 Cytologia **73**(3): 283–291, 2008

Differences and Resemblances in Banding Patterns and Ribosomal DNA Distribution in Four Species of Paullinieae Tribe (Sapindaceae)

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Received June 11, 2008; accepted September 12, 2008

Summary The tribe Paullinieae (Sapindaceae) is exclusively neotropically distributed, and is characterized by apomorphic characters and considered a monophyletic natural group. Recently explored cytogenetical aspects suggest that the disploid chromosomal reduction, the increase in the chromosomal size and the diversification of highly repetitive DNA sequences are associated with the karyotypic evolution of this tribe. This work compares patterns of chromosome banding and the distribution of ribosomal DNA 18S-5.8S-26S in *Cardiospermum grandiflorum* Sw., *Pullinia elegans* Cambess., *Urvillea chacoensis* Hunz. and *U. ulmacea* Kunth. The studied species share the presence of a pattern of terminal C-Giemsa bands, differentiated for characteristics of heterochromatic regions. Terminal AT-rich bands occurred in *C. grandiflorum* ($2n=2x=20$) and *U. chacoensis* ($2n=2x=22$). Differing from the others, *U. chacoensis* presented prominent bands in the majority of chromosomes. The polyploid cytotype of *U. ulmacea* ($2n=8x=88$) possessed terminal bands CMA^+ and $DAPI^+$, forming heterochromatic blocks constituted by GC- and AT-rich repetitive DNA. On the other hand, *P. elegans* ($2n=2x=24$) presented a pattern of neutral bands after staining with CMA_3/DAPI. The presence of GC-rich regions associated with 45S rDNA sites was a common characteristic in the studied species, nevertheless, variations in the NOR number might be useful for the differentiation of some species. Our results on karyological differences and resemblances of the studied species are discussed in relation to the systematics of the Paullinieae tribe.

Key words Banding patterns, Heterochromatin, Karyosystematics, Paullinieae, rDNA genes, Sapindaceae.

The tribe Paullinieae (Sapindaceae), with nearly 430 species, is exclusively neotropically distributed, with important distribution centres in the Southeast of Mexico, Amazonian, and Brazilian Central and Coast Plateau (Acevedo-Rodríguez 1993). In relation to other tribes of Sapindaceae, Paullinieae is characterized by a set of apomorphic characters and is considered a monophyletic natural group (Acevedo-Rodríguez 1993). The monophyletic nature of Paullinieae tribe is confirmed by means of molecular phylogentics studies, emphasizing the close systematic relationship with the tribe Thouinieae (Harrington *et al.* 2005). According to the classification proposed by Radlkofer (1931), the *Cardiospermum* L., *Houssayanthus* Hunz., *Lophostigma* Radlk., *Paullinia* L., *Serjania* Mill., *Thinouia* Triana & Planch and *Urvillea* Kunth genera are included in the Paullinieae tribe, however Acevedo-Rodríguez (1993) suggested the inclusion of *Thinouia* in the Cupanieae tribe.

Some cytogenetical studies showing numbers, morphology and chromosomal length permit

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discussion of the karyotipyc evolution in Paullinieae relative to other tribes of Sapindaceae (Ferrucci 2000, Urdampilleta *et al.* 2006, 2007). These studies suggested that the disploid chromosomal reduction and an increase in the chromosomal size would be processes that intervened in the karyotypic evolution of this tribe (Hemmer and Morawetz 1990, Lombello and Forni-Martins 1998). On the other hand, in Sapindaceae, studies involving meiotic analysis, C-, NOR-, DAPI/CMA_3-banding and *in situ* hybridization to physically locate repetitive DNA are limited. Previous results demonstrate a diversification in the quantity, type and location of heterochromatin segments in eight species of *Cardiopermum*, *Paullinia* and *Urvillea*, nevertheless, a similar pattern of terminal heterochromatin was observed in *C. grandiflorum* Sw. (Hemmer and Morawetz 1990), *U. chacoensis* Hunz., *U. ulmacea* Kunth (Urdampilleta *et al.* 2006) and *P. elegans* Cambess. (Urdampilleta *et al.* 2007).

In the present study, within the framework of chromosomal differentiation in Sapindaceae, we compare the karyotypes of four Paullinieae species with terminal heterochromatin, characterizing the differences and resemblances in the banding patterns. For this purpose *C. grandiflorum*, *P. elegans*, *U. chacoensis* and *U. ulmacea* were analyzed using fluorochrome banding with CMA_3/DAPI and fluorescence *in situ* hybridization by using ribosomal DNA as probes. The results obtained permitted continued discussion about chromosomal differentiation in Paullinieae.

Materials and methods

The species and populations studied (*C. grandiflorum*, *P. elegans*, *U. chacoensis* and *U. ulmacea*) are included in Table 1 and vouchers were deposited at FUEL (Herbarium of the Universidade Estadual de Londrina, PR, Brazil), UEC (Herbarium of the Universidade Estadual de Campinas, SP, Brazil) and CTES (Instituto de Botánica del Nordeste, Corrientes, Argentina).

Chromosome preparations and karyotype elaboration were made from root tips pretreated with 2 mM 8-hydroxyquinolein for 4–5 h at 15°C, fixed in 3 : 1 absolute ethanol and acetic acid for 24 h and stored at −20°C until use. Conventional analysis of chromosomes was performed using the HCl/Giemsa technique (Guerra 1983): root tips were hydrolyzed for 10 min in 1 N HCl at 60°C and stained in 2% Giemsa at room temperature for 5–10 min. Chromosome measurements were obtained from at least five metaphases to determine karyotype formula (KF), diploid set length (DSL) and karyotype symmetry index (TF%) (Huziwara 1962).

For Giemsa C-banding, a procedure slightly modified according to Schwarzacher *et al.* (1980) was used. Root tips were digested with an enzyme mixture of 4% cellulase and 40% pectinase at 37°C; the chromosome spread was made in a drop of 45% acetic acid and the coverslip was subsequently removed with liquid nitrogen. After three days, slides were treated with 45% acetic acid at 60°C for 10 min, 5% $Ba(OH)_2$ at 25°C for 10 min, and 2×SSC at 60°C for 1 h 20 min. Some slides were stained with 2% Giemsa and mounted with Entellan (Merck) and other slides were aged for three more days at room temperature and sequentially stained with 0.5 mg/ml CMA_3 for 1.5 h and 2 μg/ml DAPI for 30 min and mounted in a medium composed of glycerol/McIlvaine buffer pH 7.0, 1 : 1 (v/v), plus 2.5 mM $MgCl_2$. The cells were photographed with Imagelink HQ ASA 25 or T-max ASA 100, both from Kodak.

Fluorescence *in situ* hybridization (FISH) was carried out according to the methods of Heslop-Harrison *et al.* (1991) and Schwarzacher and Heslop-Harrison (2000) with minor modifications using p*Ta*71 probe containing the 18S-5.8S-26S rDNA (Gerlach and Bedbrook 1979). This probe was labelled with biotin-14-dUTP (BioNick, Invitrogen) by nick translation. Slides were incubated in 100 μg/ml RNase, post-fixed in 4% (w/v) paraformaldehyde, dehydrated in a 70–100% graded ethanol series and air-dried. In each slide 30 μl of hybridization mixture containing 100–200 ng of labelled probe, 50% formamide (15 μl), 50% polyethylene glycol (6 μl), 20×SSC (3 μl), 100 ng of calf thymus DNA (1 μl), and 10% SDS (1 μl) was applied. This hybridization mixture was previ-

Table 1. Karyotypic description, banding patterns and number of rDNA 45S sites in the all four studied species

Species	2*n*	Karyotype formulae	DSL (σ)	Size chromosome (μm)		TF (%)	Banding pattern			rDNA 45S	Locality
				Mean	Range		AT rich	GC rich	Neutral		
C. grandiflorum	20	6m+2sm+2st	36.6 (4.7)	1.8	2.3–1.3	36.2			++	2	Argentina. Misiones. Urdampilleta *et al*. 202, Posadas (FUEL). Brasil. Paraná. Urdampilleta *et al.* 213, Paiquerê (FUEL).
P. elegans	24	4m+4sm+4st	43.7 (4.4)	1.8	2.6–1.2	34.5			++	3	Argentina. Misiones. Urdampilleta *et al.* 147, Posadas (FUEL 34728). Brasil. Paraná. Urdampilleta *et al.* 131, 1° de Maio (FUEL 34715). São Paulo, Obando *et al.* 289, Campinas (UEC).
U. chacoensis	22	11m	35.7 (2.7)	1.6	2.1–1.2	41.7	+++			3	Bolivia: Dpto. Chuquisaca. Ferrucci *et al.* 1763, Prov. Calvo (CTES); Dpto. Santa Cruz. Ferrucci *et al.* 1912, Prov. Chiquitos (CTES).
U. ulmacea	88	15m+29sm	122.9 (3.2)	1.4	2.1–0.7	34.0	++	++		~12	Brazil: Paraná. Urdampilleta 192, Londrina (FUEL); Urdampilleta *et al.* 246, Paiquerê (FUEL).

DSL: diploid set length (μm). TF %: karyotype asymmetry index.

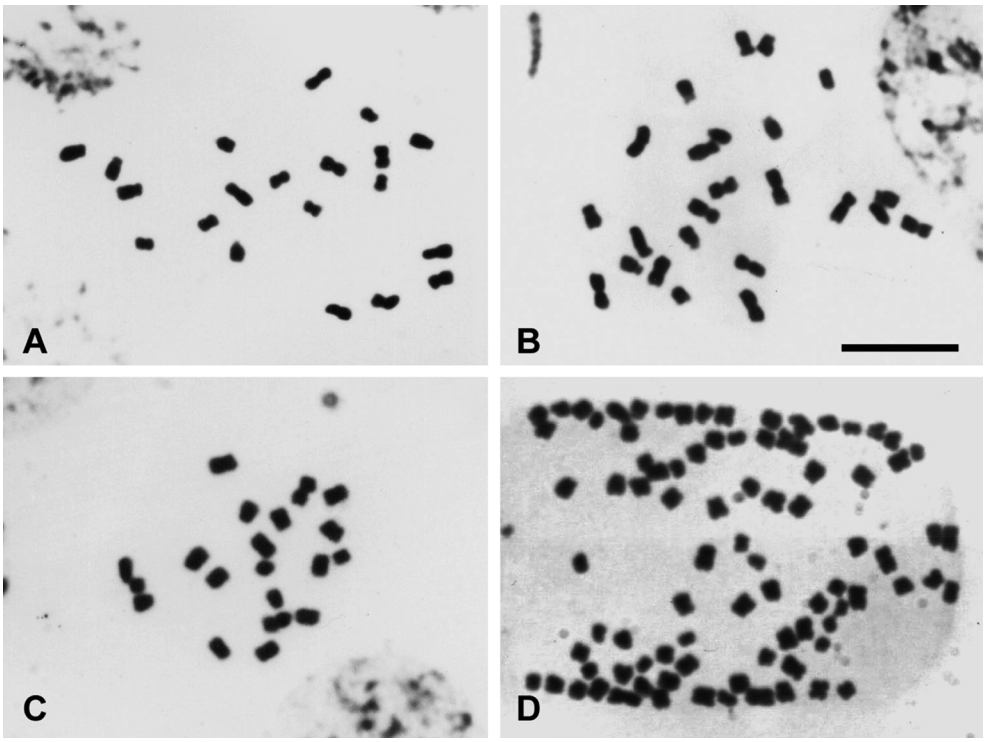

Fig. 1. Conventional staining in Paullinieae species. A, *C. grandiflorum* ($2n=20$); B, *P. elegans* ($2n=24$); C, *U. chacoensis* ($2n=22$); D, *U. ulmacea* ($2n=88$). Bar=10 μm.

ously denatured at 70°C for 10 min and immediately chilled on ice. Chromosome denaturation/hybridization was done at 90°C for 10 min, 48°C for 10 min, and 38°C for 5 min, using a thermal cycler (Mastercycler, Eppendorf), and slides were placed in a humid chamber at 37°C overnight. The hybridization sites of rDNA were detected with avidin-FITC and slides were counterstained and mounted with 25 μl of a solution of 50% antifade VectaShield (Vector Laboratories) and 50% glycerol/McIlvaine buffer, pH 7.0/2.5 mM $MgCl_2$ solution (1 : 1, v/v), plus 1 μl of 2.5 μgml^{-1} propidium iodide. Metaphases were photographed with Fuji Color 200 ISO film.

Results

Cardiospermum grandiflorum, with $2n=20$ (Fig. 1A), had the lowest chromosome number in relation to studied species in this work, and it is the only species in the genus with $x=10$ (Table 1). In *Paullinia elegans* $2n=2x=24$ was observed (Fig. 1B), however in the *Urvillea* species studied $x=11$ was seen, with $2n=22$ being observed in *U. chacoensis* (Fig. 1C) and $2n=88$ in *U. ulmacea* (Fig. 1D).

The karyotypes of the studied species demonstrated some differences (Table 1, Fig. 3). The mean chromosomal sizes were similar in *C. grandiflorum* and *P. elegans*. Whereas *U. chacoensis* and *U. ulmacea* presented relatively smaller chromosomes. All four species were characterized by the absence of acrocentric chromosomes. The karyotypes of all four species were moderately asymmetric, and TF % presented a variation from 34 to 41.7%. *Urvillea chacoensis* is the species with the most symmetric karyotype (TF %=41.7), having only metacentric chromosomes. *Urvillea ulmacea* had a more asymmetric karyotype, with minor TF value (TF %=34.0) and metacentric

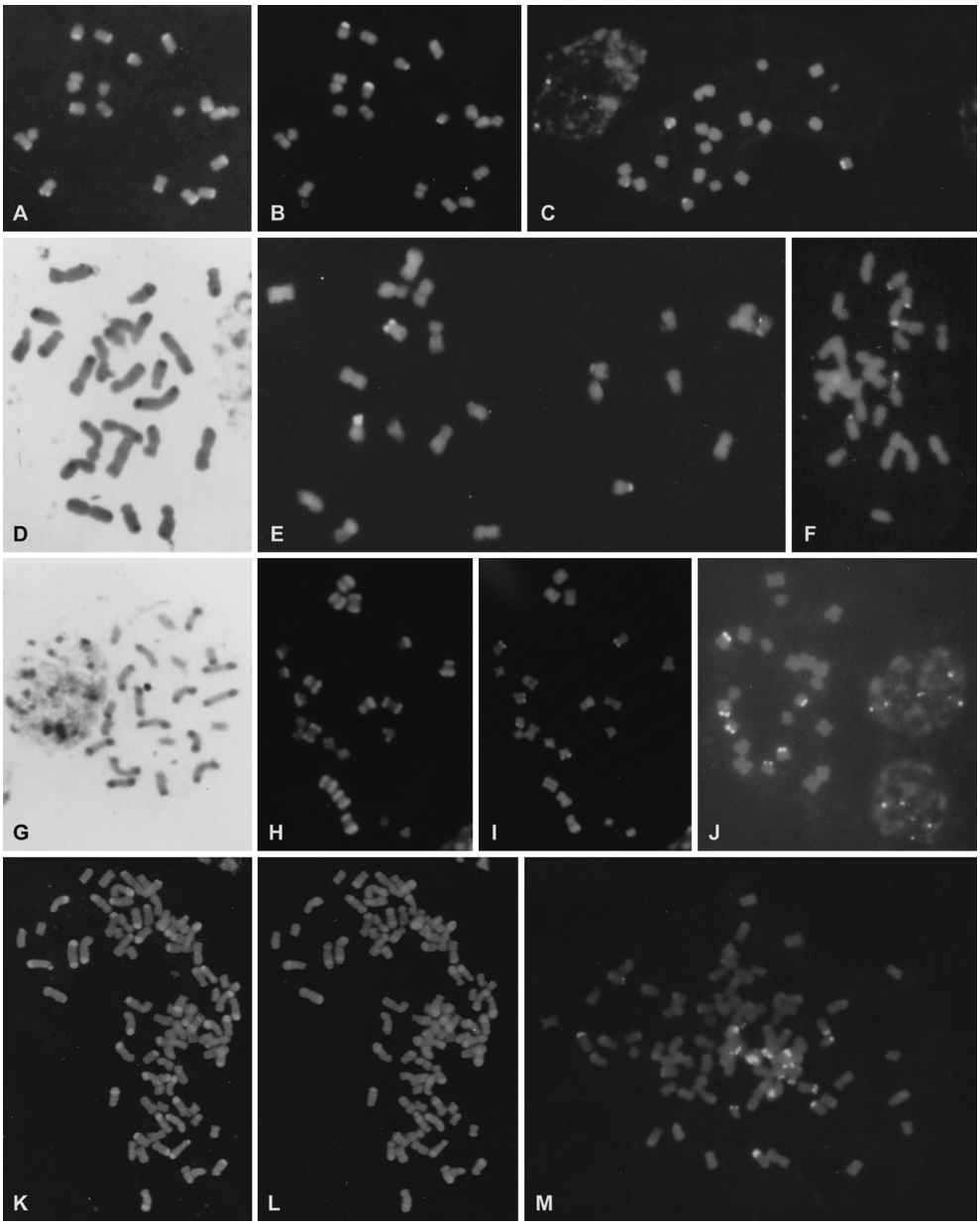

Fig. 2. Chromosome banding and FISH in Paullinieae species. C-DAPI banding (A), C-CMA_3 banding and FISH with p*Ta*71 probe (C) in *C. grandiflorum*. Giemsa C-banding, (D), C-CMA_3 banding (E) and FISH with p*Ta*71 probe (F) in *P. elegans*. Giemsa C-banding, (G), C-DAPI banding (H), C-CMA_3 banding (I) and FISH with p*Ta*71 probe (J) in *U. chacoensis*. C-DAPI banding (K), C-CMA_3 banding (L) and FISH with p*Ta*71 probe (M) in *U. ulmacea*. Bar = 10 μm.

and submetacentric chromosomes (Table 1, Fig. 3). Some submetacentric chromosomes were also observed in *C. grandiflorum* and *P. elegans*.

In *C. grandiflorum*, terminal bands in long arms of seven chromosomal pairs were observed after the C-Giemsa banding. These bands corresponded to AT-rich heterochromatin observed after

the staining with DAPI (Fig. 2A). On the other hand, GC-rich bands were located in terminal regions of short arms in two chromosomal pairs (Figs. 2B and 3). Several C-Giemsa bands were observed in the *P. elegans* karyotype. These bands were equilocally distributed in terminal regions of the long arms of the majority of the chromosomes, standing out in pair 1 and 2, which possessed bands in the terminal regions of both arms (short and long), and pair 7 that possessed a subterminal band (Figs. 2D and 3). This heterochromatin behaved neutrally after the CMA_3/DAPI staining. On the other hand, GC-rich bands were recognized in the terminal regions of short arms of three chromosomal pairs associated with the NOR (Figs. 2E and 3). A large amount of heterochromatin was found in the *U. chacoensis* karyotype: six chromosomal pairs presented C-Giemsa terminal bands in the short and long arms, and in another two pairs they are presented only in the long arm (Fig. 2G). The heterochromatin, in this species, was constituted by AT-rich repetitive DNA sequences, demonstrated with DAPI staining and it was also possible to detect minute $DAPI^+$ bands in minor chromosomes (Fig. 2H). Nevertheless, other GC-rich repetitive DNA sequences were recognized as $CMA3^+$ terminal bands in the short arms of three chromosomal pairs (Figs. 2I and 3). *Urvillea ulmacea* also presented terminal and some proximal heterochromatin blocks, and several GC-rich bands (Fig. 2K) are associated with other AT-rich bands (Fig. 2L) forming heterochromatin blocks (Fig. 3).

Differences were detected for the number of NOR in the studied species. Two pairs of chromosomes carrying 45S rDNA were found in *C. grandiflorum*, whereas *P. elegans* and *U. chacoensis* were differentiated by possessing three pairs of chromosomes carrying NOR. On the other hand, the *U. ulmacea* octoploid cytotype presented approximately 24 45S rDNA sites. The 45S rDNA sites were associated with GC-rich DNA regions in the studied species.

Discussion

Chromosome number and karyotypes

The chromosome numbers reported in this work confirm previous results in Paullinieae (Ferrucci 1981, 1989, 2000, Urdampilleta *et al.* 2006). According to previous chromosomal counts, the basic number in the tribe Paullinieae varies among $x=7$ and $x=14$ ($x=8$ and $x=13$ are absent), always with the conservation of $2n=24$ ($x=12$) in *Houssayanthus* and *Serjania* (Ferrucci 2000).

The reduction of the basic number of chromosomes and the increase in chromosomal size are processes that characterize the tribe Paullinieae, separating it from other tribes of Sapindaceae (Hemmer and Morawetz 1990, Lombello and Forni-Martins 1998), and also represent important chromosomal derivative characters that can be used in phylogenetic studies. *Cardiospermum* and *Urvillea* are very closely related, and this is reflected in their morphological and chromosomal characters. These genera are the only ones that present inflated septifragal capsules with a membranous pericarp and a dry aril (Weckerle and Rutishauser 2005), as well as a diversification in the basic chromosome number ($x=7$, 9, 10 and 11 in *Cardiospermum* and $x=11$ and 12 in *Urvillea*) in relation to other genera of the tribe. The occurrence of polyploid series is infrequent in Sapindaceae. *Paullinia* and *Urvillea* are the only genera of Paullinieae in which a polyploid series was reported. Species with 2×, 4× and 8× were found in *Urvillea* (Ferrucci 1981, Urdampilleta *et al.* 2006) and $2n=210$ was observed in *P. cupana* (Freitas *et al.* 2007).

Heterochromatin and banding patterns

The banding pattern in the species studied can be classified into two major groups: the first group, with CG rich regions associated with the nucleolar organizer regions (NOR) observed as CMA^+ bands and detected with the pTa71 probe, which localize 18S-5.8S-26S rDNA, and the second group, with terminal heterochromatin blocks observed with C-Giemsa and CMA_3/DAPI banding not associated with NOR. The CG rich regions associated to NOR contain gene sequences that

the term "heterochromatin constitutive" should not be applied to, since they could be active in interphase.

The CG rich regions associated with the nucleolar organizer regions (NOR) were detected with a p*Ta*71 probe, which contains 18S-5.8S-26S rDNA of wheat. This relation was already confirmed in other species of *Paullinia* and *Urvillea* (Urdampilleta *et al.* 2006, 2007). In plants, frequently NOR have being associated with GC-rich DNA regions (Guerra 2000). Although the location and the constitution were preserved, differences in the numbers of NOR were detected. The octoploid cytotype *U. ulmacea* presented approximately 24 sites of 18S-5.8S-26S rDNA and the non-elimination of rDNA loci suggest that this population corresponds to a recent polyploid event (Urdampilleta *et al.* 2006).

The terminal heterochromatin presented in the species studied varied in quantity and affinity for CMA_3/DAPI fluorochromes, which allowed for the distinguishing of the four species studied. Our results agree with the observations of Hemmer and Morawetz (1990) for *C. grandiflorum*, that found AT-rich bands in terminal regions of seven chromosome pairs. Otherwise, a large amount of AT-rich heterochromatin was also found in the majority of chromosomes in *U. chacoensis*. The banding pattern in *U. ulmacea* is particularly complex, since GC- and AT-rich regions were contiguous, and it was the only species that presented GC-rich regions not associated with NOR. The heterochromatin bands in *P. elegans* were visible with C-Giemsa banding, however no affinity was detected for CMA_3/DAPI fluorocromes which were named neutral bands by other authors (Guerra 2000).

Repetitive DNA can be a major component of the genome of plants, and quantitative changes of these sequences could be responsible for important modifications in the genome size of closely related species (SanMiguel and Bennetzen 1998). Variations in repetitive DNA are a frequent source of changes in chromosomal size and structure, contributing to the karyotypical differentiation of species (Flavell 1986). The heterochromatin is formed by tandem, highly repeated DNA sequences, called satellite DNA, and their monomers, generally with 150–360 bp, present 10^6–10^7 copies in the haploid genome (Macas *et al.* 2002). Different families of satellite DNA can be present in the genome of a species (Kubis *et al.* 1998, Sharma and Raina 2005). Frequently these heterochromatin blocks occupy similar chromosomal positions (patterns of equilocal bands), suggesting that sequences of repetitive DNA might be accumulated preferentially in regions where homologous and non-homologous chromosomes interact physically. Particularly, repetitive DNA is characterized by non-Mendelian heredity, with "rolling circle", "slippage replication" and "unequal crossing-over" being some of the mechanisms proposed for the amplification of these sequences (Flavell 1986). On the other hand, Schweizer and Loidl (1987) proposed a heterochromatin dispersion model based on amplification and translocation of repetitive DNA between non-homologous chromosomes, which takes place during interface and favoured by Rabl orientation.

The studied species shared a similar pattern of equilocal bands, with the terminal regions being preferential sites for the accumulation of repetitive DNA. Studies in *Koelreuteria* Laxm. (Koelreuterieae) species demonstrated the existence of GC-rich satellite DNA in a pattern of equilocal bands that promoted the karyotypic differentiation of species (Urdampilleta *et al.* 2005). In *U. chacoensis* and *U. ulmacea*, as well as in other species of the Paullinieae tribe, the presence of different heterochromatin types could be a result of the accumulation of different satellite DNA families (Urdampilleta *et al.* 2006). Hemmer and Morawetz (1990) admitted that AT-rich regions could represent a character derived in the tribe. In addition, our results demonstrated differences and resemblances in the pattern of bands in Paullinieae, whereas heterochromatin blocks happened preferentially in terminal regions, but different families of repetitive DNA: AT-, GC-rich and neutral, intervened in the karyological differentiation of species in Paullinieae.

In summary, *C. grandiflorum*, *P. elegans*, *U. chacoensis* and *U. ulmacea* showed the terminal banding pattern and 45S rDNA loci associated with GC-rich regions, however, in these species

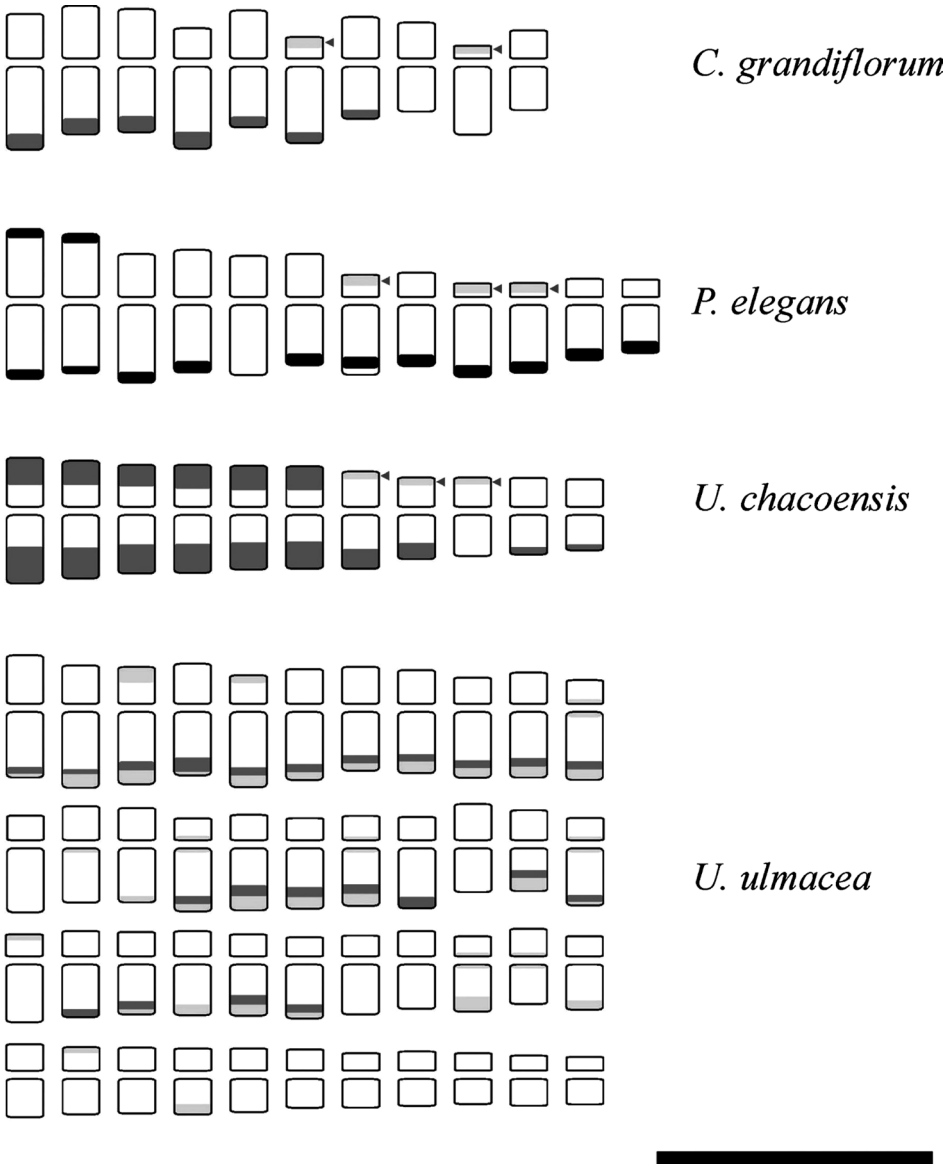

Fig. 3. Idiograms of the species of Paullinieae studied: ■=AT rich regions; ■=GC rich regions; ■=neutral C-bands; ▲=45S rDNA sites. Bar=5 μm.

there are significant differences in quantity and type of heterochromatin. The results obtained in this work suggest that the chromosomal location of repetitive DNA sites can be conserved by existing changes in the constitution and number of chromosomes, and that the dispersion of repetitive DNA occurred between non-homologous chromosomes by making an equilocal band pattern.

Acknowledgments

The authors are grateful to Ben Machado for improving the English version and the Brazilian agencies CAPES, CNPq e FAPESP for financial support.

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