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# Karyotype characterization reveals active 45S rDNA sites located on chromosome termini in *Smilax rufescens* (Smilacaceae)

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**ABSTRACT.** The genus *Smilax* (Smilacaceae) includes species of medicinal interest; consequently, their identification is important for the control of raw material used in the manufacture of phytotherapeutic products. We investigated the karyotype of *Smilax rufescens* in order to look for patterns that would be useful for comparative studies of this genus. To accomplish this, we developed procedures to grow plants and optimize root pretreatment with mitotic fuse inhibitors to obtain metaphase spreads showing clear chromosome morphology. The karyotype, analyzed in Feulgen-stained preparations, was asymmetric, with N = 16 chromosomes gradually decreasing in size; the larger ones were subtelocentric and the smaller chromosomes were submetacentric or metacentric. Nearly terminal secondary constrictions were visualized on the short arm of chromosome pairs 7, 11, and 14, but they were clearly detected only in one of the homologues of each pair. The

 

nucleolus organizer regions (NORs) were mapped by silver staining and fluorescent *in situ* hybridization of 45S rDNA probes. Silver signals (Ag-NORs) colocalized with rDNA loci were detected at the termini of the short arm of 6 chromosomes. The secondary constriction heteromorphism observed in Feulgen-stained metaphases suggests that differential rRNA gene expression between homologous rDNA loci can occur, resulting in different degrees of chromatin decondensation. In addition, a heteromorphic chromosome pair was identified and was interpreted as being a sex chromosome pair in this dioecious species.

**Key words:** *Smilax*; Karyotype; NOR; Silver staining; FISH; 45S rDNA

## INTRODUCTION

The genus *Smilax* L. (Smilacaceae) has about 300 species distributed in the tropical regions of both hemispheres. This genus, which is now included in the family Smilacaceae, was often assigned to the family Liliaceae up to some decades ago, but for the past 20 to 30 years, botanists have accepted Smilacaceae as a distinct family belonging to the order Liliales (APG III, 2009). The genus *Smilax* comprises the largest number of species within the Smilacaceae family, of which 32 species occur in Brazil (Andreata, 1997, 2009).

The medicinal importance of some *Smilax* species (sarsaparillas) has been recognized in folk medicine and in several studies. Roots have been used for several purposes, for example, as antisyphilitic, antiinflammatory and antimicrobial remedies or as an antioxidant agent (Andreata, 1997; de Souza et al., 2004; Cox et al., 2005). Problems in the taxonomic identification of *Smilax* species have been reported, and the unequivocal characterization of sarsaparillas of medicinal interest is very important for the control of the raw material used in the manufacture of phytotherapeutic products. In studies of Brazilian *Smilax* species, Andreata (2009) has described morphological features for their identification with emphasis on leaf morphology. Reports on the morphoanatomy of vegetative organs and molecular phylogeny have also contributed to species knowledge (Fu et al., 2005; Martins and Appezzato-da-Glória, 2006).

Few studies have been published on *Smilax* cytogenetics, where most of them have reported chromosome numbers and some on the karyotype morphology. Chromosome numbers of N = 16 were recorded for most species, but N = 13 and N = 15 were also found (Speese, 1939; Mehra and Sachdeva, 1976; Vijayavalli and Mathew, 1989; Fu and Hong, 1990; Kong et al., 2007). A few polyploids (N = 32, 48, 64) have been found in Asian species (Vijayavalli and Mathew, 1989; Fu and Hong, 1990; Kong et al., 2007). The karyotypes of the species so far analyzed are asymmetric, with most chromosomes being subtelocentric or submetacentric, and all of them gradually decrease in size (Vijayavalli and Mathew, 1989; Fu and Hong, 1990; Kong et al., 2007). Secondary constrictions and satellites have been visualized only in the karyotypes described by Vijayavalli and Mathew (1989).

The comparison of plant karyotypes using conventional and cytomolecular techniques has contributed to taxonomy and has provided insights into genome organization and evolution in several plant genera. For example, a karyotype analysis of *Crotalaria* species (Oliveira and Aguiar-Perecin, 1999) contributed to the visualization of species characteristics within bo-

 

tanical sections. Moreover, the mapping of ribosomal DNA sites by *in situ* hybridization and heterochromatin staining with GC- and AT-specific fluorochromes discriminated the karyotypes of *Crotalaria* species (Mondin and Aguiar-Perecin, 2011; Morales et al., 2012).

Here, we investigated the karyotype characteristics of *Smilax rufescens* Grisebach, an important species of the genus that occurs in the northern, northeastern, southern, and southeastern regions of Brazil, mainly in the "Restinga" environment. It is characterized by coriaceous leaves with elliptic shape, apiculated apices, 3 main veins, and prickle-like structures at the leaf margin. This sarsaparilla species has been used as folk medicine against syphilis and rheumatism (Andreata, 1997). We report here the procedures used to germinate wild-collected seeds to obtain plants providing roots for cytogenetic research. Root pretreatments for the analysis of somatic chromosomes were evaluated and the karyotype was analyzed in Feulgen-stained metaphases. The nucleolus organizer regions (NORs) were mapped by silver staining (Ag-NOR technique) and fluorescent *in situ* hybridization (FISH) using 45S rDNA (18S-5.8S-26S rRNA genes) probes. We aimed not only to characterize the *S. rufescens* karyotype, but also to recognize patterns that could be useful for further comparative karyotype analyses in *Smilax* species.

## MATERIAL AND METHODS

Seeds from *S. rufescens* plants collected in sandy soil of "Restinga" in Ilha do Cardoso (São Paulo State, Brazil) were used. The plants were identified by Dr. R.H.P. Andreata (Universidade Santa Ursula, Brazil), and specimens were incorporated into the ESA herbarium (ESALQ, USP) under No. 107648.

A germination experiment using 100 seeds for obtaining plant materials was carried out in plastic boxes containing wet *Sphagnum* moss, at 27°C. The seedlings were transferred to plastic pots with vegetable soil + vermiculite and maintained under screenhouse conditions, with temperature varying from 20° to 32°C. Slide preparation was carried out according to Bertão and Aguiar-Perecin (2002), with modifications. Roots excised from young plants (Figure 1) were pretreated with 8-hydroxyquinoline combined with cycloheximide, a protein synthesis inhibitor that induces chromosome condensation. Two treatments were evaluated: combinations of 300 mg/L 8-hydroxyquinoline with 20 mg/L or 1.25 mg/L cycloheximide at 28°C for 2.5 and 4 h, respectively. The roots were then fixed in 3:1 ethanol:acetic acid and kept at 4°C. For karyotype analysis, chromosomes of 10 Feulgen-stained metaphase spreads were measured. Chromosomes were identified according to their relative length (percent of haploid length) and arm ratio; chromosome types were designated according to Levan et al. (1964).

Active NORs were detected in metaphase chromosomes employing the silver-staining technique according to Stack et al. (1991), with minor modifications. Squash preparations were made using roots fixed in 3:1 ethanol:acetic acid for 24 h. The slides were then heated for 2 h at 60°C, incubated for 8 min in 2X SSC at the same temperature, washed with distilled water (3 x 5 min) and air-dried. Next, 50 µL 100% silver nitrate solution was added to the preparation that was then covered with a nylon coverslip and incubated at 60°C for 10 min on a Petri dish with moist filter paper. Coverslips were removed in tap water, and the slides were washed in distilled water, air-dried, and mounted in Entellan (Merck, Germany).

 

*In situ* hybridization was carried out as previously described (Mondin et al., 2007). Briefly, the probe used for 45S rDNA localization was the 9.1-kb maize 45S rDNA repeating unit labeled with biotin-14-dATP by nick translation (Bionick Labeling System, Invitrogen, USA). Biotin was detected with mouse anti-biotin followed by rabbit anti-mouse and swine anti-rabbit both conjugated with TRITC (DAKO, Denmark). The probe (5 ng/µL) was added to the hybridization mixture and denatured by heating at 95°C for 10 min, cooled and dropped onto the slide preparation that was denatured in a thermocycler at 92°C for 10 min. Hybridization was then carried out at 37°C for 20 h. Post-hybridization steps followed the protocol previously described by Mondin et al. (2007). The slides were counterstained with 1 µg/mL DAPI in Vectashield (Vector, USA).

All preparations were examined with a Zeiss Axiophot-2epifluorescence microscope with appropriate filters, and the images were acquired by a CCD camera using the Ikaros software to analyze the Feulgen-stained metaphases, and the ISIS software for FISH images (Meta-Systems, Germany). Silver-stained chromosomes were photographed using Fujicolor Superia 100 film (Fuji Photo Film, Brazil). All images were processed with Adobe Photoshop 6.0.

## RESULTS AND DISCUSSION

In the germination experiment using wild-collected seeds, 71% germinated after 40-50 days, and the maintenance of growing plants (Figure 1) under the conditions described above was quite successful for obtaining roots with high mitotic index required for karyotype analysis.


**Figure 1.** *Smilax rufescens*. **A.** Plant grown in plastic pot to provide roots for karyotype analysis; bar = 5 cm. **B.** Detail of a branch with leaves; bar = 1 cm.

The pretreatments evaluated resulted in adequate metaphase spreads for chromosome counting (Figure 2A and B), but they induced different degrees of chromosome condensation. The treatment with 300 mg/L 8-hydroxyquinoline and 1.25 mg/L cycloheximide for 4 h was most efficient in accumulating metaphases showing clear chromosome morphology (Figure 2A). The karyotype observed was asymmetric with N = 16 chromosomes gradually decreasing in size as shown in the karyograms displayed in Figure 2C and D. The chromosome sizes ranged from 5.62 to 1.84 µm, and the total length of the haploid set was 54.24 µm. The largest chromosomes were subtelocentric and the medium-sized and smaller ones were submetacentric or metacentric (Table 1). All cells examined showed a heteromorphic chromosome pair, classified as number 10, in which the homologues were almost the same size but differed in centromere position.

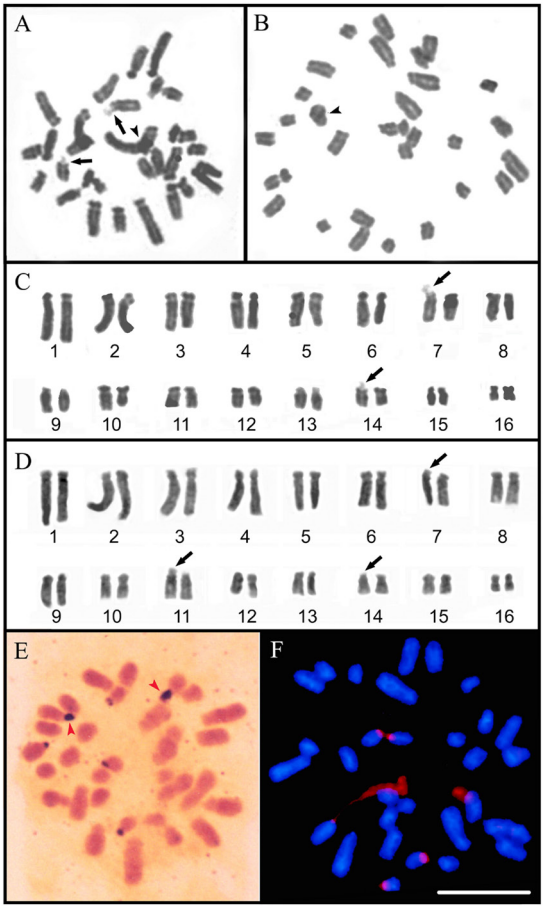


**Figure 2. A. B.** Feulgen-stained mitotic metaphase chromosomes (2n = 32) after different pretreatments: combination of 300 mg/L 8-hydroxyquinoline with 1.25 mg/L cycloheximide for 4 h and 20 mg/L cycloheximide for 2.5 h. **C. D.** Karyograms showing secondary constrictions on 2 and 3 chromosome pairs. **E.** Silver-stained metaphase with 6 positive signals; note 2 larger ones on non-homologous chromosomes (red arrowheads). **F.** FISH signals of 45S rDNA (red). Arrows indicate secondary constrictions in **A**, **C** and **D**. Arrowheads point to overlapped chromosomes in **A** and **B**. Note the heteromorphic pair 10 in **C** and **D**. Bar = 10 µm.

**Table 1.** Karyotype morphometric analysis of *Smilax rufescens*.

| | General karyotype features | | |
|---|---|---|---|
| | Range (μm) 5.62-1.84 | Haploid set (μm) 54.24 | |
| | Chromosome features | | |
| | RL | AR | Type |
| 1 | 10.37 | 5.28 | st |
| 2 | 9.64 | 4.13 | st |
| 3 | 8.70 | 4.46 | st |
| 4 | 8.23 | 3.41 | st |
| 5 | 7.55 | 3.28 | st |
| 6 | 7.08 | 3.00 | st |
| 7 | 6.36 | 3.30 | st |
| 8 | 6.03 | 2.56 | sm |
| 9 | 5.82 | 2.82 | sm |
| 10a* | 5.06 | 2.17 | sm |
| 10b* | 4.06 | 1.67 | sm |
| 11 | 4.75 | 2.15 | sm |
| 12 | 4.57 | 1.99 | sm |
| 13 | 4.35 | 1.92 | sm |
| 14 | 4.09 | 1.84 | sm |
| 15 | 3.87 | 1.53 | m |
| 16 | 3.41 | 1.24 | m |

RL = relative length; AR = arm ratio; st = subtelocentric; sm = submetacentric; m = metacentric. *Heteromorphic pair 10.

Nearly terminal secondary constrictions were detected on 3 chromosome pairs in some metaphase cells. It remained unclear whether small satellites were visible. In the karyogram shown in Figure 2C (derived from Figure 2A), secondary constrictions can be seen on the short arm of chromosomes 7 and 14, and in Figure 2D, they are on the short arm of chromosomes 7, 11, and 14. However, in both karyotypes, they are evident only in 1 homologue of each chromosome pair. This apparent variability in the number of secondary constrictions may be due to their terminal location, which makes their visualization difficult, depending on the degree of chromosome condensation. In fact, in preparations treated with a higher concentration of cycloheximide, secondary constrictions were not detected (Figure 2B). It is interesting to note that Vijayavalli and Mathew (1989) reported the presence of one satellite-bearing chromosome pair (SAT pair) in 4 species, except for *S. aspera* in which 2 SAT pairs were recognized, with one of them showing size heteromorphism. In 2 species, the chromosome pair 7 was also identified as an SAT pair.

In the present study, silver staining revealed positive signals at the termini of the short arm of 6 chromosomes, as can be seen in Figure 2E, in which larger signals were observed on 2 non-homologous chromosomes.

*In situ* hybridization detected 45S rDNA sites also on 6 chromosomes. Figure 2F shows the major loci at the termini of a medium-sized chromosome pair, with one of the homologues displaying a highly stretched NOR; minor sites can be visualized on 2 smaller chromosome pairs.

Secondary constrictions have been correlated with NORs, in which rRNA genes occur in tandem arrays that can be localized by *in situ* hybridization using 45 rDNA probes. The detection of active ribosomal genes in metaphase chromosomes by the Ag-NOR technique allows the visualization of the fraction of the NOR chromatin (rRNA genes) that was transcribed in the previous interphase, for it has been shown that silver binds to proteins that are components of the transcription machinery and remains at the NORs throughout metaphase and anaphase (see review by Pikaard, 1999; Caperta et al., 2002).

Therefore, in the present study, silver staining revealed active NORs located at sites of both homologues of 3 chromosome pairs corresponding to the 6 45S rDNA loci detected by FISH. Thus, this means that all the 45S rDNA loci are active and due to their location on chromosome termini, they form secondary constrictions that could be difficult to visualize in Feulgen preparations. On the other hand, as mentioned above, the visualization of the secondary constriction on the small chromosomes (pairs 11 and 14) was variable, according to the degree of chromosome condensation. It is also interesting to note that the secondary constriction heteromorphism between homologues observed in Figure 2D can result from differential expression of their rDNA loci. In the FISH preparation displayed in Figure 2F, the NOR located on the medium-sized chromosome pair was highly stretched in one of the homologues, and this suggests that this site is more active than in its homologous, thus forming the conspicuous secondary constriction visualized on chromosome 7 in Feulgen preparations. Additionally, the presence of non-homologous chromosomes displaying larger silver signals (Figure 2E) also suggests differential rRNA gene expression. Several studies of homologous NORs in plants have shown that usually they show identical behavior, and a direct correlation between the number of ribosomal cistrons in each NOR and the level of transcription has been demonstrated (see review in Carpeta et al., 2002). Interestingly, in *Secale cereale*, a species with only one pair of homologous NORs, a size heteromorphism between homologous rDNA loci with equivalent number of rRNA genes was revealed by silver staining of metaphase chromosomes, thus providing evidence of differential expression at these sites (Carpeta et al., 2002). It has been demonstrated in plant species that cytologically, NORs include regions that are highly condensed and regions that are decondensed, with the latter corresponding to regions at which associated proteins stain with silver and where active rRNA transcription occurs. In *S. cereale*, these domains are very clear, and it has been demonstrated that rRNA gene transcription, silver staining and NOR chromatin decondensation are interrelated (Caperta et al., 2007). Therefore, the findings reported in the present research should be further investigated in studies addressing NOR behavior in *Smilax* species.

Also, the presence of the heteromorphic pair 10 is an interesting finding, considering that one of the characteristics of *Smilax* species is the occurrence of unisexual flowers (Hutchinson, 1973). The heteromorphic SAT pair reported by Vijayavalli and Mathew (1989) was observed in male plants, and, based on this, the authors assumed an XY (male)-XX (female) type of sex chromosome complex for *S. aspera*. In the present investigation, the homologues of the heteromorphic pair differed in their centromere positions and were not NOR-bearing chromosomes. As we used young plants, we had no information on their flower morphology, so we can only speculate that this heteromorphic pair is a sex chromosome pair.

The results presented contribute to the optimization of methods aimed at the analysis of the karyotype of *S. rufescens*, and provide additional evidence that karyotype asymmetry is a characteristic of *Smilax* species. Our data on chromosome sizes are quite similar to those described for the species analyzed by Vijayavalli and Mathew (1989). In addition, the present study allowed the visualization of nearly terminal secondary constrictions on 3 chromosome pairs corresponding to silver-staining signals and to the 45S rDNA sites revealed by FISH. The finding of secondary constrictions clearly detected only in one of the homologues of each chromosome pair suggests differential rRNA gene expression, resulting in different degrees of chromatin decondensation. A heteromorphic chromosome pair was identified and could be interpreted as being a sex chromosome pair, but this requires further studies involving the gender identification of the plants studied. All these observations represent important patterns for further cytogenetic comparative studies of *Smilax* species.

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