Variation in Chromosome Numbers, CMA Bands and 45S rDNA Sites in Species of Selaginella (Pteridophyta)

ADRIANA BUARQUE MARCON, IVA CARNEIRO LEÃO BARROS and MARCELO GUERRA*

Universidade Federal de Pernambuco, Centro de Ciências Biológicas, Departamento de Botânica,
Rua Nelson Chaves s/n, Cidade Universitária, 50.670-420, Recife, Pernambuco, Brasil

Received: 16 January 2004  Returned for revision: 1 May 2004  Accepted: 15 August 2004  Published electronically: 26 November 2004

INTRODUCTION

Selaginella P. Beauv. is a heterosporous genus of the family Selaginellaceae, comprising approx. 750 species distributed throughout the tropical regions, including 250 species in the Americas (Tryon and Tryon, 1982; Kramer and Green, 1990). They are generally found in humid and shady forests, although they can also be present in dry forests, swamps, on damp rocks along riverbanks or near waterfalls, and even in cold regions such as the Alps, or in rocky deserts (Tryon and Tryon, 1982).

Polyplody and hybridization have occurred numerous times in the evolution of the pteridophytes, resulting in the characteristic high chromosome numbers seen in this group (Walker, 1984). Nonetheless, high chromosome numbers are rare among the heterosporous pteridophytes. Selaginella, the largest heterosporous genus, has only a few polyploid species, although a large dysploid variation has been reported. The chromosome number ranges between 2n = 14 and 2n = 60, and different authors have reported different basic chromosome numbers for the genus: x = 7, 8, 9, 10, 11 and 12 (Kuriachan, 1963; Jermy et al., 1967; Takamiya, 1993).

In spite of several cytotaxonomical studies, the karyological evolution of this group remains poorly understood. Cytological investigations have been based primarily on chromosome counts and, in a few cases, on chromosome morphology. Meanwhile, in angiosperms and gymnosperms the karyological studies in the last two decades have turned to more specific methods such as banding methods and fluorescent in situ hybridization (FISH) (Greilhuber, 1995; Stace and Bailey, 1999). Heterochromatic bands in plants have been analysed mainly by C-banding or by base-specific fluorochrome staining (Guerra, 2000). Fluorochrome banding has the advantage of being a simpler, more reproducible and less destructive method, as compared with C-banding (Guerra, 1993). The fluorochromes chromomycin A (CMA) and 4’6-diamidino-2-phenylindole (DAPI) exhibit preferential staining for GC and AT-rich DNA sequences, respectively, allowing the identification of different types of heterochromatin. Distamycin A (DA) has also been used to enhance the contrast between CMA and DAPI (Schweizer and Ambros, 1994; Marcon et al., 2003). The 45S rDNA sites are generally positively stained with CMA and negatively stained with DAPI. In many species, the rDNA sites are the only positively stained CMA bands (see, for example, Melo and Guerra, 2003). The variation in number and position of the rDNA sites has been

* For correspondence. E-mail mguerra@ufpe.br
demonstrated to be an additional important karyotype feature to the cytotoxic analyses of some angiosperm genera, such as *Clivia* (Ran et al., 1999) and *Sanguisorba* (Mishima et al., 2002). Silver nitrate staining is another method that allows the visualization of nucleolus organizer regions (NORs), which are also related to chromosomal secondary constrictions and rDNA sites, but after the silver nitrate staining only those sites activated in an interphase are stained in the subsequent prophase or metaphase (Moscone et al., 1995). In general, the maximum number of silver-stained sites in prophase or metaphase chromosomes and the maximum number of nucleoli in interphase nuclei correspond to the number of 45S rDNA sites (see, for example, Moscone et al., 1995). This relationship seems to be true also for homosporous pteridophytes (Kawakami et al., 1999; Marcon et al., 2003).

In order to better understand the cytogenetics and evolution of this genus, the chromosome numbers were analysed in seven Brazilian species of *Selaginella* and in four of them the distribution of 45S rDNA sites was observed. Additionally, the heterochromatic blocks stained with CMA/DAPI and the number of nucleoli per nucleus were investigated, as an indication of the number of NORs. The results are discussed in relation to the chromosomal evolution of the genus.

### MATERIALS AND METHODS

The species investigated, with their respective collection sites, chromosome numbers and previous chromosome counts are listed in Table 1. Part of the material collected was prepared for the herbarium, and the vouchers were deposited in the UFP herbarium (Federal University of Pernambuco, Brazil). Another part of the material was maintained at the experimental garden of the Department of Botany, at the Federal University of Pernambuco, for cytogenetic analysis.

Leaf buds were collected and pretreated in 0.002 M 8-hydroxyquinoline for 1 h at room temperature, followed by 23 h at 10 °C. Subsequently, they were fixed in Carnoy (ethanol : acetic, acid 3 : 1) for 24 h at room temperature, and stored at −20 °C.

For conventional chromosome staining, the fixed leaf buds were washed twice in distilled water for 5 min, hydrolysed in 5 N HCl for 30 min, and then the meristem was isolated and squashed in 45 % acetic acid (Guerra, 1983). The preparation was stained with Giemsa 5 % and mounted with Entellan. Chromosome measurements were performed on amplified photographs with the aid of a pachymeter.

The triple staining with the fluorochromes CMA, DAPI and DA was performed according to Schweizer and Ambros (1994). Leaf buds were washed twice in distilled water for 5 min, digested in 2 % cellulase/20 % pectinase for 2 h at 37 °C, and squashed in 45 % acetic acid. After coverslip removal the slides were aged for 3 d at room temperature, stained with CMA (0.5 mg mL⁻¹, 1 h), counterstained with distamycin A (0.1 mg mL⁻¹, 30 min), stained with DAPI (2 µg mL⁻¹, 30 min) and mounted in Mcllvaine’s buffer–glycerol (v/v, 1:1) containing 2.5 mM MgCl₂. CMA/DAPI staining without distamycin A produced a poorer differentiation.

Leaf buds without pretreatment were fixed directly into Carnoy and used for silver nitrate staining. Slides were prepared using the same enzyme treatment employed for fluorochromes, except that they were not aged before staining. A small drop of 50 % silver nitrate diluted in formic acid was placed over the cells, covered with a coverslip and incubated in a moist chamber at 60 °C (Rufas et al., 1987) for approx. 10 min or until adequate staining was attained.

To locate the 45S rDNA sites, probes SK18S and SK25S were used, containing 18S and 25S rDNA of *Arabidopsis thaliana* (Unfried et al., 1989; Unfried and Gruendler, 1990), kindly provided by Prof. D. Schweizer of the University of Vienna, and labelled by nick translation with biotin-11-dUTP (Sigma) or digoxigenin-11-dUTP (Roche). The probe was detected with mouse anti-biotin monoclonal antibody (Dakopatts no. M743) and visualized with rabbit anti-mouse antibody conjugated to tetramethyl rhodamine isothiocyanate (TRITC) (Dakopatts no. R270) or detected

---

**Table 1. Species of Selaginella analysed, with their respective voucher number, provenance, chromosome number and previous counting**

<table>
<thead>
<tr>
<th>Species</th>
<th>Voucher number</th>
<th>Provenance</th>
<th>Chromosome number (2n)</th>
<th>Previous countings (2n)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. muscosa</em> Spring</td>
<td>36588</td>
<td>Bonito</td>
<td>18</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. simplex</em> Baker</td>
<td>36412</td>
<td>Gravatá</td>
<td>18</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. wilsonii</em> (Desv. ex Poiret) Baker</td>
<td>36589</td>
<td>Recife</td>
<td>18</td>
<td>18</td>
<td>Jermy et al. (1967); Fabri (1963); Kuriachan (1963)</td>
</tr>
<tr>
<td><em>S. producta</em> Baker</td>
<td>–</td>
<td>Itamaracá</td>
<td>18</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. plana</em> (Desv. ex Poiret) Hieron</td>
<td>36410</td>
<td>Paulista</td>
<td>20</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>36411</td>
<td>Igarassá</td>
<td>20</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>36591</td>
<td>Bonito</td>
<td>20</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. plana</em> (Desv. ex Poiret)</td>
<td>36409</td>
<td>Recife</td>
<td>20</td>
<td>20</td>
<td>Jermy et al. (1967); Fabri (1963); Kuriachan (1963)</td>
</tr>
<tr>
<td><em>S. convoluta</em> (Arnott) Spring</td>
<td>36590</td>
<td>Rio de Janeiro</td>
<td>20</td>
<td>20</td>
<td>Jermy et al. (1967); Fabri (1963); Kuriachan (1963)</td>
</tr>
<tr>
<td></td>
<td>36408</td>
<td>Petrolina</td>
<td>24</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

All the samples were collected in the Brazilian state of Pernambuco, except *S. plana*, which is from the state of Rio de Janeiro.
with sheep anti-digoxigenin antibody conjugated to fluorescein isothiocyanate (FITC) (Boehringer Mannheim no.1207741) and FITC-conjugated rabbit anti-sheep (Dakopatts F135, DAKO). The technique was based on Moscone et al. (1996), with some modifications (denaturation at 80 °C and the post-hybridization washes in 0.1× SSC at 42 °C). The hybridization mixture contained 60 % formamide, 5 % dextran sulfate, 2× SSC, 0.01 % salmon sperm DNA, and probe at a final concentration of 1–3 ng μL⁻¹. In most hybridization experiments, a 5S rDNA probe obtained from total genomic DNA of Passiflora edulis Sims (Melo and Guerra, 2003) was added to the mixture but no clear signal was observed. Slides were stained with DAPI (2 μg mL⁻¹) and mounted in Vectashield (Vector).

The best cells were captured with a CCD Cohu camera on a Leica DMLB microscope, or photographed using Kodak Imagelink HQ ASA 25 film for bright field, or Kodak ASA 400 film for fluorescence photography.

RESULTS

The seven species of Selaginella analysed in the present work showed the following chromosome numbers: 2n = 18 [S. muscosa Spring, S. simplex Baker, S. willdenowii (Desv. ex Poiret) Baker and Selaginella sp.], 2n = 20 [S. producta Baker and S. plana (Desv. ex Poiret) Hieron.] and 2n = 24 [(S. convoluta (Arnott) Spring] (Fig. 1A–G).

Among the species with 2n = 18, S. willdenowii and Selaginella sp. exhibited the largest chromosomes, most of them meta- to submetacentric. The chromosomes in S. willdenowii varied between 1.44 and 2.48 μm (Fig. 1A), and showed up to six satellites: two on the short arms and four on the long arms of submetacentric chromosomes. In Selaginella sp. the chromosome size varied between 1.35 and 2.32 μm and three satellites were observed: two on a metacentric pair, and a third on a submetacentric chromosome (Fig. 1B). On the other hand, S. simplex and S. muscosa had much smaller chromosomes, varying, respectively, between 0.85 and 1.26 μm and 0.80 and 1.62 μm (Fig. 1C and D).

The species with 2n = 20 had smaller chromosomes, ranging from 0.70 to 1.04 μm in S. plana, and 0.78 to 1.43 μm in S. producta (Fig. 1E and F). The position of the centromere could not be observed in any of these four species, mainly due to the small size of their chromosomes. Satellites were also not clearly identified.

In the only species with 2n = 24, S. convoluta, the morphology of the chromosomes seemed to vary from

---

**Fig. 1.** Chromosome complement (A–G), interphase nuclei (H and I) and number of NORs and nucleoli (J–L) observed in species of Selaginella: (A) S. willdenowii (2n = 18) with two satellites (arrows); (B) Selaginella sp. (2n = 18) with three satellites (arrows); (C) S. simplex (2n = 18); (D) S. muscosa (2n = 18); (E) S. plana (2n = 20); (F) S. producta (2n = 20); (G) S. convoluta (2n = 24); (H and I) chromocentric interphase nuclei of S. plana and S. willdenowii, respectively; (J) prometaphase cell showing NORs (arrows); (K and L) prometaphase cells showing chromosomes associated with nucleoli (arrows) in S. plana (K) and S. convoluta (L). The bar in A = 5 μm.
with nucleoli were observed (Fig. 1K and L).

In all species analysed, the interphase nucleus structure was granulated, with small chromocentres, corresponding to the chromocentre type, according to the classification of Tanaka (1971). Some species showed larger and more sharply outlined chromocentres (Fig. 1H and I).

In situ hybridization with silver nitrate revealed that the maximum number of nucleoli per nucleus varied among species (Table 2). In S. producta the maximum number of nucleoli per nucleus was two, whereas in the other three species investigated, the maximum number ranged between six and ten, although most nuclei exhibited only three nucleoli.

Selaginella willdenowii exhibited a variation of one to ten nucleoli per interphase nucleus and up to ten NORs were observed in prometaphase (Fig. 1J). In S. convoluta and S. plana, it was not possible to identify NORs, but six to ten prophase or prometaphase chromosomes associated with nucleoli were observed (Fig. 1K and L).

A high number of metaphase cells was obtained from S. willdenowii, S. plana, S. producta and S. convoluta making it possible to analyse the chromosomes with fluorochromes and in situ hybridization. Staining with CMA++DAPI revealed differentiation of bands only in S. willdenowii and S. convoluta. The chromosomes of the other species stained deeply and homogeneously with both CMA and DAPI, even when counterstained with distamycin A. In prometaphase cells of S. willdenowii, up to eight CMA+/DAPI− terminal bands were observed (Fig. 2A and B). In S. convoluta (2n = 24), six terminal CMA+/DAPI− bands were localized in three chromosome pairs (Fig. 2D and E). No DAPI+ bands were observed in these two species, while the CMA+ bands were often negatively stained with DAPI.

In situ hybridization with 45S rDNA revealed the presence of terminal sites on the long arms of two chromosome pairs and on the short arms of another two chromosome pairs (Fig. 2C). In two populations of S. plana analysed, five submetacentric pairs were labelled terminally: three pairs on the long arms and two pairs on the short arms (Fig. 2H and I). While in one of the populations it was not possible to observe clearly the smaller site, for this stains weakly, in the another population analysed the ten sites of 45S rDNA were clearly labelled (Fig. 2I). In S. convoluta there were four sites on two apparently acrocentric pairs and two sites on a submetacentric pair (Fig. 2F). In S. producta (Fig. 2G and J) only one chromosome pair was labelled, at the terminal position, in the two populations. These two signals were always remarkably distinct in interphase nuclei (Fig. 2K), whereas up to ten signals were observed in the interphase nuclei of S. plana (Fig. 2L).

**DISCUSSION**

The chromosome numbers and the structure of interphase nuclei reported in the present work are compatible with the karyological data known for the genus *Selaginella* (Kuriachan, 1963; Jermy *et al.*, 1967; Ghatak, 1977; Takamiya, 1993). Of the seven species examined, only *S. plana* and *S. willdenowii* had been analysed previously. The chromosome numbers observed for these species agreed with the previous counts.

With regard to chromosome size and morphology, there is a strong trend to conserve the karyotype symmetry, with predominance of metacentric and submetacentric chromosomes. The species with 2n = 20 analysed in this sample were more stable in chromosome size than those with 2n = 18. Takamiya (1993) found similar results among species with x = 10 and x = 9. A higher number of meta- and submetacentric chromosomes was observed in the karyotype of all species analysed, and appears to be a common trend in this genus (Takamiya, 1993), and in other heterosporous pteridophytes (Kuriachan, 1979, 1994). On the other hand, acro- and telocentric chromosomes are more frequent among homosporous pteridophytes (Kawakami, 1982; Takamiya *et al.*, 1992; Marcon *et al.*, 2003).

In situ hybridization with 45S rDNA revealed the most prominent structural variation between species. One species with 2n = 20, *S. plana*, displayed ten sites of 45S rDNA whereas another one with the same chromosome number, *S. producta*, exhibited only two. On the other hand, *S. willdenowii* with 2n = 18, showed more rDNA sites (eight) than *S. convoluta* (six) with 2n = 24. In angiosperms a similar variation has been widely reported. In *Passiflora*, for instance, the number of 45S rDNA sites varied from one to three pairs among diploid species (Melo and Guerra, 2003). In *Paeonia* (Zhang and Sang, 1999), five diploid species (2n = 10) exhibited three to ten pairs of rDNA sites. Variation in the number of rDNA sites among angiosperm species of the same ploidy level has been attributed to chromosome rearrangements, transpositional events and gene silencing (Moscone *et al.*, 1999). Likewise, similar mechanisms may be acting in *Selaginella* species.

In *S. plana*, *S. convoluta* and *S. producta* there was a perfect correlation between the maximum number

<table>
<thead>
<tr>
<th>Species</th>
<th>2n</th>
<th>Number of rDNA sites</th>
<th>No. of cells analysed</th>
<th>Range</th>
<th>Most frequent numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. willdenowii</em></td>
<td>18</td>
<td>8</td>
<td>1015</td>
<td>1–10</td>
<td>3 (29.5%) and 4 (21.1%)</td>
</tr>
<tr>
<td><em>S. plana</em></td>
<td>20</td>
<td>10</td>
<td>800</td>
<td>1–10</td>
<td>2 (27.8%) and 3 (31.7%)</td>
</tr>
<tr>
<td><em>S. producta</em></td>
<td>20</td>
<td>2</td>
<td>308</td>
<td>1–2</td>
<td>1 (94.2%)</td>
</tr>
<tr>
<td><em>S. convoluta</em></td>
<td>24</td>
<td>6</td>
<td>528</td>
<td>1–6</td>
<td>2 (28.0%) and 3 (35.7%)</td>
</tr>
</tbody>
</table>
of nucleoli (10, 6 and 2, respectively) and the number of 45S rDNA sites. The wide variation in the number of nucleoli per nucleus observed in all species examined, with a higher frequency of nuclei with few nucleoli, is probably due to the fusion of nucleoli, as observed in angiosperms (Moscone et al., 1995).

Curiously, *S. willdenowii*, although having up to ten NORs, displayed only eight 45S rDNA sites and six
chromosomes with satellites. This apparent lack of correlation between NORs and rDNA sites may be due to a pair of rDNA sites very reduced in size, not detected by FISH. The number of satellites observed, on the other hand, is often lower than the number of rDNA sites (see, for example, Guerra et al., 1996), and in some cases they are not observed at all, as in S. convoluta, S. plana and S. producta.

The CMA/DAPI staining revealed only CMA*-DAPI bands in S. wildenowii and S. convoluta. The number of CMA* bands was positively correlated with the number of rDNA sites and the maximum number of nucleoli observed in these species. Similar results were found in the homosporous genus Acrostichum (Marcon et al., 2003), suggesting that these three features are also positively correlated in pteridophyte chromosomes. No previous report of CMA bands in pteridophytes was found in the literature.

Jermy et al. (1967) proposed that the primary basic number of Selaginella would be \( x = 10 \), characteristic of species growing in dense tropical and subtropical forests (see also Kuriachan, 1963). However, later karyological analyses (Takamiya, 1993), as well as morphological, anatomical and paleobotanical data (Mukhopadhyay, 1998) suggest that the basic number of the genus would be \( x = 9 \), generating successive dysploids with \( n = 10, 11 \) and 12 on one hand and \( n = 8 \) and 7 on the other. Takamiya (1993) proposed that dysploidy might have occurred repeatedly in different evolutionary lines within each subgenus. Indeed, the remarkable variation in chromosome number and in size and number of rDNA sites, seems to indicate that structural variation is rather common in the genus and that its karyological evolution may be far more complex, with each basic number arising two or more times.

ACKNOWLEDGEMENTS

The authors are very greatful to Dr Iván Valdespino from University of Panama, for species identification, and to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) for financial support.

LITERATURE CITED


