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# Molecular cytogenetic insights into the evolution of the epiphytic genus *Lepismium* (Cactaceae) and related genera

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Changes in chromosome structure and number play an important role in plant evolution. This was investigated in the Neotropical epiphytic cacti: all *Lepismium* spp. and some related *Rhipsalis* spp. Both genera have species with disjunct distributions between the paranas of south-eastern Brazil and north-eastern Argentina and the yungas forests of the eastern Andes. Karyotypes, fluorescent banding and fluorescence *in situ* hybridization (FISH) studies using rDNA probes were performed. A time-calibrated phylogenetic tree was generated to place the karyological information and biogeographical history in an explicit evolutionary context. All species were $2n = 22$ and showed symmetrical karyotypes comprising only metacentric chromosomes of similar sizes. The heterochromatin bands were always associated with chromosome satellites coinciding with the location and number of the 18S–5.8S–26S rDNA loci. The 5S rDNA loci had more heterogeneous profiles with one or two loci per haploid genome. Phylogenetic analysis suggested an ancient duplication event of the 5S rDNA loci and more recent post-speciation translocation and deletion events. These genome restructurings are estimated to have occurred approximately 13.98 Mya in the middle Miocene, after *Lepismium* and *Rhipsalis* diverged. The ancestor of *Lepismium* may have had a similar karyotype to *L. lumbricoides* and the *Rhipsalis* spp. (i.e. one 5S locus on chromosome 2). Both genera hypothetically originated in the yungas (north-eastern Argentina and southern Bolivia), but diversification of the *Lepismium* crown group probably originated from populations with duplicated 5S loci in the parana forests of south-eastern Brazil (8.70 Mya in the late Miocene). Two migration events between the yungas and parana forests were suggested to explain the extant distribution of *Lepismium* spp. These results make *Lepismium* a model system for the study of the complex chromosomal evolution in plants.  *Botanical Journal of the Linnean Society*, 2015, **177**, 263–277.

ADDITIONAL KEYWORDS: 18S–5.8S–26S and 5S ribosomal genes – divergence time – historical biogeography – karyotype – molecular phylogeny – Neotropical flora – *Pffeifera* – *Rhipsalis*.

## INTRODUCTION

The South American genus *Lepismium* Pfeiff. includes six epiphytic species from rain forests (Hunt, Taylor & Charles, 2006; Korotkova *et al*., 2010) that have a disjunct distribution: (i) south-eastern Brazil and (ii) north-eastern Argentina and southern Bolivia and north-western Argentina. Its members are characterized by mesotonic branching, indeterminate stem segments, absence of apical composite areoles, small, white, laterally positioned flowers, angled pericarpels and naked dark red fruits (Korotkova *et al*., 2011). It is monophyletic and is included in the epiphytic tribe Rhipsalideae (subfamily Cactoideae) with *Hatiora* Britton & Rose, *Rhipsalis* Gaertn. and *Schlumbergera* Lem. (Korotkova *et al*., 2011). Traditionally, *Pfeiffera* was included in Rhipsalideae, but Korotkova *et al*. (2010) transferred the genus to Echinocereeae, as an independent epiphytic lineage. Cactoideae, the largest subfamily of cacti, has a great diversity of growth forms adapted to different environments, from arid to temperate and tropical regions (Wallace & Gibson,

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2002; Godínez-Álvarez, Valverde & Ortega-Baes, 2003). Cactoideae are estimated to have originated *c*. 24.4 Mya, but the radiation of extant species is much younger (8–6 Mya; Arakaki *et al*., 2011).

Cactaceae are highly conserved in some chromosomal characteristics, such as basic chromosome number ($x = 11$) and symmetric karyotypes (e.g. Ross, 1981; Pinkava & Parfitt, 1982; Cota & Wallace, 1995; Bandyopadhyay & Sharma, 2000; Las Peñas, Bernardello & Kiesling, 2008). Chromosome numbers of most Rhipsalideae are known, but karyotypes have been described only for *Schlumbergera truncata* (Haworth) Moran and *Schlumbergera* × *buckleyi* (T.Moore) Tjaden (Ortolani, Mataqueiro & Moro, 2007), *Lepismium houlletianum* (Lem.) Barthlott, *Rhipsalis floccosa* Salm-Dyck ex Pfeiff. subsp. *pittieri* (Britton & Rose) Barthlott & N.P.Taylor, and *Hatiora epiphylloides* (Porto & Werderm.) P.V.Heath (Pankin, 2005). In addition, the distribution of heterochromatin and physical mapping of ribosomal genes have not previously been examined in Rhipsalideae.

Despite the apparently invariable chromosome morphology, studies using molecular cytogenetic techniques suggest a dynamic picture of chromosome restructuring in *Pyrhocactus* A.Berger (Las Peñas *et al*., 2008). The mobility and distribution patterns of rDNA genes can therefore be used as species-specific karyotype markers (Hasterok *et al*., 2006; Chacón *et al*., 2012). Interpreting changes in the number and location of rDNA loci in related species in a phylogenetic framework can be a powerful approach to enable a clearer understanding of the mechanisms and directions of chromosomal changes and their impact on plant evolution (Lim *et al*., 2000; Clarkson *et al*., 2005; Weiss-Schneeweiss *et al*., 2008).

Biogeographical patterns in some Rhipsalideae are interesting. Both *Lepismium* and *Rhipsalis* have intracontinental disjunct distributions; in addition, *Rhipsalis baccifera* (J.S.Muell.) Stern is the only cactus with an inter-continental distribution (Barthlott & Taylor, 1995). The intra-continental disjunct distributions may be explained either by long-distance dispersal or vicariance. Donoghue (2011) indicated that to distinguish between these two, molecular dating approaches are important to establish a timeline of divergence to determine whether orogenic or climatic events have caused the separation or extinction of species in certain areas. Popp, Mirré & Brochmann (2011) suggested the possibility of integrating not just reliable age estimates into likelihood-based biogeographical analyses, but also a variety of other biologically relevant factors, with the overall aim of eventually discovering general broad evolutionary patterns.

The aim of this study was to provide the first characterization of ten members of Rhipsalideae (six *Lepismium* and four *Rhipsalis*). In addition, two *Pfeiffera* spp. (Echinocereae) were analysed. The study analysed the karyotypes, amount of heterochromatin and physical location of ribosomal genes. The evolutionary dynamics of the karyotypes in *Lepismium* in a phylogenetic context are also discussed. Lineage-specific rearrangements leading to different chromosome structures were tested using ancestral state reconstruction approaches based on maximum-likelihood methods to propose insights into chromosome evolution in Rhipsalideae. Additionally, seven plastid sequences were analysed to investigate the origin of the geographical disjunctions in species distributions and to evaluate the role of vicariance and long-distance dispersal.

## MATERIAL AND METHODS

### Plant material

Collection data of the 12 species and 41 localities studied are given in Table 1. Voucher specimens were deposited in the herbarium of the Museo Botánico de Córdoba (CORD).

### Pretreatment, fixation and chromosome preparations

For the preparation of metaphase chromosomes, adventitious roots from potted plants were pretreated with 2 mM 8-hydroxyquinoline for 24 h at 4 °C and fixed in 3:1 ethanol/acetic acid. For slide preparation, root tips were washed twice in distilled water (10 min each), digested with a solution of 2% cellulose (Sigma-Aldrich) and 20% pectinase (from *Aspergillus niger*; SigmaAldrich) for 45 min at 37 °C and squashed in a drop of 45% acetic acid (Schwarzacher, Ambros & Schweizer, 1980). After coverslip removal in liquid nitrogen, the slides were stored at −20 °C.

### Karyotype analysis

Slides were stained with Giemsa (Guerra, 1983) and permanent mounts were made with Entellan (Merck). Ten metaphases from different individuals per species were photographed with a phase-contrast optical Zeiss Axiophot microscope and a Leica DFC300FX camera. Photographs were taken to enable 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 that of its chromosome arm. The arm ratio ($r = l/s$) was then calculated and used to classify the chromosomes following Levan, Fredga & Sandberg (1964). In addition, mean chromosome length (*C*), mean total haploid chromosome length of the karyotype based on the mean chromosome lengths (*tl*) and mean arm

**Table 1.** Taxa studied and karyotype characters obtained from ten metaphases per species: mean chromosome length (*C*), mean total haploid chromosome length (*tl*), ratio between the largest and the smallest chromosomes in the complement (*R*), mean arm ratio (*r*), intrachromosomal asymmetry index ($A_1$) and interchromosomal asymmetry index ($A_2$) (Romero Zarco, 1986), and amount of heterochromatin expressed as a percentage of the haploid karyotype length (*%Ht*)

| Taxa | 2*n* | *C* (μm) | *tl* (μm) | *R* | *r* | $A_1$ | $A_2$ | *%Ht* | Collection data |
|---|---|---|---|---|---|---|---|---|---|
| *Pfeiffera* | | | | | | | | | |
| *Pfeiffera ianthothele* (Monv.) F.A.C.Weber | 22 | 1.77 | 19.48 | 2.13 | 1.22 | 0.17 | 0.17 | 2.40 | Salta, Dpto. La Caldera, *Barboza et al. 2204.*<br>Salta, Dpto. La Caldera, *Barboza et al. 2207.*<br>Tucumán, Dpto. Chicligasta, *Barboza et al. 2132.* |
| *P. monacantha* (Griseb.) P.V.Heath | 22 | 1.61 | 17.69 | 1.83 | 1.12 | 0.10 | 0.15 | 2.33 | Jujuy, Dpto. Libertador Gral. San Martín, *Barboza et al. 2248*. |
| Rhipsalideae | | | | | | | | | |
| *Lepismium aculeatum* (F.A.C.Weber) Barthlott | 22 | 2.13 | 23.48 | 1.77 | 1.20 | 0.17 | 0.15 | 1.99 | Salta, Dpto. Oran, *Moreno & Amarilla s.n.*<br>Santiago del Estero, Dpto. Guasayán, *Amarilla & Demaio 30.*<br>Tucumán, Dpto. Graneros, *Kiesling R. s.n.* |
| *L. cruciforme* (Vell.) A.Cast. | 22 | 1.90 | 20.90 | 1.91 | 1.21 | 0.17 | 0.15 | 1.01 | Corrientes, Dpto. Mercedes, *Moreno & Amarilla s.n*.<br>Corrientes, Dpto. Santo Tome, *Barboza et al. 2088*.<br>Misiones, Dpto. Apóstoles, *Moreno & Iglesias 9.*<br>Misiones, Dpto. Guaraní, *Barboza et al. 2095.*<br>Misiones, Dpto. Obera, *Moreno & Iglesias 23.*<br>Misiones, Dpto. San Javier, *Moreno & Iglesias 17.*<br>Misiones, Dpto. 25 de Mayo, *Moreno & Iglesias 25*. |
| *L. houlletianum* (Lem.) Barthlott | 22 | 2.04 | 22.43 | 1.64 | 1.22 | 0.18 | 0.13 | 1.65 | Misiones, Dpto. Guaraní, *Moreno & Iglesias 27.*<br>Misiones, Dpto. San Pedro, *Kiesling R. s.n.* |
| *L. lorentzianum* (Griseb.) Barthlott | 22 | 1.40 | 15.45 | 1.64 | 1.22 | 0.18 | 0.14 | 1.06 | Jujuy, Dpto. Ledesma, *Barboza et al. 2259.*<br>Tucumán, Dpto. Yerba Buena, *Bernardello 922*. |
| *L. lumbricoides* (Lem.) Barthlott | 22 | 2.08 | 22.92 | 1.73 | 1.22 | 0.17 | 0.14 | 1.91 | Corrientes, Dpto. Mercedes, *Moreno & Amarilla s.n*.<br>Entre Ríos, Dpto. Colon, *Chiarini 712.*<br>Jujuy, Dpto. Gral. Manuel Belgrano, *Moreno s.n.*<br>Misiones, Dpto. Apóstoles, *Moreno & Iglesias 8.*<br>Misiones, Dpto. Obera, *Moreno & Iglesias 24.*<br>Salta, Dpto. Capital, *Bernardello 926.*<br>Tucumán, Dpto. Yerba Buena, *Bernardello 924.* |

**Table 1.** *Continued*

| Taxa | 2*n* | *C* (μm) | *tl* (μm) | *R* | *r* | $A_1$ | $A_2$ | *%Ht* | Collection data |
|---|---|---|---|---|---|---|---|---|---|
| *L. warmingianum* (K.Schum.) Barthlott | 22 | 1.83 | 20.16 | 1.85 | 1.17 | 0.14 | 0.16 | 1.29 | Misiones, Dpto. Guaraní, *Barboza et al. 2112.*<br>Cultivada, *Kiesling s.n.* |
| *Rhipsalis baccifera* (J.S.Muell.) Stearn | 22 | 2.43 | 26.76 | 2.22 | 2.21 | 0.17 | 0.18 | 1.39 | Corrientes, Dpto. Capital, *Amarilla 29.*<br>Misiones, Dpto. Apóstoles, *Moreno & Iglesias 7.*<br>Misiones, Dpto. Capital, *Moreno & Iglesias 2.*<br>Misiones, Dpto. Concepción, *Moreno & Iglesias 14.*<br>Salta, Dpto. Oran, *Moreno & Amarilla s.n.*<br>Salta, Dpto. Oran, *Moreno & Amarilla s.n.* |
| *R. cereuscula* Haw ex Phil. | 22 | 1.99 | 21.86 | 1.89 | 1.18 | 0.15 | 0.16 | 1.35 | Corrientes, Dpto. Mercedes, *Moreno & Amarilla s.n.*<br>Misiones, Dpto. Concepción, *Moreno & Iglesias 16.*<br>Misiones, Dpto. Obera, *Moreno & Iglesias 21.* |
| *R. floccosa* Salm-Dyck ex Pfeiff. var. *hoenahuensis* (F.Ritter) Barthlot & Taylor | 22 | 1.24 | 13.66 | 1.92 | 1.16 | 0.13 | 0.16 | 2.01 | Misiones, Dpto. Guaraní, *Barboza et al. 2111.*<br>Salta, Dpto. La Caldera, *Barboza et al. 2206.* |
| *R. floccosa* var. *tucumanensis* (F.C.A.Weber) Bartholt & Taylor | 22 | 1.28 | 14.10 | 1.73 | 1.19 | 0.16 | 0.14 | 1.35 | Tucumán, Dpto. Chicligasta, *Barboza et al. 2133.*<br>Tucumán, Dpto. Yerba Buena. *Bernardello 924.*<br>Tucumán, Dpto. Yerba Buena, *Barboza et al. 2268.* |

ratio (*R*) were calculated. Idiograms were based on the mean values. 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).

### CMA/DAPI BANDING

Slides were stained with a drop of 0.5 mg $mL^{-1}$ chromomycin $A_3$ (CMA) for 90 min, then stained with 2 μg $mL^{-1}$ 4′-6-diamidino-2-phenylindole (DAPI) for 30 min (both stains from Sigma-Aldrich) and mounted in McIlvaine's buffer/glycerol (1:1) (Schweizer, 1976; Schweizer & Ambros, 1994). For five metaphases per species, relative lengths of short and long chromosome arms (data not shown) and bands were calculated (considering haploid karyotype length = 100%). The amount of heterochromatin was expressed as a percentage of the total length of the haploid karyotype.

### FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

This protocol was developed using the methodology described by Schwarzacher & Heslop-Harrison (2000). To identify the rDNA 18S–5.8S–26S loci the p*Ta*71 probe was used (Gerlach & Bedbrook, 1979), labelled with biotin-14-dUTP by nick translation (Bionick, Invitrogen) and subsequently detected with avidin–fluorescein isothiocyanate (Sigma). For analysis of the 5S rDNA loci, a specific probe was obtained from the genome of *Pereskia aculeata* Mill. by PCR (Las Peñas, Kiesling & Bernardello, 2011). These fragments were labelled with digoxigenin-11-dUTP (DIG Nick translation mix; Roche) and detected with anti-DIG-

rhodamine (Roche). The slides were mounted with antifade Vectashield (Vector Laboratories) containing DAPI.

### Taxon sampling, DNA regions and alignment

Phylogenetic analysis and divergence time estimates were performed with a 61-accession dataset including all species of Rhipsalideae (taken from Korotkova *et al*., 2011). These samples were considered in a wider phylogenetic context to root the phylogenetic tree and to allow the inclusion of calibration points. The outgroup taxa, selected after Korotkova *et al*. (2011), included *Calymmanthium substerile* F.Ritter, *Browningia hertlingiana* (Backeb.) Buxb. and *Echinopsis aurea* Britton & Rose. Phylogenetic analyses used for the ancestral chromosomal character analysis were performed with all *Lepismium* spp., with *Echinopsis aurea*, *Pfeifera monacantha* (Griseb.) P.V.Heath, *Rhipsalis baccifera*, *R. cereuscula* Haw ex Phil. and *R. floccosa* subsp. *tucumanensis* (F.C.A.Weber) Bartholt & Taylor used as outgroup taxa. Data for seven plastid DNA regions (*trnK*, *matK*, *rbcL*, *rps3-rpl16*, *rpl16*, *psbA-trnH* and *trnQ-rps16*) were downloaded from the nucleotide database of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). GenBank accession numbers are given in Supporting Information, Table S1. The sequences were pre-aligned in Mega version 5 (Tamura *et al*., 2011) using the Muscle algorithm with default settings; subsequently, sequence alignments were manually checked and optimized.

### Phylogenetic analysis and molecular divergence time

Bayesian inference analysis was used to infer the phylogenetic tree of the plastid data set, and two schemes were used: (1) seven partitions based on DNA region identity; and (2) the DNA regions were concatenated and analysed without partitioning. Best-fitting nucleotide substitution models for the concatenated matrix and each nucleotide sequence partition were determined with jModelTest version 0.1.1 (Posada, 2008), using the Akaike information criterion. The T92+G model was selected for the *rps3-rpl16*, *psbA-trnH*, *trnQ-rps16* and *rbcL* partitions and the HKY+Γ model was selected for the *rpl16* intron partition. The GTR+Γ+I model was selected for the remaining DNA regions and the unpartitioned dataset. Bayesian reconstructions were performed using MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The Bayesian analyses were performed with four independent Metropolis-coupled Markov chain Monte Carlo (MCMC) runs. Each search used three incrementally heated and one cold Markov chain, had a temperature parameter setting of 0.16, and was run for 10 million generations and sampled every 10 000 generations. Parameters for character state frequencies, substitution rates of nucleotide substitution models and rate variation among sites were unlinked across partitions. Convergence was assessed by using the standard deviation of split frequencies as convergence index with values < 0.005 interpreted as indicating good convergence. Tracer version 1.5 (Rambaut & Drummond, 2007; http://tree.bio.ed.ac.uk/software/tracer/) was used to determine whether the MCMC parameter samples were drawn from a stationary distribution, and adequate effective sample sizes for each parameter (ESS>200) were reached. Stationarity of posterior probabilities of splits within runs and convergence of posterior probabilities of splits between different runs were visually checked using the Cumulative and Compare functions in AWTY (Nylander *et al*., 2008). The initial 25% of samples of each MCMC run were discarded as burn-in, and the post-burn-in samples were summarized as a 50% majority-rule tree with nodal support summarized as posterior probabilities. Overall performance of analyses of unpartitioned and partitioned nucleotide datasets was assessed with Bayes factor comparison implemented in Tracer version 1.5. The criterion of 2ln Bayes factor of ≥ 10 was used as a benchmark, indicating strong evidence in favour of one strategy over another (Kass & Raftery, 1995). In the Bayesian analyses, partitioning considerably improved the mean $-\ln L$ values (mean $-\ln L_{\text{unpartitioned}}$ = 67 652, mean $-\ln L_{\text{partition}}$ = 67 420). Bayes factor comparison indicated that the analysis using the unpartitioned dataset (BF = 3.1) provided a better explanation of the data than the analysis using seven partitions based on DNA region identity (BF = 2.7). Thus, all Bayesian analyses shown are based on trees derived from analyses using the unpartitioned dataset.

The time of divergence for *Lepismium* was estimated using a Bayesian approach implemented in BEAST version 1.6.1 (Drummond & Rambaut, 2007) with the identical matrix data used for the Bayesian inference. For this analysis, an uncorrelated normal relaxed clock model and a Yule speciation process were used. The Rhipsalideae node was constrained as monophyletic and the divergence dates were calibrated using a normal prior distribution. The Rhipsalideae node was calibrated as 16 Mya [95% highest posterior density (HPD) 16.82–15.18 Mya; mean 16, SD 0.5] and the *Rhipsalis* node as 10 Mya (95% HPD 10.82–9.17 Mya; mean 10, SD 0.5) according to divergence time estimates by Arakaki *et al*. (2011). The analyses were run using an uncorrelated relaxed-clock model assuming a lognormal distribution of

rates (UCLD) with GTR+Γ+I model. Four MCMC analyses were run, each with 100 million generations and sampling every 10 000 generations. Time series plots of all parameters were analysed in Tracer version 1.5 to check for adequate effective sample sizes (ESS > 200) and convergence of the model likelihood and parameters between each run. Trees were combined in Log Combiner version 1.6.1 (Drummond & Rambaut, 2007), setting the burn-in to 25% of the initial samples of each MCMC run. Post-burn-in samples were summarized using the maximum clade credibility (MCC) tree option in Tree Annotator version 1.6.1 (Drummond & Rambaut, 2007). The single tree was visualized with FIGTREE version 1.5.4 (http://tree.bio.ed.ac.uk/software/figtree/).

### Ancestral chromosome character and area reconstructions

Ancestral character state reconstructions with maximum-likelihood criterion (Mk1 model, in which all changes are equally probable) were conducted in Mesquite version 2.75 (Maddison & Maddison, 2011). Identical sequences were pruned from the concatenated (*trnK*, *matK*, *rbcL*, *rps3-rpl16*, *rpl16*, *psbA-trnH* and *trnQ-rps16*) data matrix, a total of 12 terminal taxa (all *Lepismium* spp. and, as outgroup, *Pffeifera monacantha*, *Rhipsalis baccifera*, *R. cereuscula*, *R. floccosa* subsp. *tucumanensis* and *Echinopsis aurea*). The input file for Mesquite consisted of the 3000 post-burn-in trees from the Bayesian inference analyses run in MrBayes (GTR model). The 50% majority rule was calculated in Mesquite. Character optimization was performed to reconstruct chromosome evolution of 5S rDNA number and location; as all chromosomes were similar, the karyotype for each species was encoded. Chromosomal data for *E. aurea* were taken from Las Peñas (2009). To infer character evolution, the following features were coded as discrete characters: one 5S site per haploid genome = 0, two 5S sites per haploid genome = 1; no syntenic ribosomal genes = 0, syntenic ribosomal genes = 1; 5S site on chromosome 2 = 0, 5S site on chromosome 8 = 1, 5S sites on chromosomes 1 and 2 = 2, 5S sites on chromosomes 2 and 3 = 3, 5S sites on chromosomes 2 and 8 = 4. The ancestral state was inferred using maximum-likelihood under the Markov *k*-state one-parameter model (Mk1), in which all changes are equally probable (for character matrix see Table S2).

Ancestral area reconstructions were conducted using RASP beta 1 software, which implements Bayesian binary MCMC (BBM) methods (Yu, Harris & He, 2011). Three areas based on the biogeographical provinces (Morrone, 2006) were coded as: A = Monte; B = Yungas; C = Parana forest. The maximum number of ancestral areas was set to three, allowing widespread ancestors. The input file for RASP consisted of the 3000 post-burn-in trees from the Bayesian inference analyses run in MrBayes (GTR model) with an identical matrix to that used for ancestral chromosomal character reconstructions. The tree files were combined in RASP to estimate the posterior probability > 0.50 of each node. The MCMC chains were run simultaneously for 5000 000 generations and the reconstructed state was sampled every 1000 generations. The fixed model JC+G (Jukes-Cantor + Gamma) was used for BBM analysis with a null root distribution.

## RESULTS

### Karyotypes

All species of *Lepismium*, *Rhipsalis* and *Pffeifera* were diploid with $2n = 22$ (Fig. 1). Chromosomes were small with the average chromosome length for all taxa being 1.81 μm (Table 1). Karyotypes were symmetrical (all taxa with 11 metacentric chromosomes), considering both centromere position ($A_1$ ranges from 0.10 to 0. 18) and chromosome size variation ($A_2$ from 0.13 to 0. 18) (Table 1).

The banding patterns always showed $CMA^+/DAPI^-$ constitutive heterochromatin associated with nuclear organized regions (NORs) in the satellited chromosomes (Fig. 1D–F). Additional $CMA^+/DAPI^-$ pericentromeric bands were found in *L. lorentzianum* (Griseb.) Barthlott in pair 2. The total amount of GC-rich heterochromatin ranged from 1.01 to 2.40% of the total karyotype length (Table 1).

The 18S–5.8S–26S rDNA sites in all species studied coincided with $CMA^+/DAPI^-$ signals associated with NOR bands described above (Fig. 2), i.e. they were located at the secondary constrictions on the distal part of the short arms.

The number of 5S sites varied between one or two per haploid genome and were proximally located only on the long arms of the chromosomes (Fig. 3). For species with just one 5S locus, the chromosome on which it was found varied: for all *Rhipsalis* spp. and *L. lumbricoides* the 5S locus was on chromosome 2 whereas in *L. cruciforme* (Vell.) A.Cast. and *L. aculeatum* (F.A.C.Weber) Barthlott it was on chromosome 8. For the remaining species studied, two 5S sites were detected: in *L. houlletianun* they were found on chromosomes 2 and 8, whereas in *P. ianthothele* (Monv.) F.A.C.Weber and *P. monacantha* the 5S loci were on chromosomes 2 and 3. In *L. lorenzianum* and *L. warmingianum* (K.Schum.) Barthlott, there were also two 5S sites: one on chromosome 2 as previously described, whereas the other on chromosome 1 co-localized with the 18S–5.8S–26S rDNA site

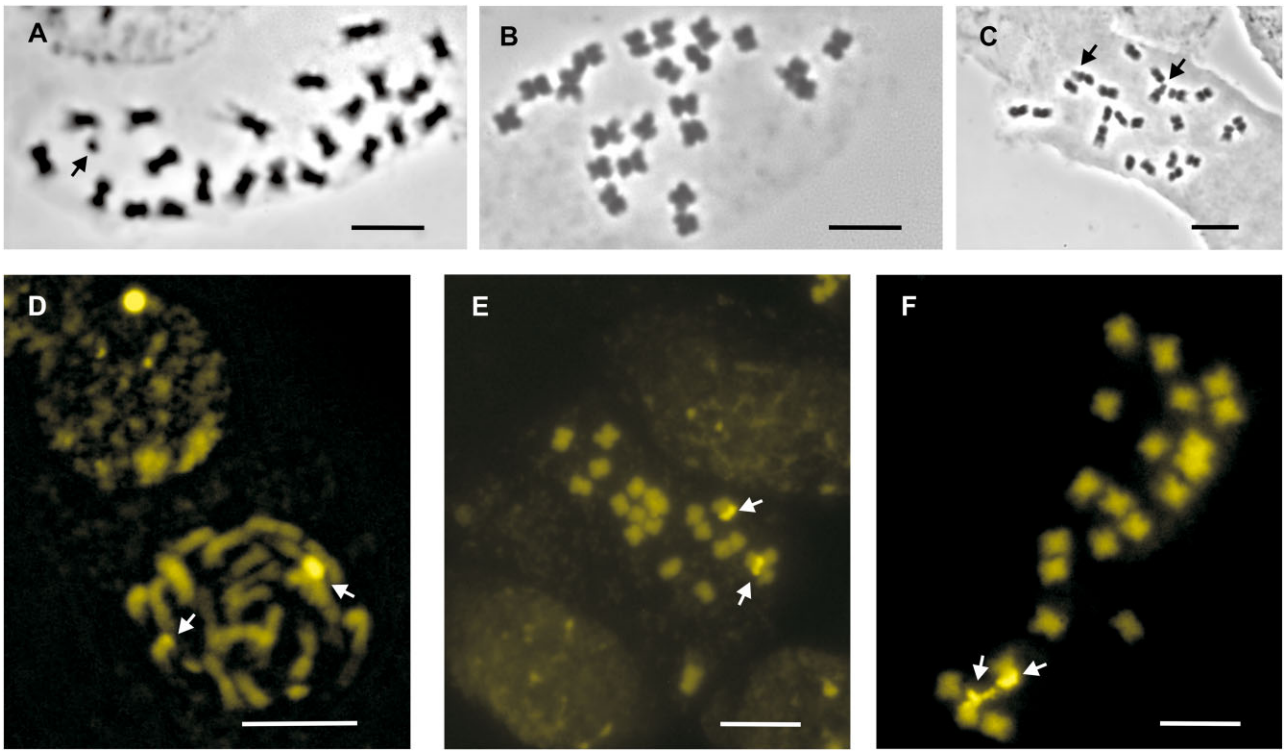


**Figure 1.** Chromosome complements of *Lepismium aculeatum* (A, D), *Pfeiffera monacantha* (B, E), *Rhipsalis cereuscula* (C, F). A–C, Giemsa staining; and D–F, CMA/DAPI fluorescence banding. Arrows show $CMA^+$ bands and satellite chromosomes. Scale bars = 5 μm.

(Fig. 3). The 5S site of chromosome 2 in *L. lorentzianum* coincided with a $CMA^+/DAPI^-$ pericentromeric band.

### Phylogenetic reconstruction and divergence time estimates

The majority-rule consensus tree resulting from Bayesian analyses was not shown because it was identical to the maximum clade credibility chronogram. The Rhipsalideae clade [posterior probability (PP) = 1.00] was monophyletic. *Lepismium* was also monophyletic (PP = 1.00). *Lepiscium aculeatum* and *L. lumbricoides* formed a clade (PP = 1.00) that was sister to the clade containing the remaining species (PP = 1.00). *Lepiscium cruciforme* and *L. houlletianum* (PP = 0.97) formed a clade that was sister to the *L. warmingianum* and *L. lorenzianum* clade (PP = 1.00) (Fig. 4; Supporting Information, Fig. S1).

The maximum clade credibility chronogram from the BEAST analysis is shown in Figure S1. According to our calibration, *Lepismium* and *Rhipsalis* diverged from their most recent common ancestor in the mid-Miocene, *c.* 13.98 ± 3.87 Mya and the diversification of the *Lepismium* crown node began in the late Miocene, approximately 8.70 ± 4.48 Mya (Fig. 4).

### Reconstruction of ancestral chromosome character and area

Results of the likelihood character and area reconstruction are summarized in Figure 4 and illustrated in the Supporting Information (Fig. S2). The most likely ancestral character states for the 5S rDNA in the common ancestor between *Lepismium* and *Rhipsalis* were one 5S site ($P = 0.57$), asyntenic ribosomal genes and 5S loci on chromosome pair 2 (Fig. S2).

After divergence, the most likely character state for the putative *Lepismium* ancestor was two 5S sites ($P = 0.56$). One 5S site seems to have been lost twice during the diversification of *Lepismium*: once prior to the divergence of the *L. lumbricoides*/*L. aculeatum* clade ($P = 0.99$) and once after the divergence of the branch leading to *L. cruciforme* ($P = 0.66$). Regarding organization of the 18S–5.8S–26S and the 5S rDNA loci, the most likely character state for the *Lepismium* common ancestor was the asyntenic state ($P = 0.99$) and it was only in the ancestor of the *L. lorentzianum*/*L. warmingianum* clade that this changed to a syntenic rDNA gene arrangement ($P = 0.98$).

The chromosomal localization of the 5S gene in the common ancestor of *Lepismium* was uncertain. The most likely character state for the *L. lumbricoides*/*L. aculeatum* clade ancestor was one 5S locus on

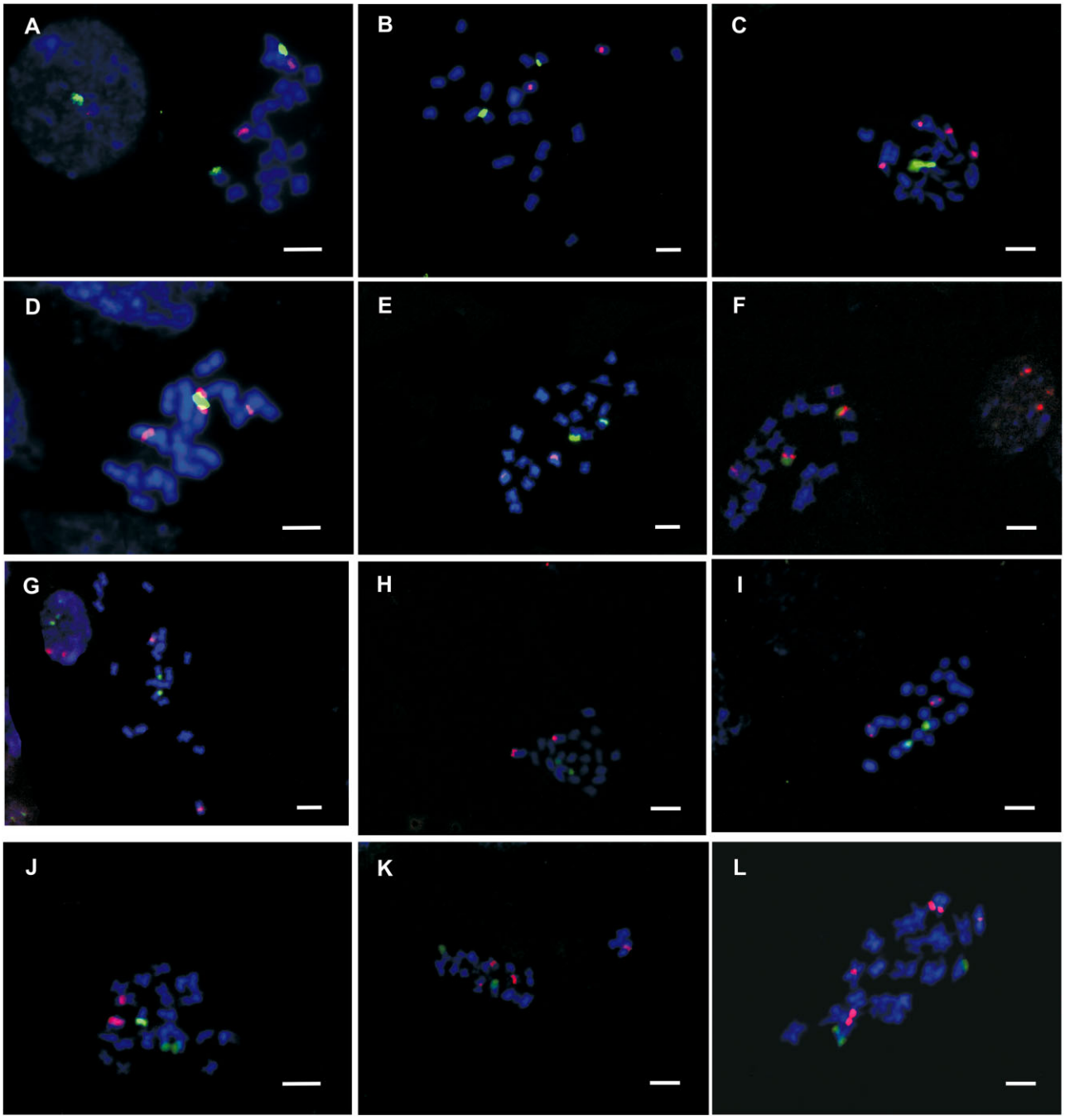


**Figure 2.** FISH of 18S–5.8S–26S and 5S rDNA to metaphase chromosomes of *Lepismium aculeatum* (A), *L. cruciforme* (B), *L. houlletianum* (C), *L. lorentzianum* (D), *L. lumbricoides* (E), *L. warmingianum* (F), *Rhipsalis baccifera* (G), *R. cereuscula* (H), *R. floccose* subsp. *Hohenauensis* (I) and subsp. *tucumanensis* (J), *Pfeiffera ianthothele* (K) and *P. monancantha* (L), simultaneously detected in all species. All scale bars = 5 μm.

chromosome 2, and for the *L.lorenzianum*/*L. warmingianum* ancestor it was two 5S loci on chromosomes 1 and 2 ($P = 0.96$).

A complex biogeographical history of *Lepismium* spp. was suggested by the BBM analysis. The results indicated that the most recent common ancestor between *Lepismium* and *Rhipsalis* originated in the yungas ($P = 0.63$) and, afterwards, some *Lepismium* spp. dispersed to the parana forests (Fig. 4). A complex biogeographical history of *Lepismium* spp. was suggested by the BBM analysis. Results indicate that the putative ancestor of the *Lepismium* clade

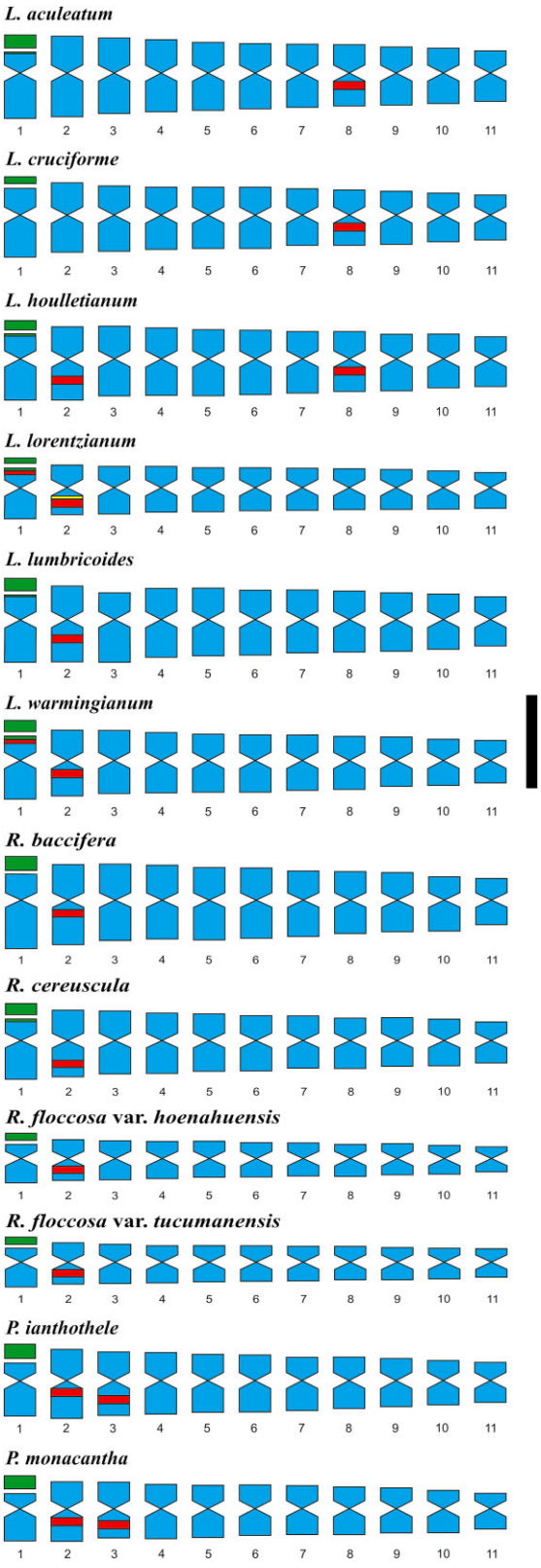


**Figure 3.** Idiograms of *Lepismium*, *Rhipsalis* and *Pfeiffera*. $CMA^+$ bands (yellow), 18S–5.8S–26S rDNA (green) and 5S rDNA (red). Scale bar = 3 μm.

had a widespread distribution range from yungas/parana forests ($P = 0.53$) and one vicariance event was recovered to this node (Fig. 4). The ancestor of the *L. lumbricoides*/*L. aculeatum* clade was in the yungas ($P = 0.58$) with one dispersal event. The areas of the ancestors of the *Lepismium* crown node and the *L. cruciforme*/*L. houlletianum* clade were in the parana forests ($P = 0.51/0.94$, respectively). The area of the ancestor of the *L. lorenzianum*/*L. warmingianum* clade was not resolved, with a probable origin in the parana forests ($P = 0.46$), in the yungas/parana forests ($P = 0.32$) and in the yungas ($P = 0.26$). Two dispersal events and one vicariance event occurred in the ancestor of the *L. lorenzianum*/*L. warmingianum* clade.

## DISCUSSION

### KARYOTYPE CHARACTERISTICS

Chromosome counts agreed with previous reports for these taxa (http://www.tropicos.org/Project/IPCN), except for two new chromosome counts (*L. lorentzianum* and *R. floccosa* subsp. *hohenauensis*). Polyploidy has been suggested as an important mechanism in the evolution of Cactaceae (Ross, 1981; Pinkava, 2002; Arakaki, Soltis & Speranza, 2007), but polyploids are rare in Rhipsalideae, except for seven tetraploid or octoploid populations of *R. baccifera* from Costa Rica, Mexico, Africa and Sri Lanka (Barthlott, 1983). For this species, we found six diploid Argentinean populations. Barthlott (1983) also found diploid populations from Brazil and Paraguay.

All species of *Lepismium*, *Rhipsalis* and *Pfeiffera* studied had small chromosomes and symmetrical karyotypes, as is typical in Cactaceae (e.g. Ross, 1981; Pankin, 2005; Das & Mohanty, 2006; Las Peñas *et al*., 2008, 2009, 2011). Among these genera, variation in karyotype composition was rare with respect to chromosome size and morphology.

The meagre data available on heterochromatin in cacti (Las Peñas *et al*., 2008, 2009, 2011, 2013; our data) show that $CMA^+/DAPI^-$ blocks are always present at a terminal position associated with the chromosome pair with secondary constrictions. Thus, this character may be conserved in Cactaceae, although more data are needed. Only *L. lorentzianum* showed a pericentromeric band. Such bands have also been reported in other cacti (Las Peñas *et al*., 2008, 2009, 2011, 2013).

The presence of 18S–5.8S–26S rDNA sites in terminal regions of the short chromosome arms is common in angiosperms (Roa & Guerra, 2012). In Cactaceae, NOR-bearing pair 1 (with 18S–5.8S–26S rDNA locus) seems to be homoeologous in all species examined thus far (Las Peñas *et al*., 2009, 2011, 2013;

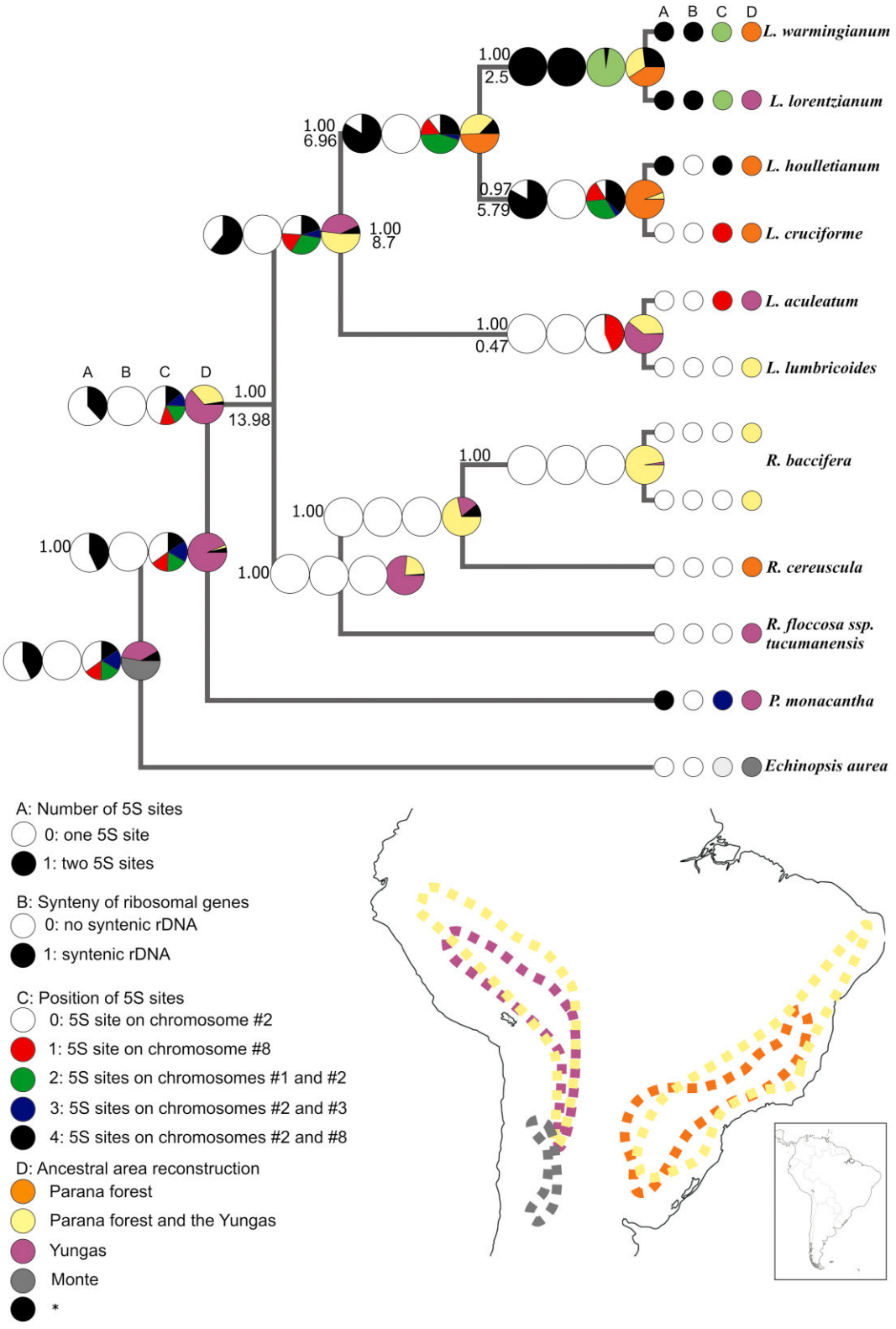


**Figure 4.** Ancestral character and ancestral area reconstructions. Character states of three chromosome characters (A–C) and current distributions (D) are indicated before the species names. Estimates of ancestral character states for internal nodes of the tree are presented in pie charts. Posterior probability (PP) values are indicated above the branches and ages (in Mya) are indicated below the branches.

this work). Unfortunately, only a few data on the localization of 5S rDNA loci in Cactaceae are available: Pereskioideae (five species), Maihuenioideae (two species) and *Setiechinopsis mirabilis* (Speg.) Backeb. ex de Haas (Cactoideae) (Las Peñas *et al*., 2011, 2013; our data). The 5S rDNA signals in the proximal region of the long arms were found in all taxa examined here, unlike other reports which have shown the 5S loci located in the proximal regions of short arms (e.g. species of *Maihuenia* Phil., species of *Pereskia* Mill., Las Peñas *et al*., 2013). It should be mentioned that the co-localization of the 5S locus with the 18S–5.8S–26S rDNA locus is the first report for this arrangement in Cactaceae. There are several hypotheses to explain this fact: (1) there is a close physical proximity of 5S and 18S–5.8S–26S S rDNA loci; (2) clusters of 5S and 18S–5.8S–26S rDNA arrays are intermingled within loci; or (3) both rRNA genes are physically linked in a single rDNA unit, as recently shown in Asteraceae and some gymnosperms (Garcia *et al*., 2009; Garcia & Kovařík, 2013). However, further work is needed to distinguish between these three possibilities.

The number of 18S–5.8S–26S rDNA loci may be indicative of ploidy (Lichtenzveig *et al*., 2000; Las Peñas *et al*., 2009, 2013). However, the number of 5S loci has not been shown to be correlated with ploidy (Las Peñas *et al*., 2011; this work). Variation in the number of 5S sites seems to be common to other plant groups (Datson & Murray, 2006; Martínez *et al*., 2010). Their variation in number and position in species with the same ploidy may reflect different mechanisms operating in different species, such as: chromosomal structural rearrangements, amplification of rDNA genes by transposon activity and ancestral increase of loci (Hall & Parker, 1995; Moscone *et al*., 1999; Cai *et al*., 2006; Datson & Murray, 2006; Pedrosa-Harand *et al*., 2006; Lan & Albert, 2011). All these processes could have operated independently or combined with no changes in overall chromosome morphology.

## Phylogenetic interpretation of 5S rDNA gene diversity in *Lepismium*

Our results support the monophyly of Rhipsalideae and *Lepismium* (Calvente *et al*., 2011; Korotkova *et al*., 2011) and agree with the phylogenetic analysis of Korotkova *et al*. (2011).

Physical chromosome mapping and high-throughput sequencing approaches have been recently shown to be useful for providing insights into several plant groups regarding the origin of diverse karyotype profiles (e.g. Lan & Albert, 2011; Buggs *et al*., 2012; Jang *et al*., 2013; Pellicer *et al*., 2013). The karyotypic similarities detected between *L. lorentzianum* and *L. warmingianum* are not unexpected because they are sister species. By contrast, *L. cruciforme* and *L. aculeatum*, although sharing several chromosome features (e.g. number and location of 5S loci) do not share an immediate common ancestor and thus these karyotypic features have probably originated independently in these two species. The increase in the number of 5S rDNA loci in *Lepismium* appears to be restricted to earlier diverging branches, whereas losses/translocations appear to be more typical in the more derived lineages.

Taking into account our study, the most recent common ancestor between *Lepismium* and *Rhipsalis* would have a similar rDNA loci distribution to *Rhipsalis* spp. This ancestral pattern was also conserved in *L. lumbricoides* (Fig. 4). We hypothesize an ancestral *Lepismium* with $2n = 22$ chromosomes and one 5S rDNA loci on chromosome 2. During the divergence of this ancestor, some populations would have duplicated the 5S loci. The ancestor of the *L. lumbricoides*/*L. aculeatum* clade would have retained the ancestral one 5S locus, whereas the *Lepismium* crown group would have originated from the populations with the duplicated 5S loci (Fig. 4). Finally, note that there was no correlation between the increase in the number of rDNA sites and the number of chromosomes or total chromosome length, as detected in some other diploid angiosperms (Rosaceae, Mishima *et al*., 2002; Brassicaceae, Hasterok *et al*., 2006; Iridaceae, Martínez *et al*., 2010; Asteraceae, Pellicer *et al*., 2013).

## Historical biogeography in *Lepismium* and species disjuntions of *Rhipsalis*

The Neotropics host *c*. 37% of global plant diversity (Richardson *et al*., 2001). This diversity has attracted the attention of scientists, especially relating to the patterns and processes responsible for generating this diversity (Pennington *et al*., 2004; Rull, 2011). Some hypotheses have been suggested to explain patterns in the geographical distribution of plant diversity in the Neotropics (see Antonelli & Sanmartín, 2011; Hughes, Pennington & Antonelli, 2013). However, only recently has it become possible to test these hypotheses explicitly with phylogenetic methods (Antonelli *et al*., 2009; Roncal *et al*., 2010; Simon *et al*., 2011). In this study, the estimated divergence times and the reconstructed ancestral geographical distributions of the Neotropical epiphytic genus *Lepismium* and some representatives of *Rhipsalis* suggest a history involving repeated colonizations of two biogeographical provinces: the yungas and parana forests. These events would have occurred over a broad time span, suggesting that several factors may have influenced the evolution of these modern epiphytic cacti. One possibility could be that hydrological changes *c*. 23–7 Mya (i.e. Parananean

sea; Ortiz-Jaureguizar & Cladera, 2006) were important for driving the diversification in this clade. Such changes have been associated with diversification in several other groups of plants (e.g. Antonelli *et al*., 2010; Roncal *et al*., 2010).

The major diversifications of Cactaceae have occurred in the Miocene (Arakaki *et al*., 2011; Hernández-Hernández *et al*., 2014). Our results suggest that the divergence between *Rhipsalis* and *Lepismium* occurred in the mid Miocene (13.98 Mya) and that the common ancestor probably inhabited the yungas. The most recent common ancestor of *Lepismium* was dated to *c*. 8.7 Mya in the late Miocene and probably lived in yungas and parana forests. The most diversified clade of *Lepismium* occurred in parana forest. Only *L. lumbricoides* and *L. aculeatum* diverged in the yungas at a later period.

Molecular clock techniques have previously demonstrated that numerous plant disjunctions are too young to have resulted from vicariance (Christenhusz & Chase, 2013; Lohmann *et al*., 2013), as here proposed for *Lepismium* and *Rhipsalis*. Two long-distance dispersal (or migration) events in *Lepismium* are proposed to explain its current distribution pattern: first from the yungas to parana forests in the mid- and later Miocene (13.98–8.7 Mya) and secondly in the opposite direction in the later Pliocene to early Pleistocene (*c*. 2.5 Ma). Both dispersal events could have been mediated by migratory birds (cf. Hawkins *et al*., 2006). Birds could have dispersed them either by endozoochory (fruits are small and juicy) or epizoochory (seeds have large amounts of sticky mucilage; Barthlott & Taylor, 1995).

South American cloud-forests were present from the Mid or Late Miocene to the Miocene–Pliocene limit; increasing aridity and decreasing temperatures then would have reduced their extent during the Pliocene–Pleistocene (Graham, Gregory-Wodzicki & Wright, 2001; Mayle, 2006). The current distribution of *Lepismium* spp. may have been influenced by the cyclical advances and retreat of glaciers that produced concurrent expansions and retreats of the humid biomes during the Pleistocene (Mayle, 2006). Xeromorphic features and physiological adaptations to balance water loss in cacti would have been advantageous strategies to tolerate the arid period. In addition, the ability to propagate vegetatively by breaking off individual phylloclades would allow tree canopies to be colonized (Cota-Sánchez & Bomfim-Patrício, 2010).

## CONCLUSIONS

Our data on the studied species suggest that Cactaceae may be chromosomally more diverse than previously supposed. Our phylogenetic analysis of *Lepismium* supported an ancestral increment of 5S loci during the late Miocene in the yungas. Biogeographical reconstructions suggest that the current disjunct distribution of *Lepismium* may be related to a few long-distance dispersal events to the parana forests. Chromosomal translocations and deletions are suggested to have occurred several times throughout the Pleistocene during the evolution of the genus. The expansion and retraction of subtropical forests, typical of this period, would have helped their fixation, facilitating *Lepismium* speciation. The diversity of Neotropical epiphytic cacti cannot be attributed to the action of one or a few events during key time intervals, but rather it appears that complex ecological and evolutionary processes including both abiotic and biotic factors are at work generating the karyotypic diversity observed (Hughes *et al*., 2013; Hernández-Hernández *et al*., 2014; this work).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Maximum clade credibility chronogram from the BEAST analysis. Branch lengths are proportional to time, with blue bars indicating 95% highest posterior densities. Values of posterior probabilities (PP) are indicated in the branches. A, last common ancestor between *Lepismium* and *Rhipsalis*; B, the crown group of *Lepismium*.

**Figure S2.** Results of the likelihood character reconstruction.

**Table S1.** Data relating to sequences taken from GenBank or EMBL; the respective citation follows the accession number. Tribal classification and accepted species names are given according to Hunt *et al*. (2006).

**Table S2.** Character matrix used in Mesquite with Mk1 model