SCIENTIA
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Chromosome banding and DNA content in tropical *Pinus* species

Bandeamento cromossômico e conteúdo de DNA em espécies tropicais de *Pinus*

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Abstract

The species *Pinus tecunumanii* Eguiluz & J. P. Perry has been the reason for a taxonomic controversy for more than 50 years. Some authors considered *Pinus tecunumanii* as a subspecies of *Pinus patula*, whereas others stated it is a distinct species closer to *Pinus oocarpa*. In the present work, the tropical species *Pinus oocarpa* Schiede ex Schltdl., *Pinus patula* Schltdl. & Cham, and *Pinus tecunumanii* Eguiluz & J.P. Perry were evaluated as to chromosome banding pattern using CMA_3 fluorochrome, to identify chromosome polymorphisms, and as to nuclear DNA content using flow cytometry, to contribute to the differentiation among the three taxa. Analysis of variance and Tukey´s test were used to verify the existence of a significative difference for nuclear DNA content. The obtained CMA_3 banding pattern evidenced that secondary constrictions are GC-rich regions and that *Pinus tecunumanii* is closer to *Pinus oocarpa* than to *Pinus patula*. Content of nuclear DNA means were significantly different, with the major mean being observed in *Pinus patula* (43.36 pg), and the minor one in *Pinus tecunumanii*, provenance Mountain Pine Ridge (40.48 pg). The intraspecific DNA content variation observed among four different provenances of *Pinus tecunumanii* was not correlated to their latitudinal origin. The DNA content variation did not allow distinction between *Pinus tecunumanii* and the remaining two species.

Keywords: *Pinus tecunumanii*, CMA banding, Nuclear DNA content

Resumo

A espécie *Pinus tecunumanii* Eguiluz & J.P. Perry é motivo de uma controvérsia taxonômica há mais de 50 anos. Alguns trabalhos consideraram o *Pinus tecunumanii* uma subespécie de *Pinus patula*, enquanto outros, uma espécie distinta e mais próxima de *Pinus oocarpa*. No presente trabalho, foram avaliadas as espécies tropicais *Pinus oocarpa* Schiede ex Schltdl., *Pinus patula* Schltdl. & Cham e *Pinus tecunumanii* Eguiluz & J.P. Perry, com relação ao padrão de bandeamento cromossômico com fluorocromo CMA_3, a fim de identificar a existência de polimorfismos cromossômicos e com relação à quantidade de DNA nuclear, por meio da citometria de fluxo, de forma a contribuir para a diferenciação dos três taxa. Os valores de conteúdo de DNA foram submetidos à análise de variância e teste de Tukey para comparação das médias. O padrão de bandas CMA_3 evidenciou que as constrições secundárias são regiões ricas em bases GC e que o *Pinus tecunumanii* é mais próximo de *Pinus oocarpa* que de *Pinus patula*, no que diz respeito a essa característica. Houve diferença significativa entre os genótipos para conteúdo de DNA nuclear sendo que a maior média foi observada em *Pinus patula* com 43,36 pg, e a menor em *Pinus tecunumanii*, na procedência Mountain Pine Ridge com 40,48 pg. A variação intra-específica observada em quatro procedências do *Pinus tecunumanii* para conteúdo de DNA não apresentou correlação com a origem latitudinal das mesmas. A variação no conteúdo de DNA não possibilitou a distinção entre *Pinus tecunumanii* e as outras duas espécies.

Palavras-Chave: *Pinus tecunumanii*, Bandeamento CMA, Conteúdo de DNA nuclear

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INTRODUCTION

Species of the genus *Pinus* were introduced into Brazil over one century ago. According to Kronka *et al.* (2005), among the 16 *Pinus* species constituting the basis of Brazilian pine culture are the tropical species *Pinus oocarpa*, *Pinus patula*, *Pinus tecunumanii* and *Pinus caribaea*.

Since its discovery, at the end of the 1940s, the taxonomic position of *Pinus tecunumanii* Eguiluz & J.P. Perry has been controversial (SCHWERDTFERGER, 1953). This species, also known as Tecun Umán pine, has already been considered either as a subspecies of *Pinus patula* (STYLES, 1985; DAVIDE and ARAÚJO, 1993b; LEÃO and DAVIDE, 1993) or a species very close to *Pinus oocarpa* (PIEDRA and PERRY, 1983; FURMAN *et al.*, 1997; SILVA-MANN *et al.*, 1999). Such statements were based on studies evaluating wood and acicula morphological and anatomical traits, terpenes, chromosome features or RAPD molecular markers.

Cytogenetic studies involving *Pinus oocarpa*, *Pinus patula* and *Pinus tecunumanii* have demonstrated discrepancies in chromosome secondary constriction position and number, total haploid set length, and chromosome banding pattern (DAVIDE and ARAÚJO, 1993a; RIBEIRO, 2001; SILVA-MANN *et al.*, 2002). *Pinus* species present very similar karyotypes with large chromosomes. The karyotype of the analyzed species consists of ten or eleven long metacentric pairs and one or two shorter submetacentric pairs. Metacentric chromosomes have similar shape and size and can hardly be differentiated through conventional karyotype analysis (HIZUME *et al.*, 1983). In view of the great uniformity of *Pinus* karyotypes, the utilization of chromosome banding and in situ hybridization techniques are essential to yield more detailed information, allowing the identification of chromosomal variations and an improved taxon delimitation for several species.

Flow cytometry has also been an efficient strategy for the identification of intra and interspecific variations within *Pinus* genus (HALL *et al.*, 2000; BOGUNIC *et al.* 2003; BOGUNIC *et al.*, 2007), which contributes to better understand the DNA plasticity mechanisms, helping to elucidate the species evolution process.

The present study aimed at identifying polymorphisms in CMA_3 (chromomycin A_3) banding pattern and variations in nuclear DNA content among the tropical species *Pinus oocarpa*, *Pinus patula* and *Pinus tecunumanii* in order to contribute to their differentiation.

MATERIAL AND METHODS

Cytological preparations and samples for flow cytometry used seeds of the following genotypes - *Pinus oocarpa* Schiede ex Schltdl.: seeds obtained from populations growing in Agudos, São Paulo State, Brazil, by the company Aracruz; *Pinus patula* Schltdl. & Cham.: seeds obtained from populations established in Camanducaia, Minas Gerais State, Brazil, by the company Melhoramentos S/A; *Pinus tecunumanii* Eguiluz & J.P. Perry: seeds from four different provenances obtained by the company Aracruz; Las Camelias, San Rafael del Norte and Yucul from Nicaragua; Mountain Pine Ridge from Belize. All provenances are considered low altitude genotypes since they are found below 1.500 m.

The seeds were germinated at room temperature. Roots of approximately 0,5 cm were collected, pretreated with 0.1 % colchicine from 6 to 8 h at room temperature, fixed with methanol:acetic acid (3:1) and stored at - 20 °C until used. Slides were prepared through the squash method. Root tips were treated with the enzyme mixture 4 % cellulose plus, 40 % pectinase for 1 h and 30 min at 37 °C.

The adopted methodology for CMA_3 banding was described by Hizume *et al.* (1983), with some modifications. Slides were pre-incubated for 15 min in McIlvaine buffer solution at pH 7.0 and treated with 0.2 mg/mL distamycin for 10 min. After a fast wash with McIlvaine buffer, they were treated with chromomycin (CMA_3) 0.1 mg/mL for 1 h and 30 min, at room temperature, in the dark, in a humid chamber. Following staining, the preparation was washed with the same buffer and air-dried. Semi-permanent slides were mounted with a 1:1 solution (glycerol:McIlvaine buffer) containing 2 % Dabco. The material was observed under a Olympus BX-60 microscope equipped with fluorescence accessory, using wavelength between 430 and 480 nm.

The analysis in flow cytometer included three samples from each of the five trees of four *Pinus tecunumanii* provenances (Las Camélias, Mountain Pine Ridge, San Rafael del Norte, and Yucul). For *Pinus oocarpa*, three samples from five trees were also used; for *Pinus patula*, only three samples from a seed pool were evaluated. To assess DNA content, approximately 20-30 mg embryos for each sample were used together with the same quantity of young leaf tissue from Pisum sativum (internal reference standard).

Samples from the species were ground in Petri dishes containing 1mL cold LB01 buffer to obtain a nuclear suspension (DOLEZEL, 1997). The ground tissue was aspirated and the nuclear suspension filtered in a 50 µm mesh. Then, the nuclear suspension received 25 µL propidium iodide and 2.5 µL RNase. For each sample, at least ten thousand nuclei were analyzed. Analysis was carried out in Facscalibur cytometer (Becton Dickinson); histograms were obtained in Cell Quest software and analyzed through the WinMDI 2.8 software.

To verify whether DNA content varied among species, analysis of variance was performed. Means were compared using Tukey's test (α=0,05). Analyses were carried out in R software (R DEVELOPMENT CORE TEAM, 2007).

RESULTS AND DISCUSSION

All three evaluated species had twelve positive CMA_3 interstitial bands coincident with secondary constrictions (Figure 1), which demonstrated that such regions are rich in GC bases and probably contain the ribosomal RNA genes. *Pinus patula* had four positive CMA_3 centromeric bands not found in the other two species. Thus, *Pinus tecunumanii* showed to be closer to *Pinus oocarpa*, considering CMA_3 banding pattern. However, as previously reported by Silva-Mann *et al.* (2002), these two taxa are quite similar to *Pinus patula*.

The number of positive CMA_3 bands obtained in the present evaluation is the same as the one reported by Silva-Mann *et al.* (2002) for these three *Pinus* species. As regards secondary constriction distribution pattern, the relation between the secondary constriction number in the present study and that previously reported by Davide and Araújo (1993a) and Ribeiro (2001) was 12:8 for *Pinus oocarpa*, 12:14 and 12:12 for *Pinus patula* and *Pinus tecunumanii*, respectively. Several authors observed CMA_3 positive bands in secondary constrictions and centromeric regions of several *Pinus* species (HIZUME *et al.*, 1983; HIZUME *et al.*, 1989; HIZUME *et al.*, 1992; DOUDRICK *et al.*, 1995; DAVIES *et al.*, 1997), which was considered an important tool for intra and interspecific differentiation.

There was a great variability in DNA content among the evaluated genotypes (Table 1 and Figure 2). The DNA content values obtained in the present study agree with those reported by Hall *et al.* (2000) – 21.92 pg, 21.74 pg and 20.49 pg DNA in haploid cells of *P. patula*, *P. oocarpa* and *P. tecunumanii*, respectively, which correspond to approximately 43.84 pg, 43.48 pg and 40.98 pg of 2C value. Those authors stated that DNA content variation was lower among species belonging to the same subsection.

Table 1. DNA content means in picograms (pg) for *Pinus oocarpa*, *Pinus patula* and four provenances of *Pinus tecunumanii*.

Tabela 1. Médias da quantidade de DNA em picogramas (pg), das espécies *Pinus oocarpa*, *Pinus patula* e de quatro procedências de *Pinus tecunumanii*.

Species	Means (pg)
Pinus patula	43,36 a
Pinus tecunumanii - San Rafael del Norte	42,98 ab
Pinus oocarpa	42,71 bc
Pinus tecunumanii - Las Camélias	42,51 c
Pinus tecunumanii -Yucul	40,66 d
Pinus tecunumanii - Mountain Pine Ridge	40,48 d

Means followed by the same letter are not significantly different (P<0,05)

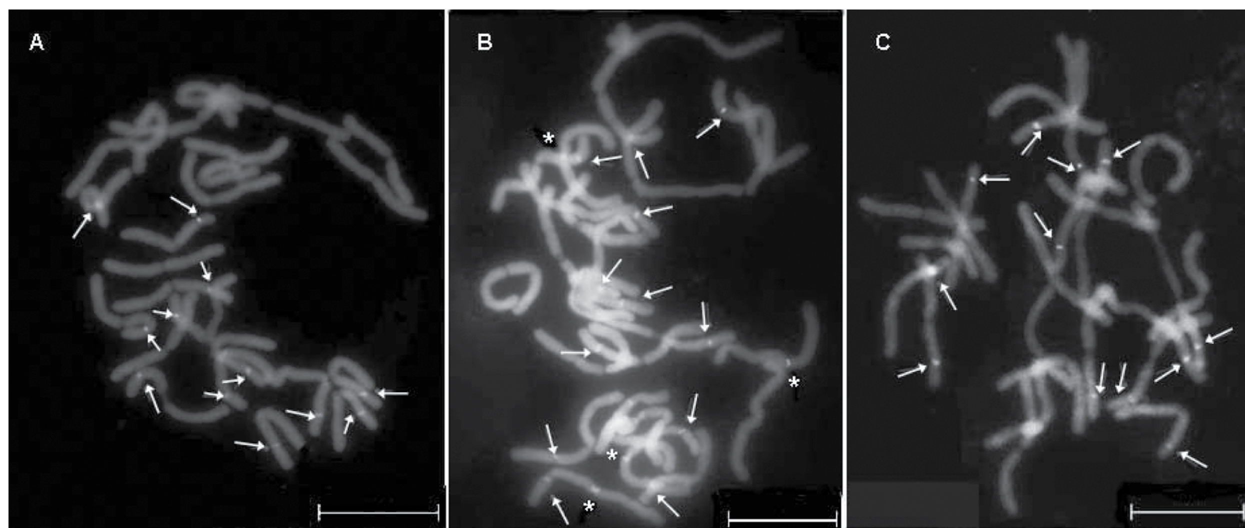

Figure 1. CMA_3 fluorescent banding in metaphases with 2n=24 chromosomes for *Pinus oocarpa* (A), *Pinus patula* (B) and *Pinus tecunumanii* (C). Arrows - interstitial bands. (*) - centromeric bands. Bar = 10 µm.

Figura 1. Bandeamento fluorescente CMA_3 em metáfases com 2n=24 cromossomos de *Pinus oocarpa* (A), *Pinus patula* (B) e *Pinus tecunumanii* (C). Setas indicam as bandas intersticiais. (*) - bandas centroméricas. Barra = 10 µm.

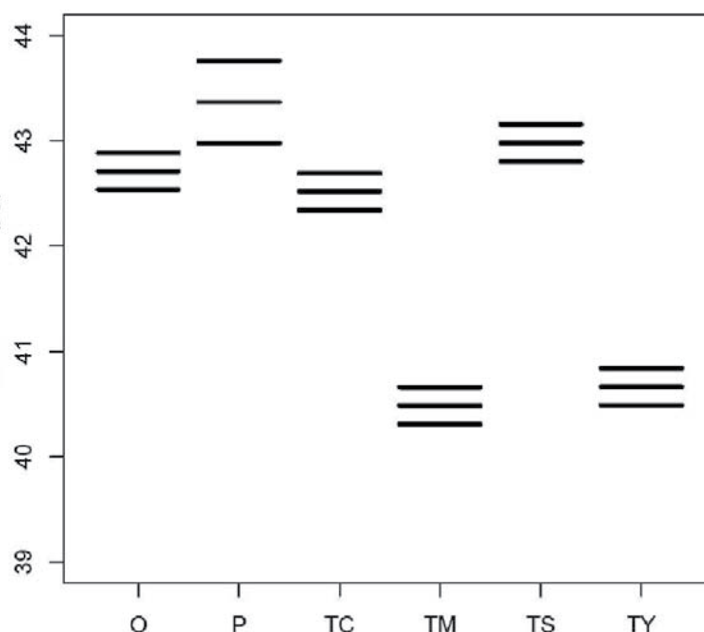

Figure 2. DNA content means in picograms (pg) and limits (upper and lower) for six *Pinus* genotypes: P – *Pinus patula*; O – *Pinus oocarpa*; TC – Las Camélias provenance of *Pinus tecunumanii*; TM – Moutain Pine Ridge provenance of *Pinus tecunumanii*; TS – San Rafael del Norte provenance of *Pinus tecunumanii*; and TY – Yucul provenance of *Pinus tecunumanii*.

Figura 2. Médias da quantidade de DNA em picogramas e os dois limites (superior e inferior) de seis genótipos de *Pinus*: P – *Pinus patula*; O – *Pinus oocarpa*; TC – procedência Las Camélias; TM – proc. Moutain Pine Ridge; TS – proc. San Rafael del Norte e TY – proc. Yucul de *Pinus tecunumanii*.

Mountain Pine Ridge and Yucul provenances of *Pinus tecunumanii* had the lowest mean DNA content values. *Pinus oocarpa* had 42.71 pg mean DNA content, which was statistically equal to that of Las Camélias provenance of *Pinus tecunumanii*. *Pinus patula* had the highest mean DNA content, 43.36 pg, which was statistically equal to that of San Rafael del Norte provenance of *Pinus tecunumanii*. The DNA content difference between Mountain Pine Ridge and San Rafael del Norte provenances was 2.5 pg. Hall *et al.* (2000) also found a great intraspecific difference, 2.18 pg, for *Pinus tecunumanii* and reported that provenances from primitively occupied regions had higher DNA content values. Differently, in the present study, there was no relation between the decrease in DNA content and the origin and latitude of provenances. Yucul, Las Camélias and San Rafael provenances had 40.66 pg, 42.51 pg and 42.98 pg DNA content, respectively, and they are from Nicaragua, a secondarily occupied region in Central America, which presents lower latitudes, between 12°55′N and 13°46′N. Mountain Pine Ridge provenance, which is from Belize, is a region of higher latitude when compared to the other three provenances, 16°58′N, had lower DNA content.

The variation in nuclear DNA content did not allow for a clear separation among the evaluated taxa. *Pinus tecunumanii*, represented by four provenances, grouped either with *Pinus patula* in the case of San Rafael del Norte provenance, presenting the highest DNA content means or with *Pinus oocarpa* in the case of Las Camélias provenance, presenting intermediate nuclear DNA values. Only Mountain Pine Ridge and Yucul provenances formed an exclusive *Pinus tecunumanii* group presenting the lowest DNA content values among the evaluated genotypes.

Bogunic *et al.* (2003) observed a significant intraspecific DNA content variation between *Pinus* nigra var. nigra and *Pinus* nigra var. dalmatica. They reported that intraspecific variation is common in species of wide distribution and high morphological differentiation, including several subspecies, like in the P. nigra complex. The existence of short repetitive sequences of low complexity, like minisatellites and telomeric sequences, may have contributed to such variation. These phenomena may also have acted in the variation observed in the evaluated taxa. The introgression, hybridization, and speciation in progress in Mexico, a primary center of species diversity, may also be the cause of intraspecific variation in the genome size (PERRY, 1991).

Localization of DNA sequences, such as ribossomal DNA (45S and 5S) and repetitive elements in *Pinus* chromosomes by Fluorescence *in situ* Hibridization (FISH) may be a valuable approach to verify either the hypothesis of genetic relationships among the tropical *Pinus* species or the hypothesis of repetitive sequences being responsible for differences in DNA content.

CONCLUSIONS

All three species had the same number of secondary constrictions. CMA_3 banding pattern indicated that secondary constrictions are GC-rich regions and that *Pinus tecunumanii* is closer to *Pinus oocarpa* than to *Pinus patula*.

There is intraspecific variation for DNA content in *Pinus tecunumanii*. Interspecific variation did not allow for a distinction among the three taxa.

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