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Karyotypes, heterochromatin, and physical mapping of 18S-26S rDNA in Cactaceae

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Abstract

Karyotype analyses in members of the four Cactaceae subfamilies were performed. Numbers and karyotype formula obtained were: Pereskioideae = *Pereskia aculeata* (2n = 22; 10 m + 1 sm), Maihuenioideae = *Maihuenia patagonica* (2n = 22, 9 m + 2 sm; 2n = 44, 18 m + 4 sm), Opuntioideae = *Cumulopuntia recurvata* (2n = 44; 20 m + 2 sm), Cactoideae = *Acanthocalycium spiniflorum* (2n = 22; 10 m + 1 sm), *Echinopsis tubiflora* (2n = 22; 10 m + 1 sm), *Trichocereus candicans* (2n = 22, 22 m). Chromosomes were small, the average chromosome length was 2.3 μm. Diploid species and the tetraploid *C. recurvata* had one terminal satellite, whereas the remaining tetraploid species showed four satellited chromosomes. Karyotypes were symmetrical. No $CMA^-/DAPI^+$ bands were detected, but $CMA^+/DAPI^-$ bands associated with NOR were always found. Pericentromeric heterochromatin was found in *C. recurvata, A. spiniflorum*, and the tetraploid cytotype of *M. patagonica*. The locations of the 18S-26S rDNA sites in all species coincided with $CMA^+/DAPI^-$ bands; the same occurred with the sizes and numbers of signals for each species. This technique was applied for the first time in metaphase chromosomes in cacti. NOR-bearing pair no.1 may be homeologous in all species examined. In Cactaceae, the 18S-26S loci seem to be highly conserved.

Fluorochrome banding (CMA/DAPI) and molecular cytogenetic methods (genomic and fluorescence in situ hybridization, GISH and FISH, respectively) have been applied to cytotaxonomical and evolutionary studies in different plant groups (e.g., Zhang and Sang, 1998; Adams et al., 2000; Schwarzacher, 2003).

The fluorochromes CMA and DAPI exhibit preferential staining for CG- and AT-rich DNA sequences, respectively, allowing the identification of different types of heterochromatin. This has been applied to chromosome analysis and has provided additional chromosome markers and informative characters for several Angiosperms (e.g., Moscone et al., 1996; Guerra, 2000; Souza and Benko-Iseppon, 2004; Gitaí et al., 2005; Urdampilleta et al., 2006).

More informative markers are often provided by FISH, a method that allows hybridization of known labeled marker sequences (probes) to homologous chromosomal targets (e.g., Adams et al., 2000; Schwarzacher and Heslop-Harrison, 2000; Schwarzacher, 2003). FISH enables the physical mapping of sequences to their location with-

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in the genome, in particular repetitive sequences that cannot be mapped easily by any other method (Schwarzacher, 2003). Repetitive sequences change rapidly during evolution, providing excellent markers for the identification of chromosomes and chromosome segments, and for following evolutionary chromosome rearrangements. The 5S and 18S-26S rDNA genes, in particular, have been used extensively to establish possible chromosomal homeologies (e.g., Moscone et al., 1999; Taketa et al., 1999; Adams et al., 2000; Cai et al., 2006; Hasterok et al., 2006). Effectively, they were commonly used as molecular-cytogenetic markers because they are abundant and highly conserved in higher plants (Schwarzacher, 2003), e.g., *Hypochaeris* (Cerbah et al., 1998), *Rhynchospora* (Vanzela et al., 2003), *Plantago* (Dhar et al., 2006), *Citrus* (Moraes et al., 2007), and *Cephalanthera* (Moscone et al., 2007).

The 18S-26S rDNA associated with nucleolar organization regions (NOR) consists of tandem repeat units, comprising coding and internal transcribed spacers (ITS) (Appels and Honeycutt, 1986). The high copy number, rapid concerted evolution, and small size of the ITS regions make them informative in determining evolutionary relationships between closely related groups (Baldwin et al., 1995; Susanna et al., 1999; Anamthawat-Jonsson and Bodvarsdottir, 2001).

The family Cactaceae has been scarcely studied cytogenetically. Cactaceae is a relatively small family with about 130 genera and 1,500–1,800 species distributed in America, from southern Patagonia to Canada (Barthlott and Hunt, 1993; Anderson, 2001; Hunt et al., 2006). Morphological and molecular evidence suggest that the family is monophyletic (Wallace, 1994) and is composed of four subfamilies (Anderson, 2001; Hunt et al., 2006): Pereskioideae (monotypic with the genus *Pereskia*), Maihenioideae (also monotypic with the genus *Maihuenia*), Opuntioideae (with 15 genera), and Cactoideae (the larger subfamily with around 110 genera).

The majority of cytological studies in cacti only provide chromosome counts, making clear that their basic chromosome number is x = 11 (e.g., Ross, 1981; Pinkava et al., 1985; Parfitt, 1987; Cota and Philbrick, 1994; Cota and Wallace, 1995; Bandyopadhyay and Sharma, 2000; Arakaki et al., 2007). On the other hand, there are few detailed karyotypic studies available (Johnson, 1980; Palomino et al., 1988; Cota and Wallace, 1995), being more rare for South American members of the family (Das and Mohanty, 2006; Las Peñas et al., 2008). Regardless of the importance of the application of chromosome banding techniques, there is only one article on the species of the exclusively Argentinean genus *Pyrrhocactus* (Las Peñas et al., 2008). There are no published reports on the use of FISH on somatic metaphase chromosomes and only one reported use of FISH on pollen mother cells to detect the ploidy status of the taxa studied (Tel-Zur et al., 2004).

Thus in this work we examined for the first time the karyotypes using CMA/DAPI chromosome banding, and FISH in six Argentinean species representing the four subfamilies of Cactaceae to correlate the banding pattern with the physical mapping of 18S-26S rDNA among the studied groups. In addition, we discuss these data in light of existing information on systematic and evolutionary relationships in Cactaceae.

Material and methods

Plant material

Collection data of the species studied are included in Table 1. Vouchers were deposited in the herbarium of the Museo Botánico de Córdoba (CORD). Specimens were planted in earthenware pots in an equal part mixture of sand and soil.

Karyotype analysis

The preparation of metaphase chromosomes was done from adventitious roots pretreated with 2 mM 8-hydroxyquinolin for 8 h at 4°C and fixed in 3:1 ethanol:acetic acid. For slide preparation, root tips were hydrolyzed with 5 N HCl for 30 min at room temperature and then washed, stained with Feulgen for 2 h, and squashed in a drop of 2% acetic carmine (Jong, 1997). Permanent mounts were made following Bowen's method (Bowen, 1956). Ten metaphases per species were photographed with a phase contrast optic Zeiss Axiophot microscope (Jena, Germany) and a Leica DFC300FX camera (Wetzlar, Germany). Photographs were used to take measurements of the following features for each chromosome pair: s (short arm), l (long arm), and c (total chromosome length); the length of the satellite was added to the respective chromosome arm. The arm ratio (r = l/s) was then calculated and used to classify the chromosomes as recognized by Levan et al. (1964). In addition, mean chromosome length (C), mean total haploid chromosome length of karyotype based on the mean chromosome length (tl), and mean arm ratio (R) were calculated. Idiograms were based on the mean values for each species. The chromosomes were arranged first into groups according to their increasing arm ratio and then according to decreasing length within each group. Karyotype asymmetry was estimated using the intrachromosomal (A_1) and interchromosomal (A_2) indices of Romero Zarco (1986).

Fluorochrome banding and FISH

Root tips were washed twice in distilled water (10 min each), digested with a 2% cellulase (Sigma-Aldrich, Vienna, Austria), 20% pectinase (from *Aspergillus niger*; Sigma-Aldrich, Vienna, Austria) solution (45 min at 37°C), and squashed in a drop of 45% acetic acid (Schwarzacher et al., 1980). Only one root tip was used in each slide. After coverslip removal in liquid nitrogen, the slides were stored at –20°C.

Table 1. Cactaceae species studied (all from Argentina). Collection data: collector and number, province, locality, date, and in brackets number of individuals, number of cells studied, respectively.

Taxa	Collection data
Pereskioideae	
Pereskia aculeata Mill.	G. Barboza et al. 1036, Misiones, Guaraní, May-15-2004 (15, 15)
Maihuenioideae	
Maihuenia patagonica (Phil.) Britton and Rose	P. S. Steibel and H. Troiani, 16213, Rio Negro, Valcheta, December-08-2004 (20, 40), G. Barboza et al. 1247, Neuquen, Barrancas, February-05-2005 (10, 30)
Opuntioideae	
Cumulopuntia recurvada Guilmes and Thomas	M. L. Las Peñas 182, San Juan, Los Medanos, April-16-2005 (14, 20)
Cactoideae	
Acantocalycium spiniflorum (K. Schum.) Backeb.	M. L. Las Peñas and D. Uñates 118, San Luis, Quines, November-30-2004 (5, 15)
Echinopsis tubiflora (Pfeiff.) Zucc.	M. L. Las Peñas and D. Uñates 278, Salta, Guachipas, January-20-2006 (7, 15)
Trichocereus candicans (Gillies ex Salm-Dyck) Britton and Rose	M. L. Las Peñas and D. Uñates 152, San Juan, Caucete, December-05-2005 (10, 25)

CMA/DAPI banding: Slides were stained with a drop of 0.5 mg/ml chromomycin A_3 (CMA) in McIlvaine buffer, pH 7.0 and distilled water (1:1) containing 2.5 mM $MgCl_2$ for 90 min and subsequently stained with 2 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) (both Sigma-Aldrich, Vienna, Austria) for 30 min, and finally mounted in McIlvaine's buffer-glycerol v/v 1:1 (Schweizer, 1976; Schweizer and Ambros, 1994). The amount of heterochromatin was expressed as a percentage of the total length of the haploid karyotype.

FISH: This technique was applied for all species, except for the cytotype diploid of *Maihuenia patagonica* for which very few individuals and cells were available. The location and number of rDNA sites were determined by FISH using as probe the p*Ta*71 containing the 18S-5.8S-26S rDNA (Gerlach and Bedbrook, 1979) labeled with biotin-14-dATP (BioNick, Invitrogen Carlsbad, USA). The FISH protocol was according to Schwarzacher and Heslop-Harrison (2000), with minor modifications. The preparations 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. On each slide 30 μl of hybridization mixture was added (4–6 ng/μl of probe, 50% formamide, 10% dextran sulfate, 3.3 ng/μl of salmon DNA, 2× SSC and 0.3% SDS), previously denatured at 70°C for 10 min. 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, Hamburg, Germany), and slides were placed in a humid chamber at 37°C overnight. The probe was detected with avidin-FITC conjugate and counterstained and mounted with 25 μl antifade (Vectashield Vector Lab., Burlingame, USA), containing 1 μl propidium iodide. Photomicrography was done with a BX51 Olympus photosystem (Tokyo, Japan) coupled with Evolution MIT CCD using Image ProPlus v4.5 software (Maryland, USA) for image capture.

Results

Karyotypes

Pereskia aculeata, *Echinopsis tubiflora*, and *Acantocalycium spiniflorum* were diploid (2n = 22), whereas *Cumulopuntia recurvata* and *Trichocereus candicans* were tetraploid (2n = 44), in all cells examined (Figs. 1, 2). On the other hand, the two populations of *Maihuenia patagonica* studied showed different ploidy levels: diploid from Neuquen province (2n = 22) and tetraploid from Rio Negro province (2n = 44) (Fig. 1a, e).

In general, the chromosomes were small, 2.3 μm being the average chromosome length for all taxa (Table 2). The karyotypes of the diploid species had one terminal satellite on the short arms of pair no.1, as also occurs in the tetraploid *Cumulopuntia recurvata* (Figs. 1, 3). The remaining tetraploid species showed four satellited chromosomes on the short arms of the longest *m* chromosomes (Figs. 1, 3). The frequency of appearance of the satellites in both homologues reached 70% of the examined cells in all taxa.

Karyotypes were symmetrical, considering both centromere position (most are *m* with 1 or 2, exceptionally 4 *sm* pairs) and chromosome size variation (there are slight differences among the chromosomes of the complement) (Table 2, Fig. 3). This is expressed by the asymmetry indices: A_1 ranges from 0.16 to 0.31 and A_2 from 0.14 to 0.26 (Table 2).

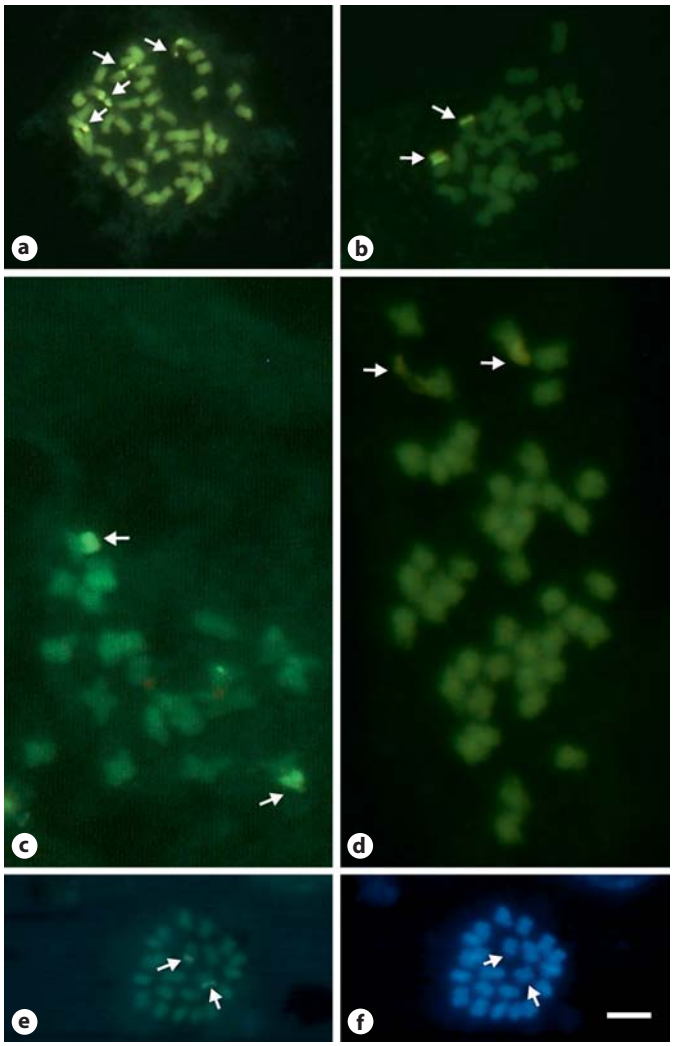

Fig. 1. Somatic metaphases with fluorochrome banding. (**a**) *Maihuenia patagonica* (2n = 44). (**b**) *Acantocalycium spiniflorum* (2n = 22). (**c**) *Pereskia aculeata* (2n = 22). (**d**) *Cumulopuntia recurvata* (2n = 44). (**e–f**) *Maihuenia patagonica* (2n = 22). (**a–e**) CMA fluorescence. (**f**) DAPI fluorescence. Arrows indicate $CMA^+/DAPI^-$ NOR-associated heterochromatin. Bar = 5 μm.

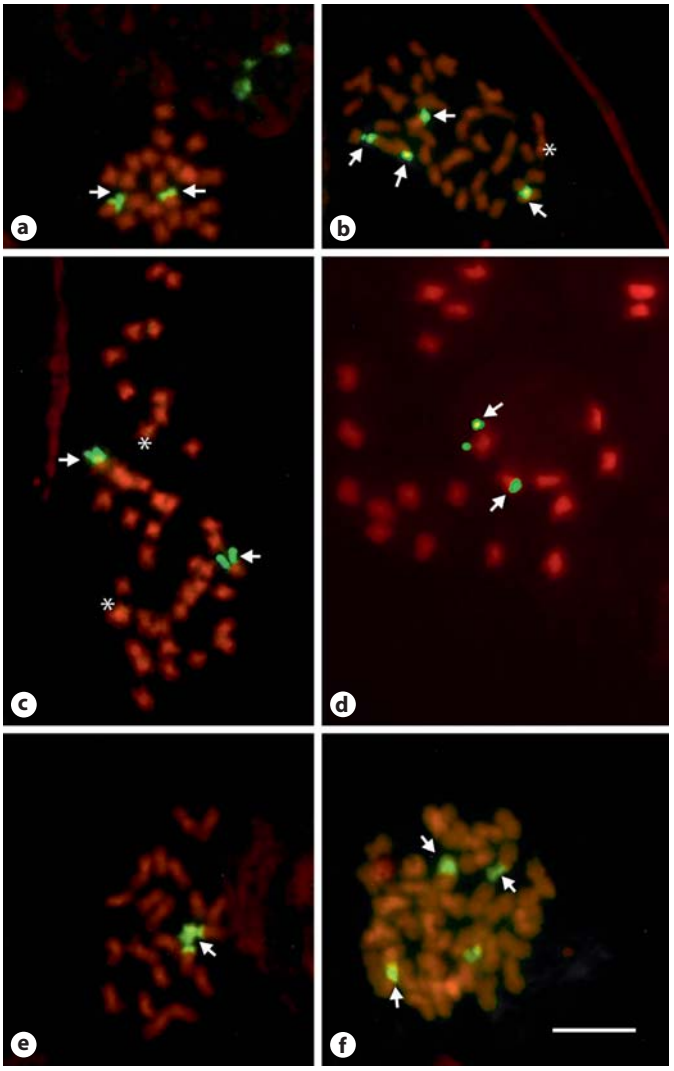

Fig. 2. Somatic chromosomes detected by FISH using 18S-26S rDNA probe. (**a**) *Pereskia aculeata*. (**b**) *Maihuenia patagonica* (2n = 44). (**c**) *Cumulopuntia recurvata*. (**d**) *Acantocalycium spiniflorum*. (**e**) *Echinopsis tubiflora*. (**f**) *Trichocereus candicans*. Arrows show 18S-26S rDNA sites and asterisks indicate hybridization in pericentromeric region. Bar = 5 μm.

Chromosome banding

The banding patterns always showed $CMA^+/DAPI^-$ constitutive heterochromatin associated with NOR (CG-rich). On the other hand, no $CMA^-/DAPI^+$ bands were detected in any species. The diploids *Echinopsis tubiflora*, *Pereskia aculeata*, *Acanthocalycium spiniflorum*, and *Maihuenia patagonica* (Neuquen population), as well as the tetraploid *Cumulopuntia recurvata*, showed a NOR-associated heterochromatin region in the satellited chromosome pair no.1. *Maihuenia patagonica* (Rio Negro population) and *Trichocereus candicans*, both tetraploid, had four NOR-associated heterochromatin regions (Fig. 1).

Additional $CMA^+/DAPI^-$ pericentromeric bands were found in different numbers: four bands in two *m* pairs in *Cumulopuntia recurvata* and two bands in one *m* pair in *Acantocalycium spiniflorum* and the tetraploid cytotype of *Maihuenia patagonica* (Figs. 1, 3). The highest and low-

Table 2. Karyotype data of Cactaceae. Abbreviations = tl: mean total haploid karyotype length; C: mean chromosome length; A_1: mean intrachromosomic asymmetry index; A_2: mean interchromosomic asymmetry index, %: amount of $CMA^+/DAPI^-$ heterochromatin expressed as percentage of the haploid karyotype length; Lengths are in μm. Chromosome nomenclature after Levan et al. (1964). An asterisk indicates that the first chromosome pair has satellites and two asterisks two satellited pairs (# 1 and 2).

Taxa	2n	Haploid karyotype formula	tl	C	A_1	A_2	%
Pereskioideae							
Pereskia aculeata	22	10 m* + 1 sm	28.80	2.60	0.16	0.24	4.96
Maihuenioideae							
Maihuenia patagonica	22	9 m* + 2 sm	29.71	2.47	0.27	0.26	3.30
	44	18 m** + 4 sm	56.00	2.45	0.30	0.26	6.20
Opuntioideae							
Cumulopuntia recurvata	44	20 m* + 2 sm	39.50	1.80	0.23	0.20	3.10
Cactoideae							
Acantocalycium spiniflorum	22	10 m* + 1 sm	28.00	2.55	0.17	0.14	5.10
Echinopsis tubiflora	22	10 m* + 1 sm	26.90	2.40	0.20	0.25	4.62
Trichocereus candicans	44	22 m**	45.90	2.10	0.25	0.14	4.82

est total amounts of $CMA^+/DAPI^-$ heterochromatin were found in two tetraploid taxa: *Cumulopuntia recurvata* with 3.1% and Rio Negro population of *Maihuenia patagonica* with 6.2% (Table 2).

Chromosomal mapping of the 18S-26S rDNA by FISH

The locations of the 18S-26S rDNA sites in all species studied coincided with $CMA^+/DAPI^-$ bands described above (Figs. 2, 3), i.e., they are located on the secondary constrictions and the adjacent satellites at telomeric positions. The same occurred with the sizes and numbers of signals for each species.

Discussion

The basic chromosome number for the family is x = 11 (e.g., Pinkava et al., 1977, 1985, 1992; Parfitt, 1987; Cota and Wallace, 1995; Bandyopadhyay and Sharma, 2000; Das and Mohanty, 2006). It was found in all taxa here, examined at the diploid or tetraploid levels.

Most numbers are here reported for the first time. The exceptions are: *P. aculeata* for which we confirmed previous reports (Leuenberger, 1986; Lombello and Forni-Martins, 1998) and *M. patagonica* for which we found a diploid population, as registered by Leuenberger (1997), as well as the novelty of a tetraploid population, a fact known for several species (Johnson, 1980; Pinkava, 2002). This tetraploid cytotype of *M. patagonica* is the first report of polyploidy in a member of subfamily Maihuenioideae. Polyploidy has been reported for ca. 25% of the cacti investigated so far (cf. Palomino et al., 1988; Cota and Wallace, 1995; Pinkava, 2002; Arakaki et al., 2007); particularly, it seems to have played an important role in the evolution of Opuntioideae where it reaches a percentage of 64%.

Although variation in karyotype composition regarding chromosome size and morphology was found, the taxa here studied and previous data show that Cactaceae has symmetrical karyotypes mostly composed of *m* and *sm* chromosomes (e.g., Pinkava et al., 1985, 1992; Parfitt, 1987; Cota and Wallace, 1995; Bandyopadhyay and Sharma, 2000; Das and Mohanty, 2006; Las Peñas et al., 2008). *St* chromosomes are rare and only reported for two species (Johnson, 1980; Las Peñas et al., 2008), whereas *t* chromosomes have never been registered.

CMA/DAPI banding has been widely used in flowering plants to characterize heterochromatin bands with respect to their highly repeated DNA composition, known also as satellite DNA. The numbers of bands and the heterochromatin amount varied, but the general patterns were relatively conserved at the generic level (Galasso et al., 1996; Moscone et al., 1996; Miranda et al., 1997; Ran et al., 1999; Marcon and Guerra, 2005; Fregonezi et al., 2006). There is a positive association between the ploidy level and the amount of heterochromatin in the cytotypes of *Maihuenia patagonica*. The scarce data available in Cactaceae are reduced to seven *Pyrrhocactus* species (Las Peñas et al., 2008) and our data on members of the four subfamilies. Both data sets showed that $CMA^+/DAPI^-$

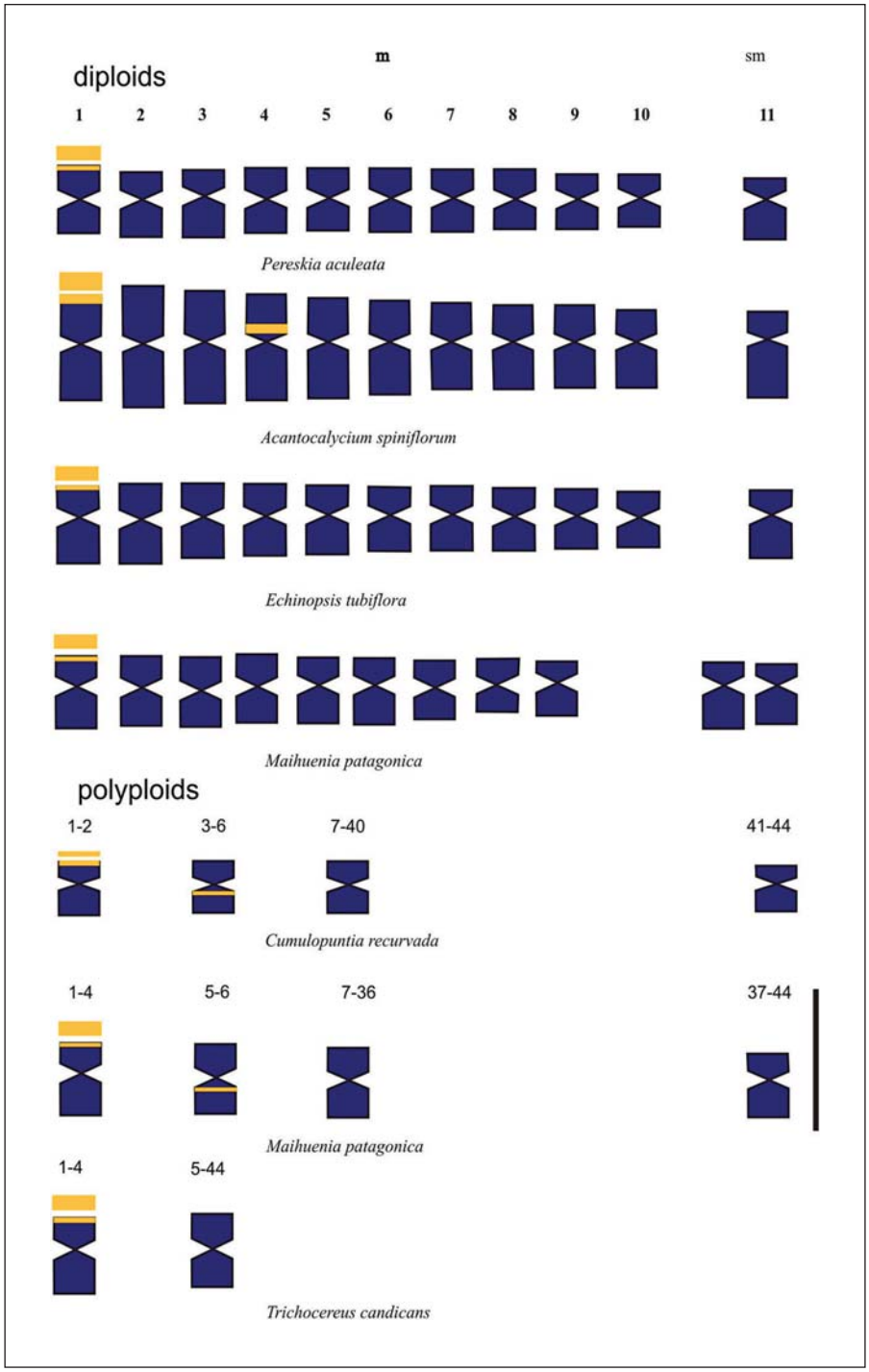

Fig. 3. Idiograms of Cactaceae studied. Blue blocks = euchromatin, yellow blocks = CMA^{+}/18S-26S rDNA sites. Bar = 5 μm.

heterochromatin blocks were always present on a terminal position associated with secondary constrictions; on the other hand, there is variability in the presence and the number of pericentromeric bands, which may be useful for evolutionary and systematic purposes.

Physical mapping by FISH of 18S-26S rDNA were here performed for the first time in metaphase chromosomes in cacti. FISH signals with p*Ta*71 do not exhibit variation in size among the different species studied. The presence of these sites in terminal regions of both chromosome

arms is frequent in Angiosperms (e.g., Zhang and Sang, 1998; Benko-Iseppon and Morawetz, 2000; Vaio et al., 2005; Urdampilleta et al., 2006).

The number of 18S-26S rDNA sites in most species studied varied from two in the diploids to four in the tetraploids; thus, they may be indicative of the ploidy level. The same numbers of sites were found on pollen mother cells to detect the ploidy level in species and hybrids of *Hylocereus* and *Selenicereus* (Tel-Zur et al., 2004). Nevertheless, the tetraploid *C. recurvata* here analyzed showed signals in only one pair. This reduction in the locus number of 18S-26S rDNA units in several polyploids has been reported for other plant species (Appels et al., 1980; Hanson et al., 1996; Adachi et al., 1997; Vanzela et al., 2003). Reduction in 18S-26S rRNA gene locus number has previously been attributed to diploidizing (Melo and Guerra, 2003; Leitch and Bennett, 2004).

In many Angiosperm species, the centromere is associated with blocks of heterochromatin that contain a core of tandem satellite repeats, many of which are species specific and show chromosome-specific variants (Heslop-Harrison et al., 2003; Schwarzacher, 2003). Diverse mechanisms have been postulated to account for the variation in size, number, and position of rDNA sites and their apparent mobility, i.e., chromosome rearrangements, homologous and nonhomologous unequal crossing-over, gene conversion, transpositional events (Schubert and Wobus, 1985; Leitch and Heslop-Harrison, 1992; Hanson et al., 1996). These processes could act alone or in combination and do not necessarily imply changes in overall chromosome morphology (Hall and Parker, 1995). In this study, pericentromeric fluorescence signals were less intense than terminal signals. A possible hypothesis explaining the presence of these pericentric sites might be that they originated as active, subtelomeric 18S-26S rRNA genes that were subsequently shifted to the centromeric region through an inversion mechanism; nevertheless, information based on additional techniques, as AgNOR staining or FISH using IGS as probe, are needed to confirm it. Frequently, intergenic spacers could organize other chromosome regions (Hemleben et al., 2007), e.g., the satellite DNA sequences of some species of *Vigna* (Fabaceae) apparently originated from subrepeats of the intergenic spacer of rDNA (Unfried et al., 1991; Macas et al., 2003). In *Nicotiana* and *Solanum* (Solanaceae), independent satellites highly homologous to rDNA intergenic spacers were observed in terminal and pericentromeric regions, respectively (Stupard et al., 2002; Lim et al., 2004), and the interspecific variation indicated a dynamic nature of the repetitive DNA.

Both fluorochrome banding and rDNA gene mapping have been valuable in the identification of homeologous chromosome pairs among plant species (e.g., Moscone et al., 1995, 1996, 2007). In Cactaceae, NOR-bearing pair no.1 may be homeologous in all species examined so far (Las Peñas et al., 2008, this study). Although more data are needed, in Cactaceae the 18S-26S loci seem to be highly conserved as detected in members of its four subfamilies. This NOR-associated heterochromatin (18S-26S rDNA locus) is the rule in plants as a whole (Sinclair and Brown, 1971; Morawetz, 1986; Benko-Iseppon and Morawetz, 2000; Urdampilleta et al., 2006).

In general, karyotype features of cacti allowed individual species to be distinguished. Chromosome variation, although not always large, accompanied evolutionary divergence of the species. In addition, our comparative study of chromosome banding patterns and data on *Pyrrhocactus* (Las Peñas et al., 2008) suggested that repeated DNA segments also contributed to karyotypic differentiation. On the other hand, our data on the 18S-26S rDNA locus implied that its physical position seems to be conserved in the family. In summary, more data are needed using these techniques in cacti, as they provided useful information in the cytogenetics of these peculiar plants.

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