S rDNA (e) with FISH. Bar = 6 μm.

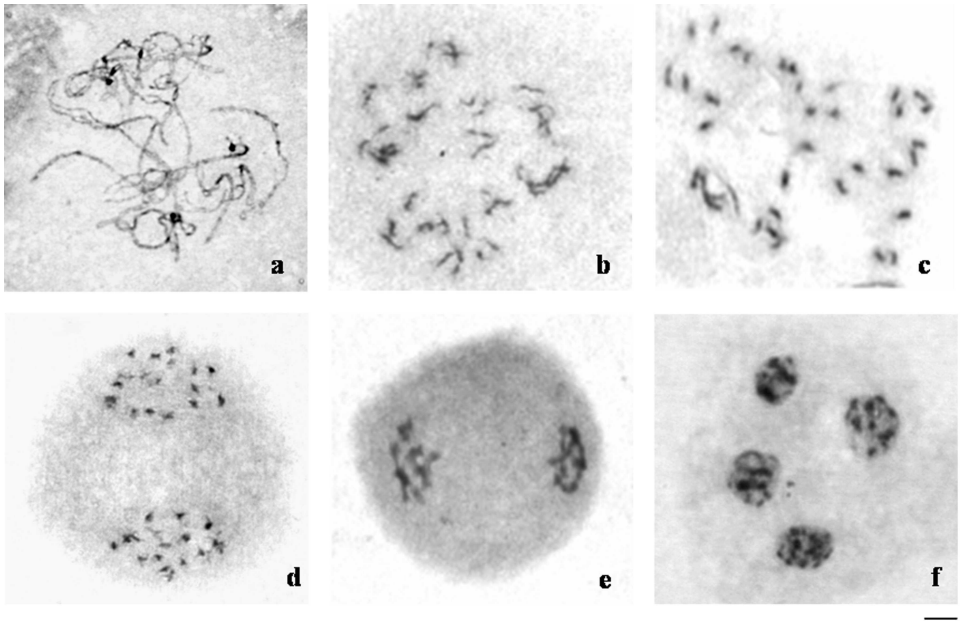

Figure 2 - Meiotic cells: middle pachytene (a), middle diplotene (2b), diakinesis (c), anaphase I (d), telophase I (e) and final telophase II (f) Bar = 8.5 μm.

DISCUSSION

Cytogenetic analysis of *A. virgata* revealed 2n = 36 small metacentric chromosomes. Previous cytogenetic studies of this genus reported chromosomal numbers of 2n = 36, 54 and 72 (Corazza-Nunes et al., 1995; Andrada et al., 1998). Since the basic number proposed for *Aloysia* is x = 9 (Corazza-Nunes et al., 1995), and since polyploidy is very common in plants (for reviews see Otto and Whitton, 2000; Stace, 1989; Wendel, 2000; Schmidt, 2002), a polyploid origin for *A. virgata* is possible. Based on this hypothesis, the 2n = 36 found in *A. virgata*, *A. ligustrina* and *A. polystachya* could be considered tetraploid, whereas the 2n = 54 in *A. grattisima* and *A. lycioides* as hexaploid and the 2n = 72 in *A. scorodonioides* as octaploid, in agreement with the basic number of x = 9 proposed by Corazza-Nunes et al. (1995).

Previous studies of seed viability, pollen fertility and meiotic chromosomal behavior in *Aloysia* revealed many abnormalities (as univalent, trivalent, tetravalent, pentavalent and hexavalent associations during prophase I, early segregation during metaphase I, telophase I and metaphase II, multiple and multipolar spindles in anaphase II, several nuclei of different sizes in telophase II and tetrads with 3 to 8 microspores of unequal size) associated with a high percentage of sterile pollen and a low index of seed germination (Corazza-Nunes et al., 1995). These alterations have been related to polyploidy. Cytological evidence for an association between sterility and a high level of ploidy was also reported by Abraham and Gopinathan (1991) for *Dioscorea alata* L. (Dioscoreaceae). Khoshoo and Mahal (1967), Natarajan and Ahuja (1957) and Raghavan and Arora (1960) found meiotic irregularities and seed sterility in *Lantana* populations in India. *Aloysia virgata* showed a regular meiotic behavior with a high percentage of seed germination (about 80%), unlike *Aloysia* species.

If *A. virgata* has tetraploid origin, the meiotic success observed here could be explained by genic control of the pairing of distinct genomes in an alotetraploid or a high degree of homology between the genomes (Riley and Chapman, 1958; Sybenga, 1996; Dagne, 2001). A regular meiotic behavior may also be indicative of polyploidy established by diploid-like behavior at meiosis (De Wet, 1979; Widen and Widen, 2000; Ozkan et al., 2001; O´Leary et al., 2004). An additional hypothesis is that natural selection may have eliminated the eventual problems caused by chromosomes with a normal meiotic behavior. FISH with rDNA probes provided an important cytogenetic marker for defining the number of

NORs and revealed only one chromosomal pair with 5S rDNA sites in *Aloysia*. This result would be possible for a tetraploid species if chromosomal reorganization and DNA elimination occurred during polyploidization, proposed by Eckardt (2001).

No report has described *Aloysia* species with 2n = 18. However, this lack of evidence does not allow to definitively discard 2n = 36 as a diploid number for *A. virgata*, with x = 18. Some authors have also suggested a high basic chromosomal number for other genera when compared with those for angiosperms (x = 7) (Raven, 1975). Other examples include *Chroranthus* and *Sarcandra* (x = 15) (Okada,1995), *Euphrasia* (x = 11) (Barker et al., 1987), *Clerodendrum* (Verbenaceae) (x = 15, 23 and 26) (Choudhary and Roy, 1983; Moldenke, 1985), *Artemisia* (x = 17) and *Brassica* (x =18) (Stace, 2000); Rosaceae (x = 17) (Ramam and Kesavan, 1963) and *Boronia* (x = 18) (Shan et al., 2003). Yang et al. (2000) described for *Pyrenaria* basic chromosomal number x = 15 and concluded that all of the species investigated were diploid. These authors suggested that polyploidization during evolution could explain the high basic number for each genus studied.

Heterochromatin is one of the most important factors in evolution (Edelman and Lin, 1995) since it can interfere with phenomena such as DNA replication, nuclear volume, cell size, chromosomal structure, gene expression, genome organization, cell cycle and developmental rate (Stace, 2000; Redi et al., 2001). Variations in the amount of heterochromatin have proven to be useful in karyosystematic and phylogenetic studies (Schwarzacher et al., 1980; Grif, 2000). As shown here, *A. virgata* had a large amount of heterochromatin. At the supra- and infra-generic levels in plants, large amounts of heterochromatin are considered to represent an accumulation during evolution (Ikeda, 1988; Roser, 1994). Studies of the longitudinal differentiation of chromosomes in some *Sesbania* species have suggested that a large amount of heterochromatin indicates evolutionary differentiation within the genus, while a small amount of heterochromatin represents an ancestral condition (Forni-Martins and Guerra, 1999). A similar conclusion was reached by Greilhuber et al. (1981) and Moscone et al. (1996) for other genera. Hence, it could be possible that the large amount of heterochromatin seen in *A. virgata* represented an apomorphic condition for this species, but it would be necessary to analyze other species of *Aloysia* genus to confirm this hypothesis.

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RESUMO

O gênero *Aloysia* reúne aproximadamente 30 espécies, porém sua circunscrição tem sido motivo de controvérsia. São poucos os estudos citogenéticos para o gênero, relatando apenas o número e o comportamento cromossômico. O presente trabalho teve como objetivo, identificar os caracteres citogenéticos de *Aloysia virgata* da flora brasileira que possam ser utilizados para um melhor entendimento e caracterização dos aspectos citogenéticos da família Verbenaceae, que possam contribuir para a organização taxonômica do grupo. *Aloysia virgata* apresentou cariótipo com 2n = 36 pequenos cromossomos, todos com o mesmo tamanho. Grande quantidade de heterocromatina foi observada com Giemsa e confirmada pela técnica de banda C. Foram detectadas quatro regiões organizadoras de nucléolo (NORs) e dois sítios de rDNA 5S pela técnica de hibridação *in situ* fluorescente (FISH). O núcleo interfásico foi classificado como semi-reticulado. Foi realizada a caracterização meiótica, na qual os cromossomos apresentaram comportamento normal, com a presença de 18 bivalentes na prófase I e na metáfase I. O número de cromossomos, de NORs e de segmentos de DNAr 5S não excluem uma possível origem poliplóide.

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