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Karyotype characterization and evolution of chromosome number in Cactaceae with special emphasis on subfamily Cactoideae

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ABSTRACT

Cactaceae species are karyotypically well-known with $x = 11$ and chromosome number variation due mainly to polyploidization. However, both assumptions are based on descriptive observations without taking an evolutionary framework of Cactaceae into account. Aiming to confirm these hypotheses in an evolutionary context, we obtained chromosome numbers for 20 species of Cactoideae, performed an extensive review of chromosome number for the family, and analyzed these data using a phylogenetic approach. The karyotypes presented here were characterized by CMA/DAPI banding, and for six species 5S and 45S rDNA sites were located. Our data, along with a survey of the literature, reinforce the long-standing hypothesis of a $x = 11$ as the base chromosome number for Cactaceae. They also reinforce the relevance of polyploidy in karyotype evolution of cacti, although polyploidy was important just after the diversification of subfamilies Maihuenioideae and Pereskioideae. Despite the homogeneous chromosome complements observed among cacti, chromosome banding and FISH techniques revealed informative characteristics, allowing the identification of chromosome synapomorphies, such as proximal CMA^+ bands in *Melocactus* and proximal 5S rDNA in *Pilosocereus,* indicating the taxonomic potential of chromosome characterization in cacti.

Keywords: base chromosome number, CMA/DAPI, dysploidy, heterochromatin, polyploidy, rDNA

Introduction

Cacti species have been important for humans for the past 9,000 years due to their frequent uses as food, medicine and, mainly and more recently, as ornamental plants (Russell & Felker 1987; Anderson 2001; García-Suárez *et al*. 2007; Aragane *et al*. 2011). Cactaceae species (Caryophyllales) are a conspicuous component of arid regions of the New World with 1,150 species distributed in 94 genera (Christenhusz & Byng 2016). Three centers of diversity are proposed (Anderson 2001; Arakaki *et al.* 2011): (1) arid regions in North America (northern Mexico and southern USA; tribes Cacteae and Pachycereae; 8-6 million years ago, Ma), (2) arid/semi-arid regions in the central Andes, comprising Peru, Bolivia, Chile, extending until Argentina (tribes Browningieae, Notocacteae and Trichocereeae) and (3) Brazil (tribe Cereae, specially subtribe Cereinae; 7.5–6.5 Ma).

Most species of Cactaceae have showy flowers and exhibit adaptations to arid environments, the so-called succulent syndrome: shallow roots, thick and waxy cuticle and CAM photosynthesis (Anderson 2001; Arakaki *et al*. 2011). The family diversified at ≈35 Ma, but the major diversification is more recent, in the Miocene-Pliocene, ≈10–2.5 Ma, a period coinciding with the global evolutionary burst of C_4 photosynthesis (Arakaki *et al.* 2011). Cactaceae monophyly

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is supported by morphological traits - inferior ovary, short shoots into areoles and shoot apical meristems organized into four zones - and molecular phylogenetic results (Downie & Palmer 1994; Nyffeler 2002). Two subfamilies, Opuntioideae and Cactoideae, are well characterized by morphological traits and molecular synapomorphies (Nyffeler 2002), but relationships among these two subfamilies and the two remaining genera, *Pereskia* and *Maihuenia*, are challenging (Anderson 2001; Nyffeler 2002; Griffith 2004; Edwards *et al*. 2005; Hernández-Hernández *et al*. 2011). Among the previous taxonomic proposals, these genera were grouped in Pereskioideae based on the absence of morphological synapomorphies (Nyffeler 2002; Edwards *et al*. 2005). The two species of *Maihuenia* were also placed in their own subfamily, Maihueniodeae, which are sister to Cactoideae (Anderson 2001; Nyffeler 2002; Edwards *et al*. 2005) or sister to both Cactoideae and Opuntioideae (Hernández-Hernández *et al*. 2011). The best currently available phylogenetic hypothesis supports both genera, *Pereskia* and *Maihuenia*, as forming a two- or three-parted grade to the rest of Cactaceae (Hernández-Hernández *et al*. 2011).

Cactaceae species are well characterized in terms of chromosome numbers. In some specific genera, *e.g*. *Opuntia*, a large cytological database was used to understand species evolution and subgeneric delimitation (Pinkava & McLeod 1971; Pinkava *et al*. 1973; 1977; 1985; 1992; Weedin & Powell 1978; Pinkava & Parfitt 1982; Weedin *et al*. 1989; Powell & Weedin 2001; 2004). However, a complete survey of chromosome data for Cactaceae is still missing, preventing a more comprehensive identification of the main chromosome rearrangements in an evolutionary context.

Symmetrical karyotypes and $2n = 22$ are conserved characteristics across the family (Stefano *et al*. 2011; Majure *et al*. 2012; Castro *et al*. 2013; Moreno *et al*. 2015). Nevertheless, the homogeneous karyotypes of cacti, when submitted to differential staining and rDNA sequence localization, reveal to be variable and typically informative. *Pyrrhocactus* species, *e.g.*, could be characterized by its variation in pericentromeric CMA^+ bands (Peñas *et al*. 2008). The genera *Opuntia* and *Brasiliopuntia* also exhibit pericentromeric CMA^+ bands, whereas the related genus *Tacinga* is characterized by the absence of such pericentromeric CMA^+ bands (Castro *et al*. 2016). Duplicated 5S rDNA sites are characteristic of most *Lepismium* clades, but this is absent in the more derived clades (Moreno *et al*. 2015).

Polyploidy and $x = 11$ in Cactaceae have been hypothesized as the main mechanism and the base chromosome number of karyotype evolution, respectively. However, these two assumptions are based just on the high frequency of $n = 11$ and multiples of chromosome numbers among Cactaceae species and have never been examined in an evolutionary framework, a defficiency this paper aims to rectify, emphasizing subfamily Cactoideae. To complete our objectives, we first generate new chromosome data for Cactoideae from South America, using classical (chromosome number and CMA/DAPI banding) and molecular cytogenetic (5S and 45S rDNA localization by fluorescent in situ hybridization, FISH) approaches for 20 and six previously unexamined species, respectively. Finally, based on an extensive compilation of Cactaceae chromosome data and close species used as outgroup, we analyzed all data in a phylogenetic context aiming to confirm (1) the base chromosome number for Cactaceae and subfamilies and (2) the main chromosome modification that have occurred in Cactaceae.

Materials and methods

Plant Material - A total of 20 species (Tab. 1) were analyzed, representing ten genera from Cactoideae covering tribes Cereae, Trichocereae and Hylocereae (Anderson 2001). Vouchers have been deposited at EAN.

Cytogenetic analysis - Chromosome number, CMA/DAPI banding, and FISH

Chromosome number - Root tips were pre-treated with 0.002 M 8-hydroxyquinoline (8HQ) for 5 h at 18 °C and subsequently fixed in 3:1 (absolute ethanol: glacial acetic acid, v:v) for 2–24 h at room temperature (RT) and stored at –20 °C. For chromosome slide preparation, material was washed twice in distilled H_2O (dH_2O) for five minutes and digested in an enzymatic solution containing 2 % cellulase (Onozuka) and 20 % pectinase (Sigma) for 1 h at 37 °C. Subsequently, they were squashed in a drop of 45 % acetic acid and the coverslip was removed in liquid nitrogen.

CMA/DAPI banding - For each voucher, the three best slides (*i.e.*, slides with at least five well-spread metaphases) were aged for three days and double-stained with the fluorochromes CMA (0.2 mg mL^{-1}) for 1 h, and DAPI (2 µg mL^{-1}) for 30 min (Schweizer 1976). Slides were mounted in glycerol/McIlvaine pH 7.0 (1:1/v:v) buffer medium. The metaphases were captured on a Zeiss epifluorescent microscope using an AxioCam MRC5 video camera and Axiovision 4.8 software. Images were processed in Adobe Photoshop CS6®. Usually, between 20 and 40 cells were analyzed per voucher. For species with more than one voucher analyzed, and presenting consistent banding pattern, the individual with best slides (*i.e.*, at least ten good metaphases) were chosen, and two slides were de-stained in 3:1 absolute ethanol: glacial acetic acid (v:v) for 30 min at room temperature, followed by absolute ethanol for 2 h at 10 °C and stored for FISH.

FISH - The D2 probe from *Lotus japonicus* (Pedrosa *et al*. 2002) and an R2 probe from *Arabidopsis thaliana* (Wanzenböck *et al*. 1997) were used to localize 5S and 45S rDNA, respectively. Both probes were labeled by nick

Table 1. Cactoideae species collected for cytogenetic analysis, with respective voucher and provenance. Somatic chromosome numbers (2*n*), CMA$^+$ bands, and number of 5S and 45S rDNA site pairs are summarized. t = terminal position; st = subterminal position; p = proximal position; i = interstitial position.

Taxon	Voucher	Provenance (country, state)	2n	CMA$^+$	Fig	rDNA		Fig
						5S	45S	
Subfamily Cactoideae								
Tribe Cereeae								
Arrojadoa dinae Buining & Brederoo	JPCastro 112	Morro do Chapéu, BA	22	1t + 1p	1A			
A. penicillata (Gürke) Britton & Rose	JPCastro 30	Senhor do Bonfim, BA	22	1t + 1p	1B			
A. rhodantha (Gürke) Britton & Rose	LPFelix 11895	Jacobina, BA	22	1t	1C			
	LPFelix 11899	Morro do Chapéu, BA	22	1t				
	JPCastro 2	Salgueiro, PE	22	1t				
Cereus jamacaru DC.	LPFelix S/N	Bayeux, PB	22	1t	1D	1p + 1i + 1st	1t	1E
	LPFelix 11893	Campo Formoso, BA	22	1t				
	LPFelix 12165	Águas Belas, PE	22	1t				
Melocactus azureus Buining & Brederoo	JPCastro 186	Morro do Chapéu, BA	44	2t + 22p	1F			
M. ernestii Vaupel	NAPorto 25	Esperança, PB	44	3t* + 6p	1G			
M. lanssensianus P.J.Braun	JPCastro 198	Jaguarari, BA	22	1t + 8p	1H			
M. levitestatus Buining & Brederoo	JPCastro 188	Morro do Chapéu, BA	22	1t + 4p	1I			
M. oreas Miq.	JPCastro 76	Jacobina, BA	44	2t + 7p	1J	2p	2t	1K
	JPCastro 139	Morro do Chapéu, BA	44	2t + 7p				
M. zehntneri (Britton & Rose) Luetzelb.	LPFelix 12189	Águas Belas, BA	44	2t + 3p	1L			
	LPFelix S/N	Triunfo, PE	44	2t + 3p				
Micranthocereus flaviflorus Buining & Brederoo	JPCastro 91	Morro do Chapéu, BA	22	1t	1M			
Pilosocereus gounellei (F.A.C.Weber) Byles & Rowley	LPFelix 11791	Carnauba dos Dantas, RN	22	1t + 10p	1N			
	JPCastro 211	Ingá de Bacamarte, PB	22	1t + 10p				
	JPCastro 109	Morro do Chapéu,BA	22	1t + 10p				
P. chrysostele (Vaupel) Byles & G.D.Rowley	LPFelix 11781	Carnauba dos Dantas, RN	22	1t	2A	2p +1p	1t	2B
	LPFelix 13180	Sumé, PB	22	1t				
P. pachycladus subsp. *pachycladus* F.Ritter	LPFelix 11894	Campo Formoso, BA	22	1t	2C			
	JPCastro 90	Morro do Chapéu, BA	44	2t	2D			
P. pachycladus subsp. *pernambucoensis* (Ritter) Zappi	NAPorto 21	Esperança, PB	44	2t	2E	2p + 2i	2t	2F
	LPFelix 12177	Pariconha, AL	44	2t				
	LPFelix 12244	Pariconha, AL	44	2t				
	LPFelix 12640	Areia, PB	44	2t				
	LPFelix S/N	Pocinhos, PB	44	2t				
P. pentaedrophorus (Cels) Byles & Rowley	JPCastro 35	Jacobina, BA	22	1t + 4p	2G	1p + 1i	1t	2H
	LPFelix 11843	Morro do Chapéu, BA	22	1t + 4p				
	LPFelix 13193	Itatim, BA	22	1t + 4p				
Stephanocereus luetzelburgii (Vaupel) N.P.Taylor & Eggli	JPCastro 140	Morro do Chapéu, BA	22	1t + 1p	2I			
Tribe Trichocereeae								
Discocactus zehntneri Britton & Rose	JPCastro 175	Morro do Chapéu, BA	22	1t	2K	1p	1t	2L
Harrisia adscendens (Gürke) Britton & Rose	JPCastro 13	Salgueiro, PE	22	1t + 4p	2J			
Tribe Hylocereeae								
Epiphyllum anguliger (Lemaire) D. Don	EMAlmeida 186	Serraria, PB	22	1t	2M			
Hylocereus setaceus (Salm-Dyck) R.Bauer	LPFelix 11777	Serraria, PB	44	2t	2N			

* - exceptional for this species, the total number of terminal bands are presented

translation (Roche Biochemicals), the 5S rDNA probe with digoxigenin-11-dUTP and the 45S rDNA probe with biotin-14-dUTP. The *in situ* hybridization mixture was 50 % (v/v) formamide, 10 % (w/v) dextran sulphate, 0.1 % (w/v) sodium dodecyl sulphate (SDS) in 2 × saline-sodium citrate buffer (SSC), and 5 ng ml-1 of each probe. The 5S rDNA probe was detected with mouse anti-biotin (Roche), and the signals were amplified with rabbit anti-mouse TRITC conjugate (Dako). The 45S rDNA was detected with sheep anti-digoxigenin FITC conjugate (Roche) and amplified with rabbit anti-sheep FITC conjugate (Dako). All preparations were counterstained with DAPI (2 mg ml^{-1}) and mounted in Vectashield (Vector). At least 20 metaphases per voucher were captured and analyzed as described in "CMA/DAPI banding".

Literature survey

The published chromosome numbers for Cactaceae were compiled from the Index to Plant Chromosome Numbers (IPCN; www.tropicos.org/Project/IPCN), Chromosome Counts Database (Rice *et al*. 2015; CCDB, http://ccdb.tau.ac.il/) and original literature (Tab. S1 in supplementary material). The scientific names follow The Plant List (http://www.theplantlist.org/) and The International Plant Names Index (www.ipni.org).

Chromosome number evolution

Following standard conventions, throughout this article we refer to '*x*' as the base chromosome number of a clade, taking in account closely related taxa; x_2, x_3. . . as the derived secondary, tertiary (. . .) base chromosome number of a clade; 2*n* as the chromosome number in somatic tissues and *n* as the haploid chromosome number observed in gametes (Stebbins 1966; 1971; Guerra 2008; 2012).

Applying the chromosome numbers obtained both here and in the literature and considering the phylogenetic hypothesis of Hernandez-Hernandez *et al*. (2011) downloaded from TreeBase (https://treebase.org/), we reconstructed the base chromosome numbers for the tree nodes with the ChromEvol v.2 software (http://www.zoology.ubc.ca/prog/chromEvol.html) (Mayrose *et al*. 2010; Glick & Mayrose 2014). This software uses likelihood-based methods to evaluate the chromosome number changes along the phylogenetic branches. Such inference employs four parameters: polyploidy (chromosome number duplication, with rate ρ), demi-duplication (fusion of gametes of different ploidy, with rate μ), and dysploidy, which could be ascending (*i.e.*, chromosome gain, with rate λ) or descending (*i.e.*, chromosome loss, with rate δ). These parameters are evaluated with constant models and linear models, which use both constant (λ*l*) and linear (δ*l*) rates for gain and loss of chromosomes. The linear models estimate the chromosome number changes depending on the current chromosome number, whereas the constant models measure the rate of changes independently of the current chromosome number. Finally, eight models (four models considering just constant rates and other four models considering both constant (λ*l*) and linear (δ*l*) rates) were tested, allowing us to infer the main mechanism for chromosome number changes according to the best model. Both model sets include a null model that assumes no polyploidization events. The best-fitted model was selected using the Akaike's information criterion (Akaike 1974), and considering this best-fitting model we re-ran the analysis using 10,000 simulations to infer the expected number of changes along the branches, as well as to infer the ancestral base chromosome number for specific nodes throughout the phylogenetic hypothesis. The haploid chromosome numbers of 70 species were recorded and plotted into a file, representing 31 % of the 226 species present in the phylogenetic analysis published by Hernández-Hernández *et al*. (2011) (Tab. S2 in supplementary material; unknown chromosome numbers were coded by "x"].

Results

Karyotypes - New chromosome counts and chromosome markers for Cactaceae

New chromosome numbers

The most frequently observed chromosome number among the 20 analyzed species was 2*n* = 22 (Figs. 1, 2; Tab. 1), with polyploidy, 2*n* = 44, found in six species: *Melocactus azureus* (Fig. 1F), *M. ernestii* (Fig. 1G), *M. oreas* (Fig. 1J), *M. zehntneri* (Fig. 1L; tribe Cereae), *Pilosocereus pachycladus* subsp. *pachycladus and* subsp. *pernambucoensis* (Fig. 2D-E, respectively; tribe Cereae) and *Hylocereus setaceus* (Fig. 2N; tribe Hylocereae). Both ploidies were observed in *Pilosocereus pachycladus* subsp. *pachycladus*, with diploid (Fig. 2C) and polyploid (Fig. 2D) populations. All species exhibited symmetrical karyotypes with metacentric-submetacentric chromosomes (Figs. 1, 2).

Chromosome markers - CMA/DAPI banding

The chromosome banding revealed heterochromatic bands positively stained with CMA ($CMA^+/DAPI^-$), *i.e.*, the observed heterochromatin was always CG-rich (for an ideogram with the position of $CMA^+/DAPI^-$ bands, see Fig. 3). The terminal $CMA^+/DAPI^-$ bands were always co-localized with 45S rDNA (see section *Chromosome markers - FISH*), and they were usually more intense when compared to proximal bands (*e.g.*, see Fig. 1I). Two terminal $CMA^+/DAPI^-$ bands were observed in diploid species with 2*n* = 22, whereas four bands were observed in the tetraploids, 2*n* = 44 (Figs. 1, 2). However, in the tetraploid *M. ernestii,* just three $CMA^+/DAPI^-$ bands were detected, one reduced in size (see arrows and details in Fig. 1G).

Besides the terminal CMA⁺ bands, one pair of proximal bands was detected in *Arrojadoa dinae* (detail in Fig. 1A) and *A. penicillata* (detail in Fig. 1B), as well as, in *S. luetzelburgii* (Fig. 2I, Tab. 1). Variable numbers of proximal CMA⁺ bands were detected in *Melocactus* (Tab. 1) from four pairs (two strong and two weak) in the tetraploid *M. zehntneri* (Fig. 1L) to all chromosomes in the tetraploid *M. azureus* (Fig. 1F). *Pilosocereus gounellei* exhibited proximal CMA⁺ bands in 10 of its 11 pairs (Fig. 1N), whereas both *P. pentaedrophorus* (Fig. 2G) and *Harrisia adscendens* (Fig. 2J) had CMA⁺ bands in four chromosome pairs.

Chromosome markers - FISH

The 5S and 45S rDNA sites were localized in six species, four diploids and two tetraploids. Although position of the 5S rDNA site was an informative chromosome marker, exhibiting variation among the analyzed species (Fig. 3), the position of 45S rDNA was conservative among species and between ploidies - two sites in diploid and four sites in polyploid species/cytotypes.

The diploid *Discocactus zehntneri* (Fig. 2L) had one pair of 5S rDNA site, and *Pilosocereus pentaedrophorus* (Fig. 2H) exhibited two pairs of 5S rDNA sites, one proximal and another adjacent to 45S rDNA, whereas the two other diploid species had 5S rDNA duplications (two sites on the same chromosome): (1) *Cereus jamacaru* (Fig. 1E) with four pairs of 5S rDNA loci - one proximal pair, one subterminal pair, plus one chromosome pair at a proximal and an interstitial site on one chromosome arm; (2) *Pilosocereus chrysostele* (Fig. 2B) with two pairs of 5S rDNA sites on the chromosome bearing the 45S rDNA site – one 5S rDNA pair is adjacent to the 45S rDNA and another is interstitial (detail in Fig. 2B), plus an additional proximal 5S rDNA pair observed on another

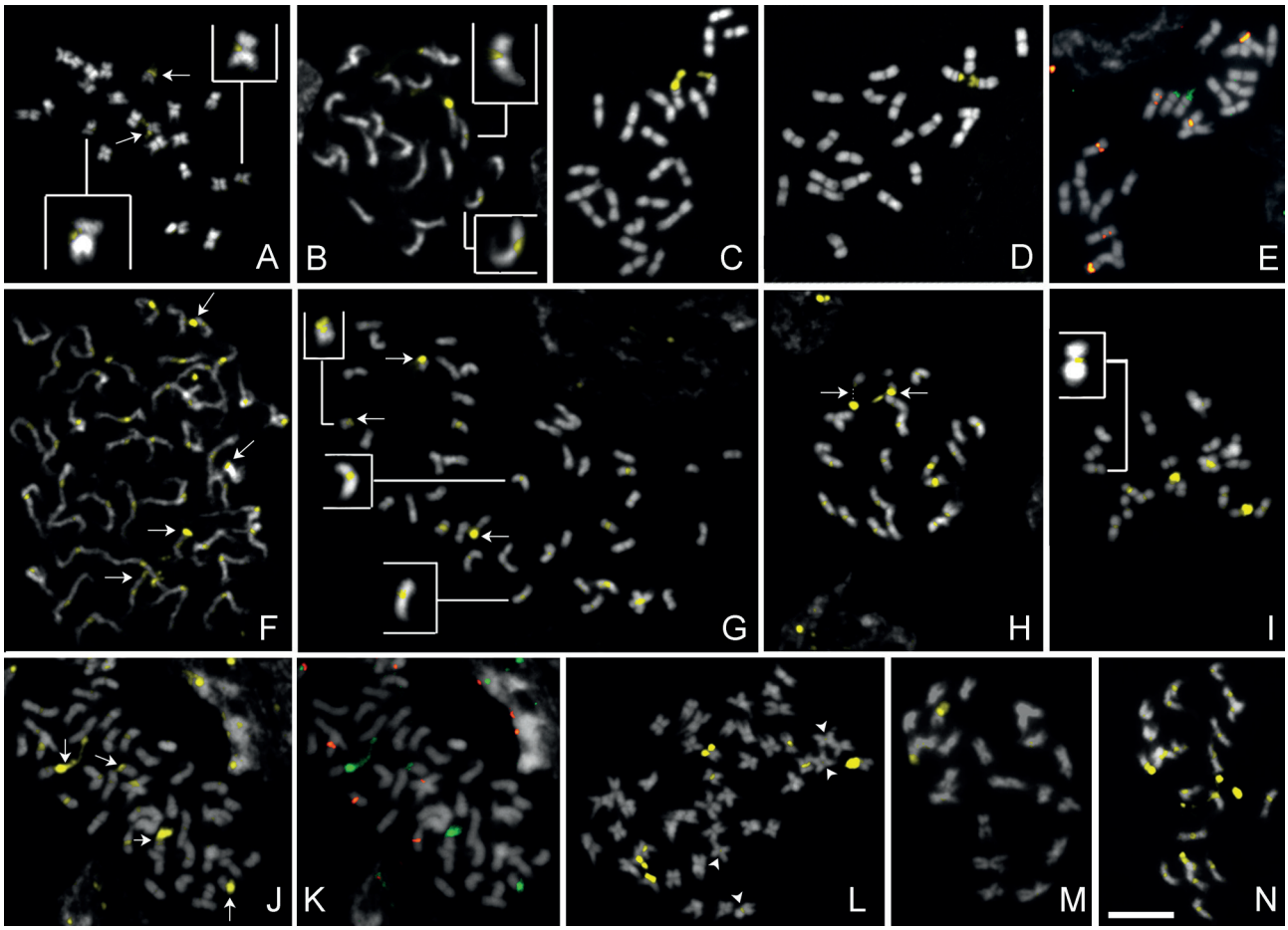

Figure 1. CMA/DAPI banding and *in situ* hybridization in Cactoideae mitotic metaphases. **(A)** *Arrojadoa dinae* (2*n* = 22)**;** **(B)** *A. penicillata* (2*n* = 22); **(C)** *A. rodantha* (2*n* = 22); **(D, E)** *Cereus jamacaru* (2*n* = 22); **(F)** *Melocactus azureus* (2*n* = 44); **(G)** *Melocactus ernestii* (2*n* = 44); **(H)** *Melocactus lanssensianus* (2*n* = 22); **(I)** *Melocactus levitestatus* (2*n* = 22); **(J, K)** *Melocactus oreas* (2*n* = 44); **(L)** *Melocactus zehntneri* (2*n* = 44); **(M)** *Micranthocereus flaviflorus* (2*n* = 22); **(N)** *Pilosocereus gounellei* (2*n* = 22). Pictures in **A - D, F - J** and **L - N** show metaphases with double fluorochrome staining banding using CMA (yellow) and DAPI (gray). Metaphases in **E** and **K** show *in situ* hybridization with 5S rDNA probe (red) and 45S rDNA probe (green). All metaphases were stained with DAPI pseudocolored in gray. Inserts in **A**, **B**, **G**, and **I** show tiny pericentromeric CMA⁺ bands. Arrows in **A, F, G, H, J** point to terminal CMA⁺ bands. Arrowheads in **L** point to tiny proximal CMA⁺ bands. Dots in **H** indicate the distended NOR. Bar in **N** is equivalent to 10μm.

chromosome pair (see asterisks in Fig. 2B). The two polyploid species, *P. pachycladus*. subsp. *pernambucoensis* (Fig. 2F) and *M. oreas* (Fig. 1K), also presented variation regarding the 5S rDNA sites; whereas the former had two pairs of proximal 5S rDNA sites plus two interstitial pairs adjacent to the 45S rDNA (four pairs in total), the latter exhibited only two proximal pairs that were co-localized with the CMA^+/$DAPI^-$ heterochromatic bands - a typical characteristic of *M. oreas* (compare Fig. 1J and K, see Fig. 3).

The 45S rDNA sites were always co-localized with terminal CMA^+/$DAPI^-$ bands. The number of sites varied from two in the diploid species, $2n = 22$ - *Cereus jamacaru* (Fig. 1E), *Pilosocereus chrysostele* (Fig. 2B), *P. pentaedrophorus* (Fig. 2H) and *Discocactus zehntneri* (Fig. 2L) to four sites in the tetraploid species, $2n = 44$ - *Melocactus oreas* (Fig. 1K) and *P. pachycladus* subsp. *pernambucoensis* (Fig. 2F).

Literature survey

The chromosome number survey showed polyploid counts for 31.32 % of Cactaceae species, whereas dysploidy was poorly represented, occurring in just 6.65 % of 677 available species (Tabs. 2 and S1 in supplementary material). Polyploidy was concentrated in Cactoideae (20 %) and Opuntioideae (65.5 %), absent in *Pereskia* and with a single record in *Maihuenia* (one species with a polyploid cytotype) (Tab. 2). However, even in Cactoideae and Opuntioideae, polyploidy is not evenly spread and is especially concentrated in some tribes, such as Cereeae (19 of 42 species, 45.24 %) and Trichocereeae (40 of 99 species, 40.40 %) (Tab. 2). The high frequency of polyploidy observed in Opuntioideae (65.5 %) is a consequence of polyploid records in *Opuntia* and *Cylindropuntia*, the two genera with the greatest number

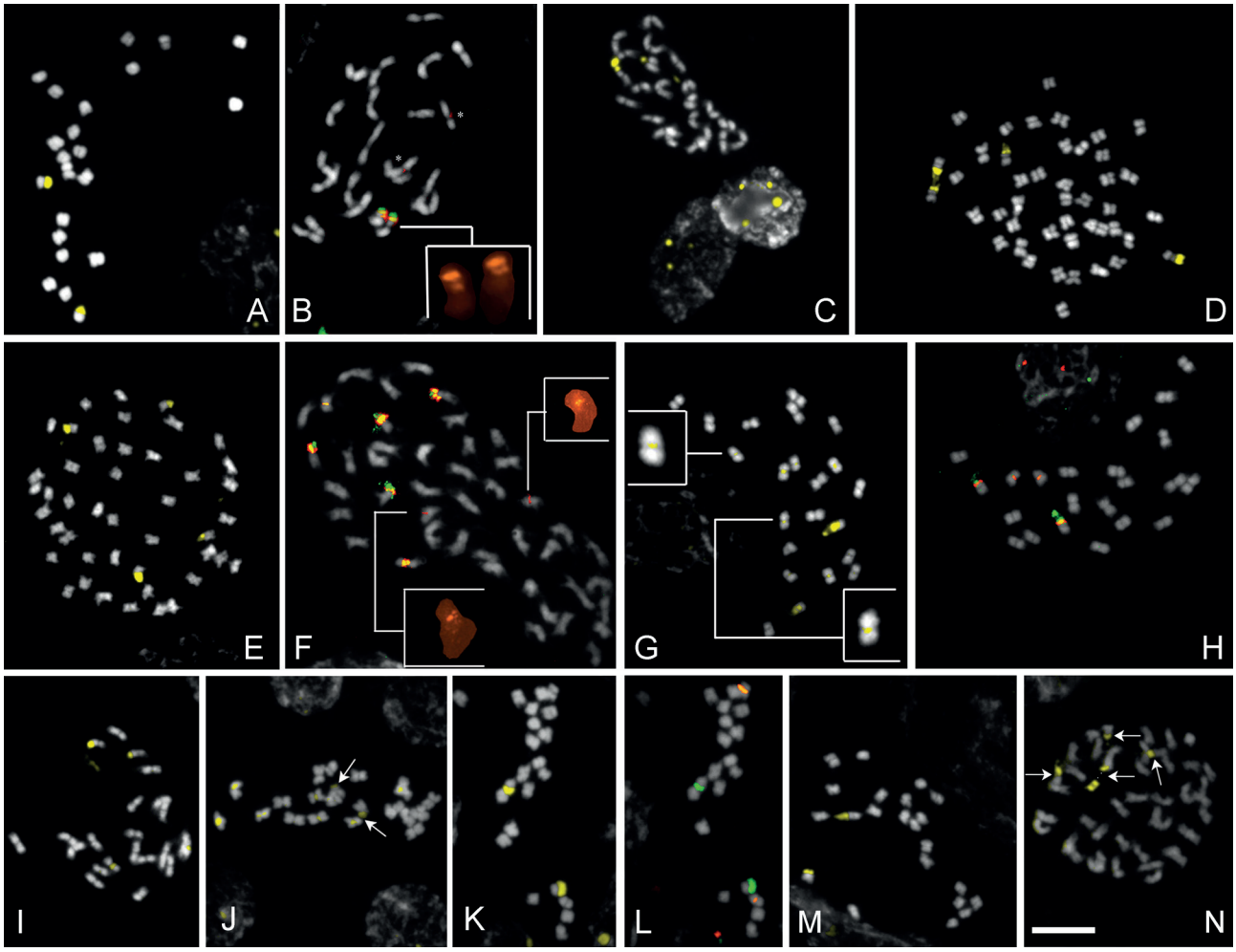

Figure 2. CMA/DAPI banding and *in situ* hybridization in Cactoideae mitotic metaphases. **(A, B)** *Pilosocereus chrysostele* ($2n = 22$); **(C, D)** *Pilosocereus pachycladus* sbsp. *pachycladus* ($2n = 22$, $2n = 44$; respectively); **(E, F)** *Pilosocereus pachycladus* sbsp. *pernambucoensis* ($2n = 44$); **(G, H)** *P. pentaedrophorus* ($2n = 22$); **(I)** *Stephanocereus luetzelburgii* ($2n = 22$); **(J)** *Harrisia adcendens* ($2n = 22$); **(K, L)** *Discocactus zehntneri* ($2n = 22$); **(M)** *Epiphyllum anguliger* ($2n = 22$); **(N)** *Hylocereus setaceus* ($2n = 44$). Metaphases in **A**, **C - E**, **G**, **I - K**, **M**, and **N** show metaphases with double fluorochrome staining banding using CMA (yellow) and DAPI (gray). Metaphases in **B**, **F** and **L** show *in situ* hybridization with 5S rDNA probe (red) and 45S rDNA probe (green). Inserts in **B** and **F** show small proximal 5S rDNA site. Inserts in **G** show tiny pericentromeric CMA^+ bands. Arrows in **J** and **N** point terminal CMA^+ bands. Dots in **N** indicate the distended NOR. Bar in **N** is equivalent to 10μm.

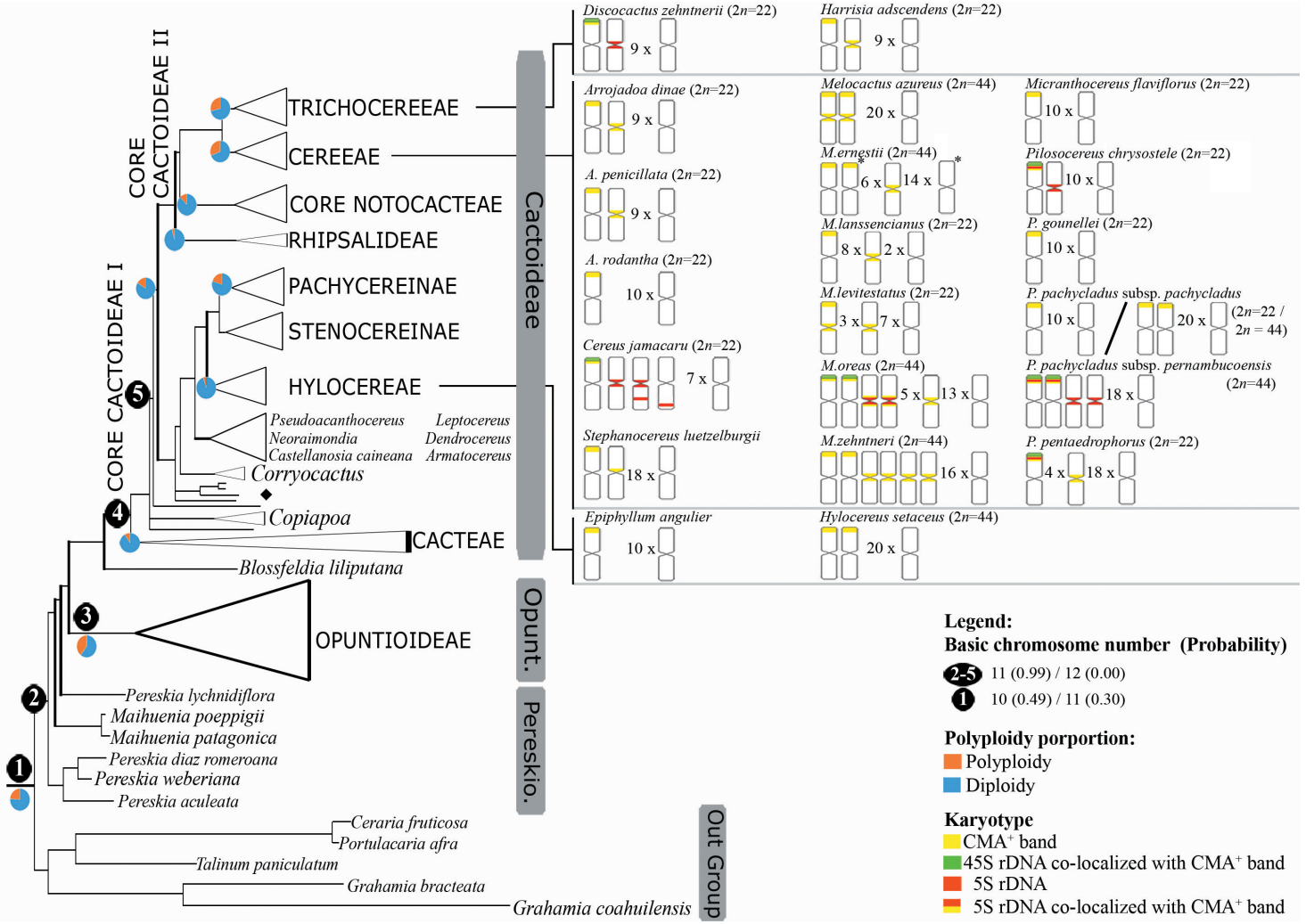

Figure 3. Basic chromosome number and numerical chromosome changes in Cactaceae. The two more likely base chromosome numbers for five selected nodes are presented, followed by their probability in parenthesis. The proportion of polyploidy is presented in the circles (blue for dyploidy, orange for polyploidy). For species analyzed by chromosome banding and FISH, the monoploid ideograms are presented with species grouped by its tribe. The CMA$^+$ bands (yellow), 5S rDNA sites (red) and 45S rDNA sites (green) are shown. The chromosomes are presented as metacentric for representative proposes, and centromeres are not precisely positioned. The chromosomes with an asterisk in *M. ernestii* indicate an odd number of chromosome and not a pair of chromosomes. The first asterisk indicates a single chromosome with terminal CMA$^+$ band and the second asterisk indicates 14 chromosome pairs plus a single chromosome (*i.e.*, a total of 15 chromosomes), all without a heterochromatic band. For the sake of simplicity, a small group of terminals is indicated by ♦ and group the following genera - *Eulychnia* (two first terminals), *Austrocactus*, *Pfeiffera*, and *Frailea*.

Table 2. Chromosome number survey in Cactaceae. Species are presented following the taxonomic proposal of Anderson (2001). The number of species with available chromosome number is presented followed by the number of species with polyploidy (polyploid species plus species with polyploid cytotype) and dysploid counts. For each tribe/subfamily and for the whole Cactaceae, the number of taxonomic groups analyzed is provided, with the total number of polyploid and dyploid species. The percentage of polyploidy and dysploidy are presented into brackets.

Family Cactaceae (677 sp., 212 polyploid species/cytotype [31.32 %], 45 dysploid species/cytotype[6.65 %])			
Subfamily Cactoideae (8 tribes, 79 genera, 491 species, 60 polyploid species, 37 polyploid cytotype, 9 dysploid species, 32 dysploid cytotype - Polyploidy is involved in 20 % of species, dysploidy is involved in 8.35% of species)			
	Number of species with *2n*	Polyploidy	Dysploidy
Tribe Hylocereeae (7 genera, 38 species, 2 polyploid species, 3 dysploid species, x=11)			
Disocactus Lindl.	13	0	2
Epiphyllum Haw.	6	0	0
Heliocereus (A.Berger) Britton & Rose	7	1	0
Hylocereus (A. Berger) Britton & Rose	7	1	0
Pseudorhipsalis Britton & Rose	2	0	0
Selenicereus (A. Berger) Britton & Rose	8	1	1
Weberocereus Britton & Rose	1	0	0
Tribe Cereeae (7 genera, 42 species, 13 polyploid species, 6 polyploid cytotypes, 1 dysploid species)			
Arrojadoa Britton & Rose	2	0	0
Cereus Mill.	12	1	2
Melocactus Link & Otto	20	16	0
Micranthocereus Backeb.	1	0	0
Pilosocereus Byles & Rowley	5	2	0
Praecereus Buxb.	1	0	0
Stephanocereus A. Berger	1	0	0
Tribe Trichocereeae (14 genera, 99 species, 26 polyploid species, 14 polyploid cytotypes, 2 dysploid cytotypes, x=11)			
Cleistocactus Lem.	6	1	0
Discocactus Pfeiff.	2	0	0
Echinopsis Zucc.	11	2	1
Espostoa Britton & Rose	4	2	0
Gymnocalycium Pfeiff.	46	17	1
Haageocereus Backeb.	11	4	0
Harrisia Britton	1	0	0
Lasiocereus F. Ritter	2	0	0
Lobivia Britton & Rose	1	1	0
Matucana Britton & Rose	1	0	0
Mila Britton & Rose	1	0	0
Rebutia K. Schum.	8	6	0
Sulcorebutia Backeb.	1	0	0
Weberbauerocereus Backeb.	4	2	0
Tribe Notocacteae (6 genera, 14 species, 2 polyploid species, 1 dysploid species, x=19, x=11)			
Austrocactus Britton & Rose	1	0	0
Blossfeldia Werderm.	1	1	0
Eriosyce Phil.	3	0	0
Parodia Speg.	2	1	0
Pyrrhocactus (A. Berger) Backeb. & F.M. Knuth	6	0	0
Notocactus (K.Schum.) Fric (x=19)	1	0	1
Tribe Rhipsalideae (6 genera, 43 species, 1 polyploid species, 2 dysploid species, 1 polyploid cytotype, x=11)			
Hatiora Britton & Rose	3	0	0
Lepismium Pfeiff	7	0	0
Pfeiffera Salm-Dyck	27	1	1
Rhipsalis Gaertn.	3	1	1
Schlumbergera Lem.	3	1	1
Zygocactus K. Schum.	1	0	0
Tribe Browningieae (1 genus, 1 species, x=11)			
Browningia Britton & Rose	1	0	0
Tribe Pachycereeae (14 genera, 47 species, 8 polyploid species, 4 polyploid cytotypes, 1 dysploid cytotype, x=11)			
Acanthocereus Britton & Rose	1	0	0

Table 2. Cont.

	Number of species with *2n*	Polyploidy	Dysploidy
Bergerocactus Britton & Rose	1	0	0
Carnegiea Britton & Rose	1	0	0
Corryocactus Britton & Rose	1	0	0
Echinocereus Engelm	29	10	1
Lemaireocereus Britton & Rose	1	0	0
Leptocereus (A.Berger) Britton & Rose	1	0	0
Lophocereus (A.Berger) Britton & Rose	1	0	0
Marginatocereus (Backeb.) Backeb.	1	0	0
Myrtgerocactus Moran	1	0	0
Myrtillocactus Console	2	1	0
Pachycereus (A.Berger) Britton & Rose	2	1	0
Rathbunia Britton & Rose	1	0	0
Stenocereus (A. Berger) Riccob.	5	0	0
Tribe Cacteae (24 genera, 207 species, 8 polyploid species, 12 polyploid cytotype, 2 dysploid species, 10 dysploid cytotype, x=11)			
Ancistrocactus (K. Schum) Britton & Rose	4	1	0
Ariocarpus Scheidw.	3	1	0
Astrophytum Lemaire	4	0	0
Coryphantha Lem.	13	0	0
Dolichothele Britton & Rose	2	0	0
Echinocactus Link & Otto	5	0	1
Echinofossulocactus	1	0	0
Echinomastus Britton & Rose	5	1	0
Epithelantha F.A.C.Weber ex Britton & Rose	1	0	0
Escobaria Rose	11	2	0
Ferocactus Britton & Rose	16	0	0
Hamatocactus Britton & Rose	1	0	0
Lophophora J.M.Coult.	1	0	0
Mammillaria Haw.	109	13	4
Neolloydia Britton & Rose	1	0	0
Neomammillaria Britton & Rose	12	1	0
Obregonia Frič	1	0	0
Pediocactus Britton & Rose	3	0	0
Pelecyphora Ehrenb.	2	0	0
Sclerocactus Britton & Rose	4	0	0
Stenocactus (K. Schum.) A. Berger ex A.W. Hill	2	0	0
Strombocactus Britton & Rose	2	0	0
Thelocactus (K. Schum.) Britton & Rose	3	1	0
Turbinicarpus (Buckeb.) Buxb. & Buckeb.	1	0	0
Subfamily Opuntioideae (16 genera, 171 species, 59 polyploid species, 53 polyploid cytotypes, 2 dysploid cytotypes, x=11 - Polyploidy is involved in 65.5 % of species, dysploidy is involved in 1.17 % of species)			
Austrocylindropuntia Backeb.	6	3	0
Brasiliopuntia (K.Schum.) A.Berger	1	0	0
Consolea Lem.	7	7	0
Corynopuntia F.M.Knuth	8	7	0
Cumulopuntia F. Ritter	1	1	0
Cylindropuntia (Engelm.) F.M.Knuth	34	18	0
Maihueniopsis Speg.	3	3	0
Micropuntia Daston	1	0	0
Nopalea Salm-Dyck	3	0	0
Opuntia Mill.	94	65	2
Pereskiopsis Britton & Rose	2	2	0
Pterocactus K.Schum.	2	1	0
Quiabentia Britton & Rose	2	1	0
Tacinga Britton & Rose	3	1	0
Tephrocactus Lem.	3	2	0
Tunilla D.R. Hunt & Iliff	1	1	0

Table 2. Cont.

	Number of species with *2n*	Polyploidy	Dysploidy
Genus *Maihuenia* (1 genus, 2 species, 1 polyploid cytotype, x=11)			
Maihuenia (F.A.C.Weber) K.Schum.	2	1	0
Genus *Pereskia* (1 genus, 13 species, 1 dysploid specie, 1 dysploid cytotype, x=11)			
Pereskia Mill.	13	0	2

Table 3. Summary of the eight ChromEvol models for the used phylogenetic tree. The Log-likelihood and the AIC values are presented, and the lowest values are indicated in bold and underlined. The best model, constant rate demi-polyploidy est, is indicated by the * and this model was re-run under Optimize Model option with 1,000 simulations. The expectation values for gain, loss, duplication, and demi-duplication under constant rate demi-polyploidy-est are presented, indicating that polyploidy is the more frequent form of chromosome number change.

	ML		Expectation of events (Tev)			
Model	Loglikelihood	AIC	gain events (ascending dysploidy)	loss events (descending dysploidy)	duplication events (polyploidy)	demi-duplications events
Constant rate	79.03	164.1				
Constant rate demi-polyploidy	67.56	141.1				
Constant rate demi-polyploidy-est*	65.3	139.1	4.13294	5.58036	17.5813	3.59838
Constant rate no duplication	253.4	510.9				
Linear rate	76.84	163.7				
Linear rate demi-polyploidy	66.44	142.9				
Linear rate demi-polyploidy-est	64.46	140.9				
Linear rate no duplication	229.9	467.7				

of species analyzed and also an elevated frequency of polyploidy (65 of 94 and 18 of 34 species, respectively) (Tab. S1 in supplementary material).

Chromosome number evolution and base chromosome number in Cactaceae

Based on the chromosome numbers obtained here in addition to those from the literature and an available phylogenetic hypothesis for Cactaceae (Hernandez-Hernandez *et al.* 2011), we performed an analysis for chromosome number evolution and determination of the base chromosome number for Cactaceae. Here, the ChromEvol analysis highlights the importance of polyploidy for cacti. Eight models were tested, and two of them did not consider polyploidy, working as a null hypothesis for this event. These two models, Constant rate no-dupl and Linear rate no-dupl, presented an elevated AIC value compared with the six remaining models that do consider polyploidy (compare the AIC for constant no-duplication, 510.9, and for linear rate no duplication, 467.7, with remainder models; see Tab. 3), reinforcing the importance of polyploidy.

The best model, estimated by the lowest AIC value, was 'Constant rate demi-*est*' (Tab. 3), a model that considers (1) gain and loss of chromosomes (ascending and descending dysploidy), (2) duplication (polyploidy; *i.e.*, a multiplication of the chromosome number by a factor of 2×) and (3) demi-duplications (demi-polyploidy; *i.e.*, a multiplication of the chromosome number by a factor of 1.5× caused by fusion of gametes with different ploidies) as causes of chromosome number variation. In this model, the rate for polyploidy and demi-polyploidy were considered as independent rates (ρ is independent of μ), and for cacti, the expectation values were different for ρ and μ (Tab. 3). Constant rate models consider that all rates were constant, *i.e.*, the changes were independent of current chromosome numbers.

Considering the base chromosome numbers, the same result was obtained in the six tested models that include polyploidy (data not shown): $x = 11$ was estimated for Cactaceae, as well as Cactoideae, Opuntioideae and *Pereskia* and *Maihuenia* (the non-monphyletic subfamily Pereskioideae) (Fig. 3). Both *Pereskia*-as ancestral and *Pereskia*-as-derived hypothesis would give the same result due to the stability of chromosome number in Cactaceae.

Gain and loss of chromosomes (ascending and descending dysploidies) are restricted to the outgroup – a gain in *Talinum paniculatum* and loss in *Grahamia bracteata* and *Portulacaria afra*, whereas polyploidy is widespread in Cactaceae (Fig. 3).

Discussion

Chromosome number and the importance of polyploidy

For three taxa, chromosome numbers were described here for the first time - *Arrojadoa dinae* and *Pilosocereus pachycladus* subsp. *pachycladus and* subsp. *pernambucoensis. For the other 18 species, our* new chromosome numbers agree with previously published data (Darlington & Wylie 1955; Pedrosa *et al.* 1999; Goldblatt & Johnson 2003; 2006; Castro *et al.* 2013; Peñas 2018; Tab. S1 in supplementary material), except for *M. zehntneri* and *M. ernestii*, which had polyploid karyotypes in our accessions, $2n = 44$, but diploid

counts, 2*n* = 22, in a previous study (Assis *et al*. 2003). Such chromosome number variation represents polyploid populations in a single species, a frequent phenomenon in Cactaceae (Pinkava & McLeod 1971; Pinkava *et al*. 1973; Castro *et al*. 2013; 2016).

Polyploidy has been long considered one of the main evolutionary phenomena in plants (Stebbins 1971; Grant 1981), and this subject still being reviewed to evaluate its importance in angiosperm diversification (Peer *et al*. 2017; Rice *et al*. 2019; Magallón *et al*. 2019). Diversification of Cactaceae fits this model well, and polyploidy is more frequent after Maihuenioideae and Pereskioideae diverged, *i.e.*, in the called 'core cacti'. This group diversified at ≈25 Ma, with subsequent increase in diversification rate (Arakaki *et al*. 2011), which seems to occur concomitant with an increased frequency of polyploidy. In a similar way, many families and genera display an association between species-richness and occurrence of polyploidy (Soltis *et al*. 2009), reinforcing the hypothesis that polyploidy has been important for angiosperm diversification. Among Cactaceae, the well-studied genus *Opuntia* (Opuntioideae) is one of the largest in the family, with 226 accepted species (following The Plant List 2019), and also present high frequency of polyploidy (70 % of species are polyploid or have some polyploid cytotypes). Nevertheless, this assumption could be biased by study effort more than a positive relationship between polyploidy and diversification. In this sense, the efforts should now be concentrated in the poorly studied groups with recent diversification, such as Cereinae (tribe Cereeae, diversification *ca*. 7.5 - 6.5 Ma; Arakaki *et al*. 2011), to achieve a more realistic scenario for the effect of polyploidy on cacti evolution and its role in diversification.

The recent polyploidy observed in Cactaceae has frequently been hypothesized to involve interspecific and intergeneric hybridization (Baker *et al*. 2009). For example, it is assumed that the morphological variation observed in *Melocactus* (Zappi 1994; Anderson 2001) could be related to hybridization and polyploidy, as observed in the sympatric zone of *Melocactus paucispinus* and *M. concinnus* where there have been hybrids detected with variable morphologies (Lambert *et al*. 2006). Here, polyploidy was detected also in sympatric populations of *Pilosocereus pachycladus* subsp. *pachycladus* (diploid and polyploid cytotypes) and subsp. *pernambucoensis* (all populations are polyploid). Both cytotypes of *P. pachycladus* subsp. *pachycladus* are terrestrial and occur in specific vegetation locally known as *carrasco* - arborescent semi-arid vegetation in northeastern Brazil. The polyploid *P. pachycladus* subsp. *pernambucoensis* occurs in Bahia State in populations sympatric with *P. pachycladus* subsp. *pachycladus*, where they could hybridize and expand to the remaining states of northern Brazil, always colonizing extremely xeric vegetation similar to *carrasco* (Zappi 1994). The wider geographic distribution of the tetraploid subsp. *pernambucoensis* reflects the positive effect of polyploidy, increasing the genetic variability and putative allowing it to colonize a wide range of extreme habitats (Leitch & Leitch 2008; Fawcett & Peer 2010; McIntyre 2012; Theodoridis *et al*. 2013; Ramsey & Ramsey 2014; Oberlander *et al*. 2016).

Chromosome markers - Heterochromatic bands and rDNA loci

The presence of both terminal and proximal CG-rich fluorescent bands detected here seems to be a common characteristic among Cactoideae species (Peñas *et al*. 2008; 2009; 2011; Moreno *et al*. 2015). *Pereskia* did not have proximal CMA^+ bands (Peñas *et al*. 2014; Castro *et al*. 2016), suggesting that the amplification of CG-rich heterochromatin occurred in the common ancestor of Opuntioideae and Cactoideae.

Variation in heterochromatic bands has long been used in karyotypic characterization among species, *e.g.* Orchidaceae (Koehler *et al*. 2008; Moraes *et al*. 2016; 2017), as well as, among varieties, as in species of *Citrus* (Guerra 1993; Yamamoto & Tomiaga 2003; Moraes *et al*. 2007). Independent of the taxonomic group, there is a tendency for heterochromatin to be CG-rich and not AT-rich. It is also true for Cactaceae, with only one report of AT-rich heterochromatin in *Pyrrhocactus* from Argentina (Peñas *et al*. 2008). The CMA^+ band pattern proved to be taxonomically informative in cacti, introducing useful chromosome markers in the stable karyotypes typical of Cactaceae. For example, in *Melocactus* two species, *M. ernestii* and *M. oreas*, have long spines but distinguishing these two species based on morphology is a challenging task. However, they can be differentiated by the number of CG-rich heterochromatin blocks. In *Arrojadoa*, the proximal CMA^+ bands are potentially informative for this small genus endemic to northern Brazil, differentiating taxa with heterochromatic bands (here, *A. dinae* and *A. penicillata*) from taxa without proximal bands (*A. rodantha*). In this sense, when studying a group with stable chromosome numbers such as cacti, the use of additional chromosome markers is mandatory, allowing us to distinguish species and supporting taxonomy studies, in addition to improving the understanding of chromosome evolution.

Fluorescent *in situ* hybridization provides additional chromosome markers for these analyses. The the physical mapping of 45S rDNA exhibited a conserved pattern, with the number of sites strictly correlated with species ploidy: two sites in the diploid species, four sites in the tetraploid species (Peñas *et al*. 2009; Moreno *et al*. 2015; Castro *et al*. 2016; result presented here). All 45S rDNA sites were terminally localized and co-localized with CMA^+ bands, in agreement with the most common situation observed in plants (Lima-de-Faria 1980; Roa & Guerra 2012). In contrast, 5S rDNA sites were variable in number and position, occupying proximal and interstitial positions and occasionally adjacent to the 45S rDNA sites, as observed in other Cactaceae such as *Lepismium* (Moreno *et al*. 2015), or

located on different chromosomes. As observed in *M. oreas*, 5S rDNA could also be detected as a heterochromatic block. The 5S consists of a transcriptional unit of 120 nucleotides separated from the next copy by a non-transcribed spacer (NTS). Although NTS varies in length and sequence, with a rapid rate of concerted evolution, the 5S exon is highly conserved (Rebordinos *et al*. 2013). Changes in the NTS region could involve shifts to AT-rich or CG-rich, and due to amplification it can be detected as a heterochromatic block by chromosome banding, whereas the remaining sites in the same karyotype and closely related species are not identified as AT- or CG-rich. In addition, 5S rDNA is known to be part of a multigene family that could be linked to different multigene families, *e.g.* transposable elements (TE) as demonstrated in animals (Rebordinos *et al*. 2013) and plants. For example, the Cassandra element, a retrotransposon associated with 5S rDNA in plants could facilitate the movement and creation of additional 5S rDNA sites throughout the genome (Kalendar *et al*. 2008). Hybridization also could favor TE-movement, a consequence of which is dynamism of 5S rDNA sites associated with TEs, as detected in *Aegilopsis* (Raskina *et al*. 2004) and some fish species (Fontdevila *et al*. 2005). It is possible that in Cactaceae the combination of hybridization and polyploidy favors the dynamic movement of retrotransposons, creating new sites with or without a relationship to ploidy, as observed with the 45S rDNA here.

Finally, the diversity of 5S rDNA sites also highlights the importance of structural chromosome rearrangement, such as inversions. This phenomenon could be responsible for the creation of two sites in the same chromosome arm, as observed in both *Cereus jamacaru* and *Pilosocereus chrysostele*. It is possible that a break point could have occurred inside the original 5S rDNA site (an event putatively favored by the TE activity), and after an inversion, inserted some copies of 5S rDNA, creating a new site while retaining some copies at the original site. Such an event has previously been suggested for some groups of plants, for example, different unrelated species of Orchidaceae (e.g., see Moraes *et al*. 2012; 2017; Lee *et al*. 2017) and *Nothorcordum* (Amaryllidaceae; Souza *et al*. 2012). In this sense, evolution of 5S rDNA sites in cacti contrasts to evolution of 45S rDNA, with 5S being more variable than 45S, which is the opposite of the commonly accepted hypothesis that position and number of 5S rDNA loci in plants are usually more conserved than those of 45S rDNA loci (Roa & Guerra 2012; 2015).

Conclusions

Except for *Pereskia*, in which there have been no records of polyploidy, this event seems to be an important evolutionary mechanism in Cactaceae, commonly associated with natural hybridization. The use of karyotype characterization in combination with phylogenetic analyses provides a useful tool in chromosome evolution studies. In our analyses, $x = 11$ was confirmed, and the important role of polyploidy in chromosome evolution in our results confirms the traditional assumptions about Cactaceae. Heterochromatin distribution and 5S rDNA positions were both considered as good cytotaxonomic markers in Cactoideae, mainly in *Arrojadoa*, *Melocactus* and *Pilosocereus*, and are likely to be useful in other genera as well. For the last two genera, both with many taxonomic problems due to the recurrent interspecific hybridization, karyotype characterization based on CMA^+ bands and 5S rDNA appears to be promising tools to aid in species delimitation.

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