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Chromosomal changes and recent diversification in the Andean genus *Jaborosa* (Solanaceae)

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With the purpose of discussing the evolutionary dynamics of the karyotypes and their relationship with key geological events, chromomycin A3/4′,6-diamidino-2-phenylindole (CMA/DAPI) banding and fluorescence *in situ* hybridization (FISH) techniques were applied to metaphase chromosomes of 13 *Jaborosa* spp. and *Sclerophylax spinescens*, and the resulting characters were reconstructed onto a dated phylogenetic tree. *Jaborosa* shows a distinctive evolutionary pathway at the chromosomal level with respect to its sister clades, Hyoscyameae, *Lycium* and *Sclerophylax*. The divergence of the common ancestor of *Jaborosa* involved an increase in chromosome size, with a slight increase in the amount of heterochromatin and chromosomal asymmetry, all these changes taking place at the diploid level. Variation in the number and position of rDNA sites showed profuse chromosomal rearrangements, including duplications and transpositions. Contrasting evolution of the 5S and 18S-5.8S-26S rDNA seems to have occurred, with the 5S site being more stable than 18S-5.8S-26S. The diversification of *Jaborosa* seems to be correlated with the most recent uplifts in the Andes in central South America and the establishment of the Patagonian steppe: the genus colonized these biomes and, at the same time, underwent a remarkable chromosomal and morphological differentiation. Although some characters were seen to be homoplasious, the combination of chromosome markers employed here allowed species discrimination.

ADDITIONAL KEYWORDS: fluorescence *in situ* hybridization – heterochromatin – karyotype – phylogeny – rDNA.

INTRODUCTION

Chromosomal characters provide key information in studies of systematics, phylogenetic analyses and evolution, and are especially helpful for understanding speciation and hybridization (Stebbins, 1971; Guerra, 2000, 2012). Structural and quantitative characteristics of karyotypes have been significant in evolutionary and taxonomic studies in many angiosperm groups, and have contributed to the establishment of linkage groups and natural classifications (e.g. Benko-Iseppon & Morawetz, 2000; Moscone *et al.*, 2007; Urdampilleta *et al.*, 2015). Chromomycin A3/4′,6-diamidino-2-phenylindole (CMA/DAPI) banding and fluorescence *in situ* hybridization (FISH) are procedures that have been shown to be useful in the detection of chromosome rearrangements involved in speciation (e.g. Hasterok *et al.*, 2006; Chacón *et al.*, 2012). CMA/DAPI staining reveals heterochromatin blocks, which are one of the most remarkable chromosome components, because of their unknown function, their apparent lack of genes and their differential stainability, thus constituting a source of variability for comparative purposes (Guerra, 2000). FISH allows homologous chromosomes in a complement to be identified, permits comparison among related species and allows chromosomal evolutionary questions to be addressed (e.g. Chacón *et al.*, 2012; Chiarini *et al.*, 2014). The most common FISH markers are ribosomal genes (5S and 18S-5.8S-26S rDNA), which are abundant and highly conserved in higher plants (Heslop-Harrison & Schwarzacher, 2011).

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Some data on the mechanisms and speed of chromosomal changes have already been accumulated in the literature, especially relating to the evolution of heterochromatin and rDNA. It is clear that heterochromatic bands and the number and position of rDNA sites are not fixed characters, but can undergo dynamic, plastic changes (e.g. Datson & Murray, 2006; Hasterok *et al.*, 2006; Heslop-Harrison & Schwarzacher, 2011; Chacón *et al.*, 2012; Morales, Aguiar-Perecin & Mondin, 2012). At the same time, the basic chromosome number can remain unchanged for some plant groups over long evolutionary timescales and the overall morphology of the karyotype can appear to be remarkably stable, despite the occurrence of large changes in the distribution and organization of DNA sequences in chromosomes (Lim *et al.*, 2000; Chacón *et al.*, 2012). The reconstruction of these chromosome characters using phylogenetic frameworks can help to determine their significance for diversification and speciation (e.g. Kitamura *et al.*, 2001; Chacón *et al.*, 2012; Chiarini *et al.*, 2014; Moreno *et al.*, 2015).

Explosive species diversification (Givnish, 2015) is an outstanding phenomenon that has been poorly studied from a cytogenetic perspective, although such an approach would certainly contribute to a better understanding of the process (Mandáková, Heenan & Lysak, 2010; Garnatje *et al.*, 2012). Studies on plant groups from different geographical regions have shown that dramatic species diversification can be accompanied by chromosomal changes at different levels (Ran, Hammett & Murray, 2001; Datson & Murray, 2006). However, most assertions about the speed of chromosomal change lack a truly dated phylogenetic framework and no causes other than concerted evolution, karyotypic orthoselection or common ancestry have been proposed to explain the uniform chromosomal patterns (e.g. Adams *et al.*, 2000; Kovařík *et al.*, 2004, 2005; Stiefkens *et al.*, 2010).

Environmental factors are one of the elements that should be considered when analysing chromosomal evolution and species diversification. For example, it has been suggested that orogenic activity can play a role in triggering evolutionary changes over a short period of time (Simpson & Todzia, 1990; Nores, 1995). In particular, several studies have noted the relationship between the Andean folding of the Miocene (*c.* 20 Mya) (Hoorn *et al.*, 1995; Hooghiemstra & Van Der Hammen, 1998) and plant (Kay *et al.*, 2005; Pennington & Dick, 2010) and animal (Bates & Zink, 1994; Bleiweiss, 1998) radiation. High-elevation areas of South America, where several endemic plant clades have diversified, are even younger (2–4 Mya in the Pliocene), coinciding with the development of arid environments in South America (Hughes & Eastwood, 2006; Luebert, Hilger & Weigend, 2011; Särkinen *et al.*, 2012). However, few studies are available that connect chromosomal change and key geological events (e.g. Deng *et al.*, 2009; Nürk, Scheriau & Madriñán, 2013). In this context, it is interesting to study what happened to these recently diversified taxa at the chromosomal level, a relationship that has been disregarded until now.

To test the hypothetical relationship between chromosome characters and key geological events, we focused our study on the South American genus *Jaborosa* Juss. (Solanaceae), which extends from southern Peru to southernmost Patagonia. *Jaborosa*, comprising 23 species, is a natural group (Särkinen *et al.*, 2013; Moré *et al.*, 2015) in the '$x = 12$ clade' (Olmstead *et al.*, 2008) and, with *Lycium* L., *Sclerophylax* Miers, *Nolana* L.f. and Hyoscyameae, forms the *Atropina* clade (Olmstead *et al.*, 2008; Tu *et al.*, 2008, 2010; Levin *et al.*, 2011). *Sclerophylax* and *Nolana* are also endemic to South America, whereas *Lycium* occurs in North and South America and the Old World (Miller *et al.*, 2011). In contrast, Hyoscyameae are exclusive to the Old World, with some genera endemic to China (Tu *et al.*, 2010).

A recent phylogenetic analysis strongly supports two major clades of *Jaborosa*: the 'lowland clade' (L), comprising three sphingophilous species distributed < 1000 m and north of 36°S latitude, and the 'Andean clade' (A), composed of the remaining species, which differ markedly in floral traits (morphology, corolla colour, architecture and the chemical composition of fragrance) and mainly occur at high elevation or high latitudes (Moré *et al.*, 2015). Karyotype data are available for 14 of the 23 *Jaborosa* spp. (Chiarini & Barboza, 2008; Chiarini *et al.*, 2010), but no species have been studied using CMA/DAPI or FISH. These techniques have been applied to several genera of Solanaceae (e.g. Lim *et al.*, 2000; Moscone *et al.*, 2007; Chiarini *et al.*, 2014), in which they have been used to test the apparent chromosomal homogeneity in the '$x = 12$ clade'. We set the following objectives in order to discuss the evolutionary dynamics of the karyotypes in *Jaborosa*: (1) to describe heterochromatin patterns in *Jaborosa* and some species from sister clades and to relate them to the classical karyotype variables; (2) to detect the number and position of the rDNA sites; and (3) to test lineage-specific rearrangements leading to different chromosome structures and propose insights into chromosome evolution in *Jaborosa*.

MATERIAL AND METHODS

Material examined

Fourteen species of the *Atropina* clade, 13 *Jaborosa* spp. and *Sclerophylax spinescens* Miers were studied

Table 1. Provenance, collectors and accession numbers of the species studied

Species	Source	Figure
J. caulescens Gillies & Hook. var. *bipinnatifida*	La Rioja prov., Famatina dpt., *Barboza et al. 3185*	3C
J. cabrerae Barboza.	Catamarca prov., Belén dpt., *Barboza et al. 2005*	2D
J. integrifolia Lam.	Corrientes prov., Goya dpt., *Barboza et al. 359*	1B, 3G
J. kurtzii Hunz. & Barboza	Neuquén prov., Collón Curá dpt., *Barboza et al. 1182*	1E
J. laciniata (Miers) Hunz. & Barboza	Mendoza prov., Las Heras dpt., *Chiarini 1041*	1F, 2F
J. leucotricha (Speg.) Hunz.	La Rioja prov., General Lavalle dpt., *Las Peñas 224*	1A, 3E
J. magellanica (Griseb.) Dusén	Santa Cruz prov, Güer Aike dpt., *A. A. Cocucci 4589* Corpen Aike dpt., *Barboza et al. 3710*	1D, 3B
J. oxipetala Speg.	Tucumán prov., Tafí dpt., *Barboza & Oberti 3045*, *Barboza et al. 773*	1H, 2A
J. reflexa Phil.	Chubut prov., Tehuelches dpt., *Barboza et al. 2389*	1G, 2B
J. rotacea (Lillo) Hunz. & Barboza	Catamarca prov., Belén dpt., *Barboza et al. 1991*	2C
J. runcinata Lam.	Entre Ríos prov., Paraná dpt., *Barboza et al. 984* – Paraná dpt., 31°38′40″S, 60°10′46″W, *Barboza et al. 1479* – Dpt. Paraná, 31°37′45″S, 60°09′26″W, *Barboza et al. 2062*	1C, 3F
J. sativa (Miers) Hunz. & Barboza	La Rioja porv., Famatina dpt., *Barboza et al. 3233* – Catamarca porv., Paclín dpt, *Barboza et al. 1956*	2E
J. volkmannii (Phil.) Reiche	Neuquén prov., Picunches dpt., *Barboza et al. 3778* – Picunches dpt., *Barboza et al. 3783*	3A
Sclerophylax spinescens Miers	Córdoba prov., Capital dpt., *Chiarini 1042*	3D

All voucher specimens are deposited at the Herbarium of Museo Botánico de Córdoba (CORD).

by means of different chromosome techniques (Table 1). The focus was on *Jaborosa*, which comprises 23 species that inhabit the Andean zone, except for *J. integrifolia* Lam. and *J. runcinata* Lam., which grow in the Paraná and Uruguay river basins (Barboza & Hunziker, 1987). *Jaborosa* spp. are frequently rhizomatous perennial herbs, hemicryptophytes or geophytes [except for *J. bergii* Hieron. and *J. sativa* (Miers) Hunz. & Barboza that are annual or biennial].

Sclerophylax is a small genus with 12 species endemic to dry regions in central, north-western Argentina, at elevations < 2600 m. As no chromosomal data are available for this genus, we performed the techniques on *S. spinescens*, to use it as an outgroup. In all cases, seeds were bulked from natural populations in the field (Table 1).

Chromosome preparations, CMA/DAPI banding and FISH

Mitotic chromosomes were examined in root tips obtained from germinating seeds following the protocol explained by Chiarini *et al.* (2014). Slides were stained with a drop of 0.5 mg mL^{-1} CMA in McIlvaine buffer, pH 7.0, and distilled water (1 : 1) containing 2.5 mM $MgCl_2$ for 90 min, subsequently stained with 2 μg mL^{-1} DAPI for 30 min and, finally, mounted in 1 : 1 v/v McIlvaine's buffer–glycerol (Schweizer, 1976; Schweizer & Ambros, 1994). The amount of heterochromatin was expressed as a percentage of the total length of the haploid karyotype.

The location and number of rDNA sites were determined by FISH using two probes: p*Ta*71 containing the 18S-5.8S-26S gene of wheat (Gerlach & Bedbrook, 1979), labelled with biotin-14-dATP (BioNick; Invitrogen, Carlsbad, CA, USA), and a 5S rDNA fragment obtained by PCR from *Solanum stuckertii* Bitter (Chiarini *et al.*, 2014), labelled with digoxigenin-11-dUTP (DIG-Nick Translation Mix, Roche, Indianapolis, IN, USA). The FISH protocol followed Schwarzacher & Heslop-Harrison (2000), with minor modifications. The preparations were

incubated in 100 μg mL^{-1} RNAase, post-fixed in 4% (w/v) paraformaldehyde, dehydrated in a 70–100% graded ethanol series and air dried. On each slide, 15 μL of hybridization mixture were added [4–6 ng μL^{-1} probe, 50% formamide, 10% dextran sulphate, 2 × saline sodium citrate (SSC) and 0.3% sodium dodecylsulphate (SDS)], previously denatured at 70 °C for 10 min. Chromosome denaturation/hybridization was performed 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 overnight in a humid chamber at 37 °C. The 18S-5.8S-26S probe was detected with avidin-fluorescein isothiocyanate (FITC) conjugate (Sigma-Aldrich®, St. Louis, MO, USA); the 5S probe was detected with antidigoxigenin-rhodamine (Roche) and then counterstained and mounted with 25 μL antifade Vectashield (Vector Lab., Burlingame, CA, USA), containing 1.5 μg mL^{-1} DAPI.

At least ten metaphases of each species from at least three different individuals were photographed with a Zeiss Axiophot microscope equipped with epifluorescence and a digital image capture system. The free software ImageJ (http://rsbweb.nih.gov/ij/) was used for the merging of images.

Phylogenetic reconstructions and estimation of divergence times

Phylogenetic analysis and divergence time estimates were performed using the former dataset of four plastid spacer regions (*trnH-pbsA*, *trnD-trnT*, *rpl32-trnL*^{UAG}, *ndhF-rpl32*) from Moré *et al.* (2015) with the inclusion of three new species: *J. volkmannii* (Phil.) Reiche, *Sclerophylax arnottii* Miers and *Sclerophylax spinescens* (Supporting Information, Table S1). DNA extraction, amplification and sequencing of the four plastid regions for these three species were performed using the same protocols as described in Moré *et al.* (2015). The ingroup comprised 13 *Jaborosa* spp.; four species of the *Atropina* clade (two species of *Lycium* and two species of *Sclerophylax*) were used as outgroups (Table S1). Sequences from both strands of each PCR product were examined, compared and corrected using the program BioEdit v.7.1.3.0 (Hall, 1999), from which a consensus sequence was generated. Sequence data were aligned using default settings in CLUSTAL W v.1.81 (Thompson, Higgins & Gibson, 1994), as implemented in BioEdit software (Hall, 1999), and then manually adjusted.

Bayesian inference was conducted to infer phylogenetic relationships in the *Jaborosa* clade using the combined plastid data from four markers with *Lycium* as outgroup. We used the Akaike information criterion (AIC), as implemented in jModelTest v.2.0.2 (Posada, 2008), to determine the best-fitting substitution model. The GTR model was selected for *trnH-pbsA*, *trnD-trnT* and *ndhF-rpl32* partitions and the GTR + G model was selected for *rpl32-trnL*^{UAG} and the unpartitioned dataset. The Bayesian analyses were performed using MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003), with four independent Monte Carlo Markov chain (MCMC) runs. Each search used three incrementally heated and one cold Markov chain, a temperature parameter setting of 0.16, run for 10 million generations and sampled every 10 000th generation. Convergence was assessed using the standard deviation of split frequencies as the convergence index, with values < 0.005 interpreted as indicating good convergence. The initial 25% of samples of each MCMC run were discarded as burn-in and the post-burn-in samples were summarized in a 50% majority-rule tree with branch support summarized as posterior probabilities.

Estimation of the divergence times was conducted in BEAST v.1.7.5 (Drummond *et al.*, 2012) using the combined plastid dataset from four markers. We performed the Bayesian analysis under a relaxed clock model with branch-specific rates following a lognormal distribution and a GTR + G model of nucleotide substitution. The analyses were completed with a randomly generated starting-tree topology and the Yule speciation process was used. We performed a secondary calibration based on a normally distributed node age of 10 Mya [95% high posterior density (HPD): 12.47–7.53 Mya; mean = 10, SD = 1.5] for the *Jaborosa* clade according to previous estimations performed by Moré *et al.* (2015). Four MCMC analyses were run, each with 70 million generations and sampling every 70 000th generation. Time series plots of all parameters were analysed in Tracer v.1.5 (Rambaut & Drummond, 2009) 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 v.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 v.1.6.1 (Drummond & Rambaut, 2007). The single tree was visualized with FIGTREE v. 1.5.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Ancestral chromosome character reconstructions

Ancestral character state reconstructions with maximum parsimony (MP) and maximum likelihood (ML) criteria were conducted in Mesquite v. 2.75 (Maddison & Maddison, 2011). Chronograms resulting from the Bayesian analysis (including branch length) were used to infer the evolution of GC-rich heterochromatin amount, asymmetry, and the number

and localization of rDNA loci. Character optimization was performed using MP and ML (for continuous and discrete characters, respectively). Additional data for chromosomal variables were taken from Chiarini & Barboza (2008) and Blanco *et al.* (2012), and *S. arnottii* chromosomal variables were taken from N. Lujea and F. Chiarini (unpubl. data). The following features were coded as discrete characters to infer character evolution: number of nucleolar organizer regions (NORs), co-localization of the 18S-5.8S-26S and 5S rRNA genes, dispersion of 18S-5.8S-26S loci, and number and position of 5S loci. The percentage of heterochromatin and the arm ratio (*r*) were coded as continuous characters (for character matrix, see Supporting Information, Table S2).

RESULTS

CMA/DAPI BANDING

The total amount of CMA^+ heterochromatin ranged from 1.58% (*J. cabrerae* Barboza) to 32.16% [*J. leucotricha* (Speg.) Hunz.] of the total karyotype length. The number and positions of the bands and percentage of heterochromatin for each species are summarized in Table 2. The chromosome banding showed two different heterochromatin types. (1) A strong pair of CMA^+ signals (corresponding to GC-rich heterochromatin regions) associated with the secondary constrictions (i.e. NOR) in the terminal position (Fig. 1); these were observed in all species analysed. (2) Additional $CMA^+/DAPI^-$ heterochromatin blocks not associated with the NOR were located in terminal or subterminal regions; these were only seen in eight species [*J. sativa*, *J. laciniata* (Miers) Hunz. & Barboza, *J. leucotricha*, *J. volkmannii*, *J. oxipetala* Speg., *J. kurtzii* Hunz. & Barboza, *J. reflexa* Phil. and *J. rotacea* (Lillo) Hunz. & Barboza, Fig. 1]. In addition, interstitial $CMA^+/DAPI^-$ blocks were detected in five species (*J. sativa*, *J. leucotricha*, *J. volkmannii*, *J. oxipetala* Speg. and *J. rotacea*, Fig. 1).

FISH

Chromosomes bearing one secondary constriction were present in all species and were strongly labelled by FISH using the 18S-5.8S-26S rDNA probe. All species showed one to three pairs of terminal sites (Figs 2, 3; Table 2), which coincided with a $CMA^+/DAPI^-$ block. The morphology of NOR-bearing chromosomes and the localization of the 18S-5.8S-26S rDNA loci were variable: the signal was located either in a metacentric or a submetacentric chromosome, and the size of this chromosome also varied among species. One species, *J. oxipetala*, showed a heteromorphic satellited pair, with one of the 18S-5.8S-26S blocks being smaller than the other (Figs 2A, 4). In six species (Table 2; Figs 2, 3), a dispersion phenomenon was observed in the distribution of the 18S-5.8S-26S signal, which consisted of small terminal fluorescent bands in some chromosomes or even on all the chromosomes of the complement.

Two, four or six hybridization signals were obtained with the 5S rDNA probe (Figs 2, 3). These signals were subterminal and/or interstitial (only terminal in *J. leucotricha*), located either on the short or long arm of a metacentric or submetacentric chromosome (Figs 2, 3). In some species, the 5S sites were clearly separated from the 18S-5.8S-26S sites, whereas, in others, they were co-localized (Table 2; Figs 2, 3). Heteromorphic pairs were seen in *J. laciniata* and *J. integrifolia*, with one chromosome bearing one 5S signal on the long arm, and the other member having one 5S signal on the long arm and another on the short arm (Figs 2F, 3G).

In addition, following FISH, it was noted that *J. sativa*, *J. rotacea* and *J. volkmannii* had DAPI bands in different positions (Figs 2C, E, 3A), which coincided with some of the heterochromatic bands seen earlier with the CMA/DAPI technique.

PHYLOGENETIC RECONSTRUCTION AND DIVERGENCE TIME ESTIMATES

Jaborosa spp. were recovered as a monophyletic clade [posterior probability (PP) = 1; Supporting Information, Fig. S1] and comprised two groups: a 'lowland clade' (L) grouping two species (*J. integrifolia* and *J. runcinata*; PP = 1), and an 'Andean clade' (A) grouping the rest of the species (PP = 1). *Jaborosa* was sister to both *Sclerophylax* spp., and *Jaborosa* + *Sclerophylax* were sister to *Lycium*.

According to our calibration, the time of divergence of the most recent common ancestor in clade A was estimated at *c*. 3.7 Mya (95% HPD: 1.8–5.8). Two subclades were recovered in clade A: A_1 and A_2 (Fig. 4); both diversified at more or less the same time [2.5 Mya (95% HPD: 1.3–4.2) and 2.6 Mya (95% HPD: 1.3–4.2), respectively] during the late Pliocene. In clade L, the diversification of *J. integrifolia* and *J. runcinata* was estimated to have occurred more recently, i.e. *c*. 0.5 Mya (95% HPD: 0.1–1.3), during the early Pliocene. The chronogram obtained is presented in Figure 4 with the karyotype features, to enable chromosomal changes in the evolutionary framework to be visualized.

Table 2. Cytogenetic features in *Jaborosa* spp. and *Sclerophylax spinescens*: SC, secondary constriction; Int, intercalary band; T, terminal band

	Number of pairs of $CMA^+/DAPI^-$ bands						Numbers of pairs of FISH signals				
Species	SC	Int	T	Total number of band pairs	Total number of chromosome pairs with bands	Percentage of heterochromatin ± SD	Main 18S-5.8S-26S loci	5S loci	DAPI after FISH bands	Dispersion of 18S-5.8S-26S present	Co-localization of rDNA genes
J. cabrerae	2	–	–	2	2	1.58 ± 0.23	2	1	–	No	Yes
J. caulescens var. *bipinnatifida*	1	–	–	1	1	2.43 ± 0.19	1	2	–	No	Yes
J. integrifolia	2	–	–	2	2	2.54 ± 0.15	2	2 (3)	–	No	Yes
J. kurtzii	3	–	1	4	4	6.26 ± 0.36	–	–	–	–	–
J. laciniata	3	–	10 (11)	13 (14)	9	9.87 ± 0.86	3	3 (4)	–	Yes	Yes
J. leucotricha	2	1	9	12	9	32.16 ± 5.0	2	2	–	Yes	No
J. magellanica	2	–	–	2	2	6.68 ± 0.39	2	1	–	No	Yes
J. oxipetala	2	1	5	8	7	4.19 ± 0.25	2	1	–	Yes	Yes
J. reflexa	2	–	2	4	4	2.39 ± 0.14	2	1	–	Yes	Yes
J. rotacea	2	1	8	11	9	8.48 ± 0.25	2	2	1	Yes	Yes
J. runcinata	2	–	–	2	2	1.84 ± 0.11	2	1	–	No	No
J. sativa	2	1	13	16	11	2.02 ± 0.31	2	1	1	Yes	Yes
J. volkmannii	2	1	3	6	6	8.28 ± 0.67	2	1	4	Yes	Yes
S. spinescens	1	–	–	1	1	1.02 ± 0.23	1	2	–	No	No

Heteromorphic bands and signals are shown in parentheses.

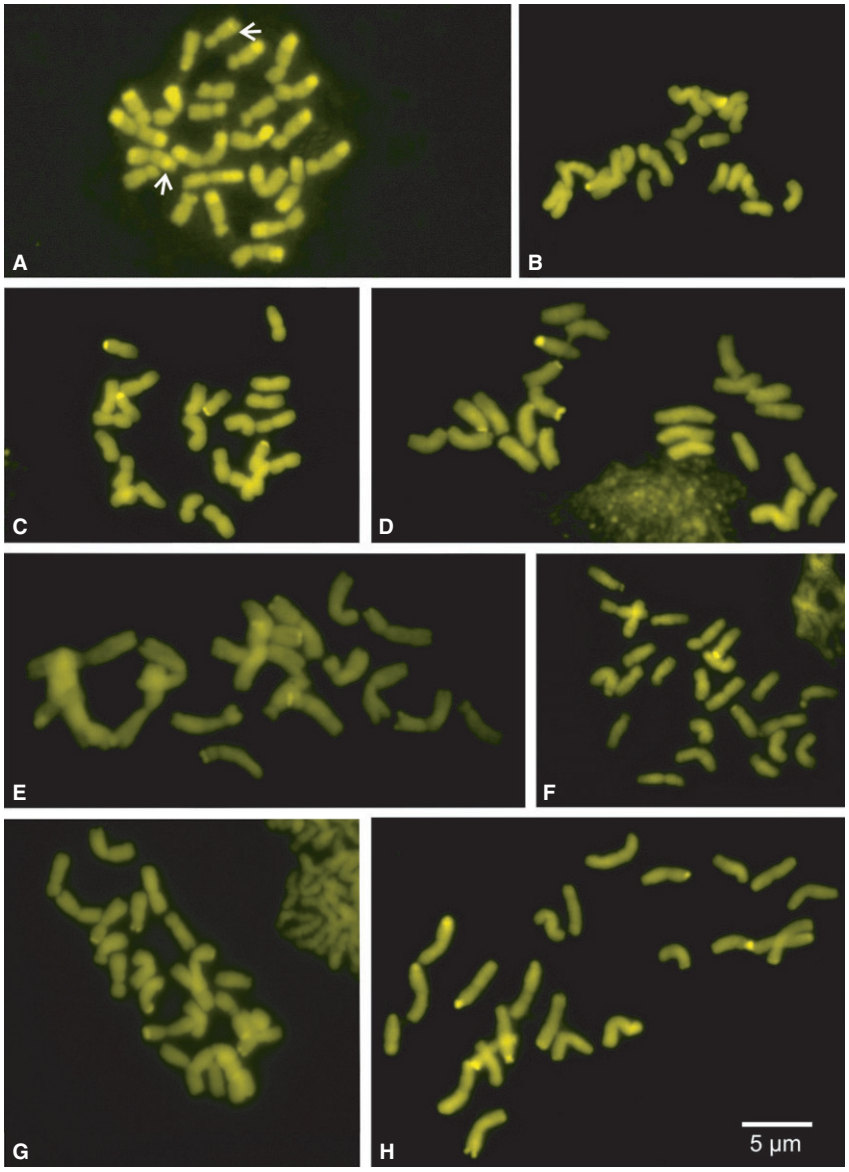


Figure 1. Fluorochrome chromosome banding (chromomycin A3, CMA) in some of the *Jaborosa* spp. studied, illustrating the heterochromatin patterns found. A, *J. leucotricha*. B, *J. integrifolia*. C, *J. runcinata*. D, *J. magellanica.* E, *J. kurtzii*. F, *J. laciniata*. G, *J. reflexa*. H, *J. oxipetala*. Arrows indicate interstitial CMA^+ bands. All photographs at the same scale.

Reconstruction of ancestral chromosome characters

Using the ancestral character state reconstruction methods, chromosome features suggested to be those most likely to be present in the common ancestor of *Jaborosa* were as follows: two pairs of NORs ($P = 0.94$, Fig. 5A); co-localized ribosomal genes ($P = 0.71$, Fig. 5B); 18S-5.8S-26S loci restricted to NORs (Fig. 5C); a low heterochromatin amount (4.75%, Fig. 5E); and a predominance of metacentric chromosomes (1.55 arm ratio, Fig. 5F).

The most likely character states of the A clade ancestor that are shared with the *Jaborosa* common ancestor after divergence are as follows: two NOR pairs ($P = 0.99$, Fig. 5A); co-localized ribosomal genes ($P = 0.99$, Fig. 5B); similar heterochromatin percentage (6.77%, Fig. 5E); and metacentric chromosomes (1.66 arm ratio, Fig. 5F). However, the 18S-5.8S-26S loci were dispersed in the genome independently of the numbers of NORs ($P = 0.74$, Fig. 5C). The dispersal of 18S-5.8S-26S loci seems to have been lost at least twice during

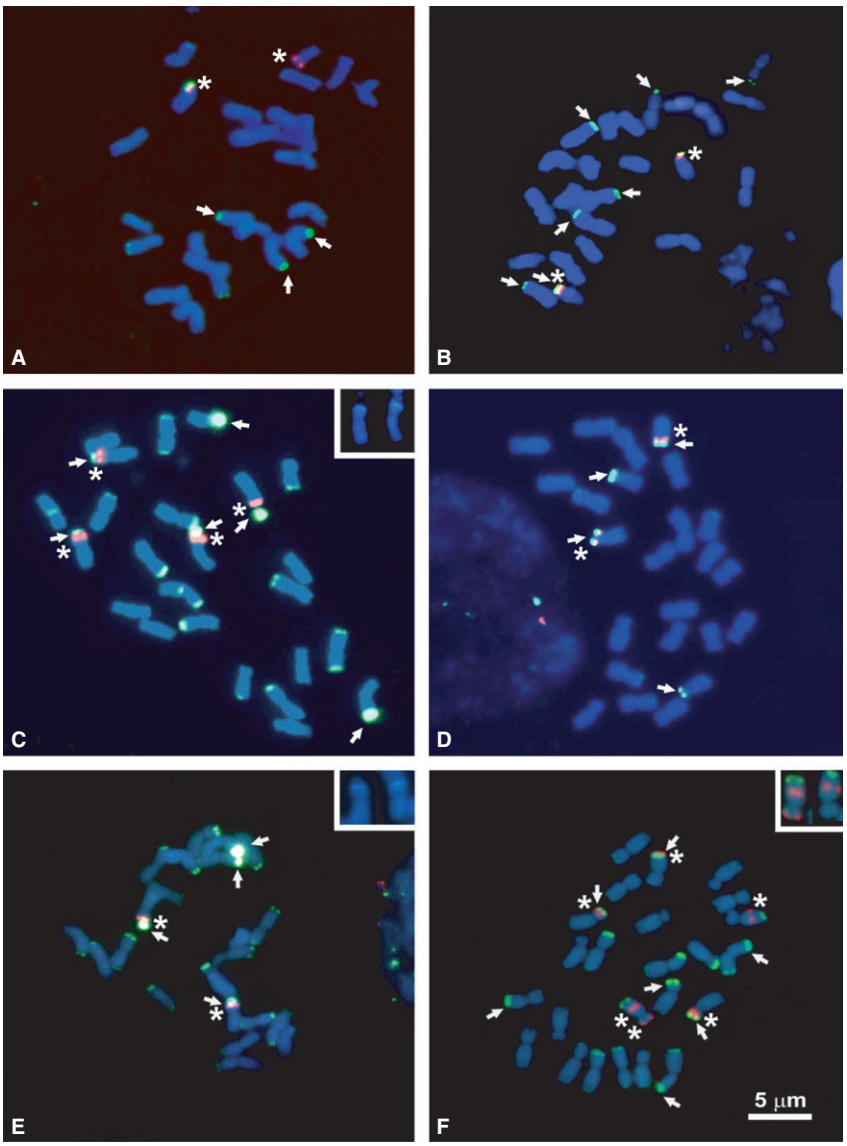


Figure 2. Fluorescence *in situ* hybridization with 5S (red signals) and 18S-5.8S-26S (green signals) rDNA probes in *Jaborosa* spp. Arrows point to chromosomes with 18S-5.8S-26S signals, and asterisks indicate chromosomes with 5S signals. The rest of the (green) signals correspond to dispersion of the 18S-5.8S-26S rDNA. A, *J. oxipetala*. B, *J. reflexa*. C, *J. rotacea*. D, *J. cabrerae*. E, *J. sativa*. F, *J. laciniata*. Close-up images: C, E, 4′,6-diamidino-2-phenylindole (DAPI) bands co-localized with 5S rDNA sites; F, heteromorphism for 5S/18S-5.8S-26S rDNA. All photographs at the same scale.

diversification of the A_2 clade: after the divergence of *J. cabrerae* and before the divergence of the *J. caulescens* Gillies & Hook. clade ancestor ($P = 0.60$, Fig. 5C). In addition, *J. caulescens* lost one pair of NORs after its divergence. The ancestral heterochromatin percentages for clades A_1 and A_2 were reconstructed as 7.70% and 6.03%, respectively (Fig. 5E).

The most likely character states for the L clade ancestor were two NOR pairs ($P = 0.99$, Fig. 5A), co-localized ribosomal genes ($P = 0.55$, Fig. 5B), 18S-5.8S-26S loci restricted to NORs ($P = 0.99$, Fig. 5C), low heterochromatin amount (2.25%, Fig. 5E) and predominance of metacentric chromosomes (1.46 arm ratio, Fig. 5F).

The loss of co-localized rDNA genes is predicted to have occurred independently twice: in *J. runcinata* and *J. leucotricha* (Fig. 5B). The position and number of 5S genes in the common ancestor of *Jaborosa* were uncertain and the change from having one pair to two pairs could have occurred several times in both directions (Fig. 5D).

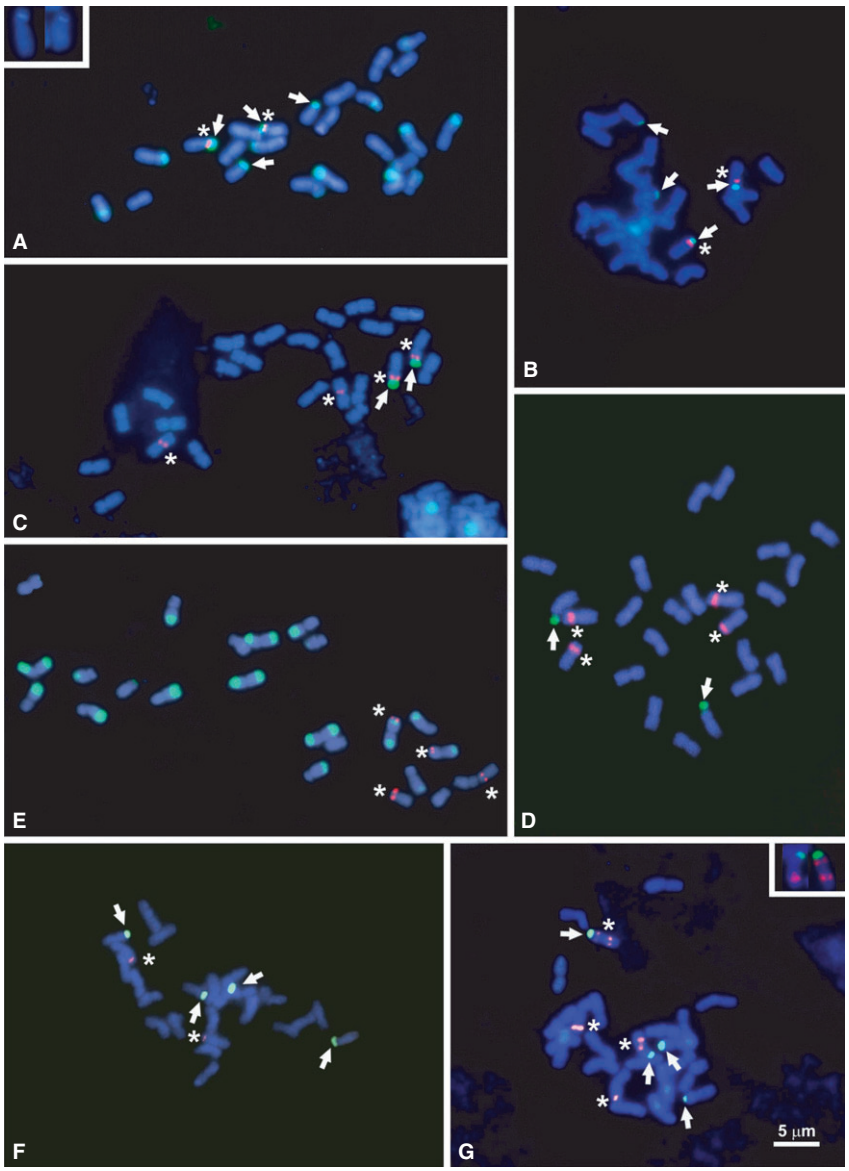


Figure 3. Fluorescence *in situ* hybridization with 5S (red signals) and 18S-5.8S-26S (green signals) rDNA probes in *Jaborosa* spp. and *Sclerophylax spinescens*. Arrows point to chromosomes with 18S-5.8S-26S signals and asterisks indicate chromosomes with 5S signals. The rest of the (green) signals correspond to dispersion of the 18S-5.8S-26S rDNA. A, *J. volkmannii*. B, *J. magellanica*. C, *J. caulescens* var. *bipinnatifida*. D, *S. spinescens*. E, *J. leucotricha*. F, *J. runcinta*. G, *J. integrifolia*. Close-up images: A, 4′,6-diamidino-2-phenylindole (DAPI) band co-localized with 5S rDNA site; G, heteromorphism for 5S/18S-5.8S-26S rDNA. All photographs at the same scale.

DISCUSSION

Heterochromatin patterns

Jaborosa did not show any extreme or discontinuous changes in the heterochromatin patterns, as occurs in several genera from different families (Guerra, 2000): percentages were relatively low and uniform, *c*. 1.5–10.0%. However, the heterochromatin patterns appeared more variable in *Jaborosa* compared with the sister clades *Lycium* and *Sclerophylax*, in which heterochromatin was restricted to the NOR-associated regions and heterochromatin percentages were low (Stiefkens, Las Peñas & Bernardello, 2009; Stiefkens *et al.*, 2010; Blanco *et al.*, 2012). The occurrence of GC-rich heterochromatin sequences adjacent to or interspersed with NORs has been frequently described for many plant species (Guerra, 2000), but not all CMA^+ bands were

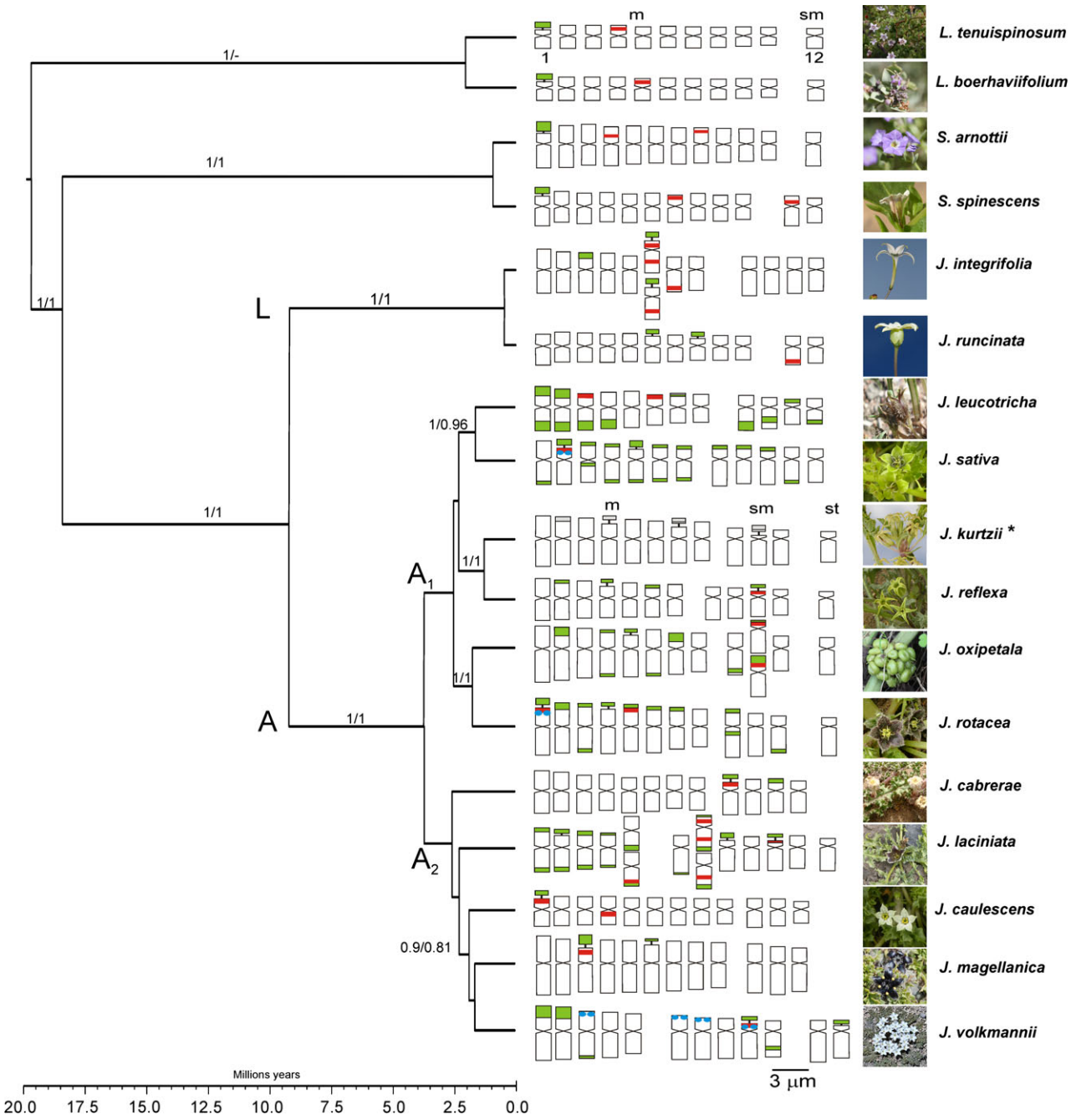


Figure 4. Idiograms of *Jaborosa* spp. and some allies (outgroups), based on mean chromosome values, placed onto the chronogram obtained from four plastid DNA sequences. Support values are shown on the branch (PP BEAST/PP Mr Bayes). All chromosomes at the same scale, ordered by categories, from metacentric (m) to subtelocentric (st). Blue circles, 4′,6-diamidino-2-phenylindole (DAPI$^+$) after fluorescence *in situ* hybridization (FISH) marks; red blocks, 5S rDNA loci; green blocks, 18S-5.8S-26S loci. In *J. integrifolia*, *J. laciniata* and *J. oxipetala*, heteromorphic chromosome pairs are shown. In *J. kurtzii*, only chromomycin A3 (CMA) bands are shown, as grey blocks.

associated with NOR in *Jaborosa*, as additional CMA$^+$ bands were detected on some chromosomes, which implies that GC-rich sequences independent of rDNA genes are also present (Jo *et al.*, 2009). However, CMA$^+$ bands and 45S rDNA sites cannot be associated unequivocally because, in our study, chromosomal techniques could not be performed sequentially.

The heterochromatic bands not associated with NORs were located in terminal positions in most species, corresponding to an equilocal pattern distribution. This agrees with the heterochromatin dispersion model proposed by Schweizer & Loidl (1987). In addition, five species presented an interstitial CMA^+DAPI^- band which could be evidence of a chromosomal rearrangement, such as an inversion or translocation. Similar patterns have been found in other genera of Solanaceae (Moscone, Lambrou & Ehrendorfer, 1996; Chiarini *et al.*, 2014).

Jaborosa runcinata and *J. caulescens*, both with symmetrical karyotypes (i.e. low asymmetry indices; Chiarini & Barboza, 2008), had simple heterochromatin patterns. In contrast, species with many heterochromatin bands (terminal and interstitial) had more asymmetrical karyotypes. This relationship between karyotype asymmetry and heterochromatin bands is more evident when comparing *Jaborosa* with *Lycium* (Stiefkens *et al.*, 2010; Blanco *et al.*, 2012) and *Sclerophylax*, and supports the suggestion that *Jaborosa* has undergone chromosome rearrangements during its recent evolution.

Another type of heterochromatin is that revealed when DAPI is applied after the denaturation/renaturation of DNA in the FISH procedure, thus detecting predominant AT-specific sequences (Bogunić *et al.*, 2011). The number of $DAPI^+$ bands after FISH may be either the same as that detected with the CMA/DAPI technique or higher (Barros e Silva & Guerra, 2010). In the species studied here, the post-FISH $DAPI^+$ bands did not coincide well with any bands visualized with the CMA/DAPI procedure, except for three species, in which the post-FISH DAPI bands coincided with the 5S signal, a fact already noticed in species from other families (Garcia *et al.*, 2007; Chang, Shii & Chung, 2009). In our case, the presence of these post-FISH DAPI bands was shown to be constant in all cells of a single individual and was therefore useful as a species-specific character.

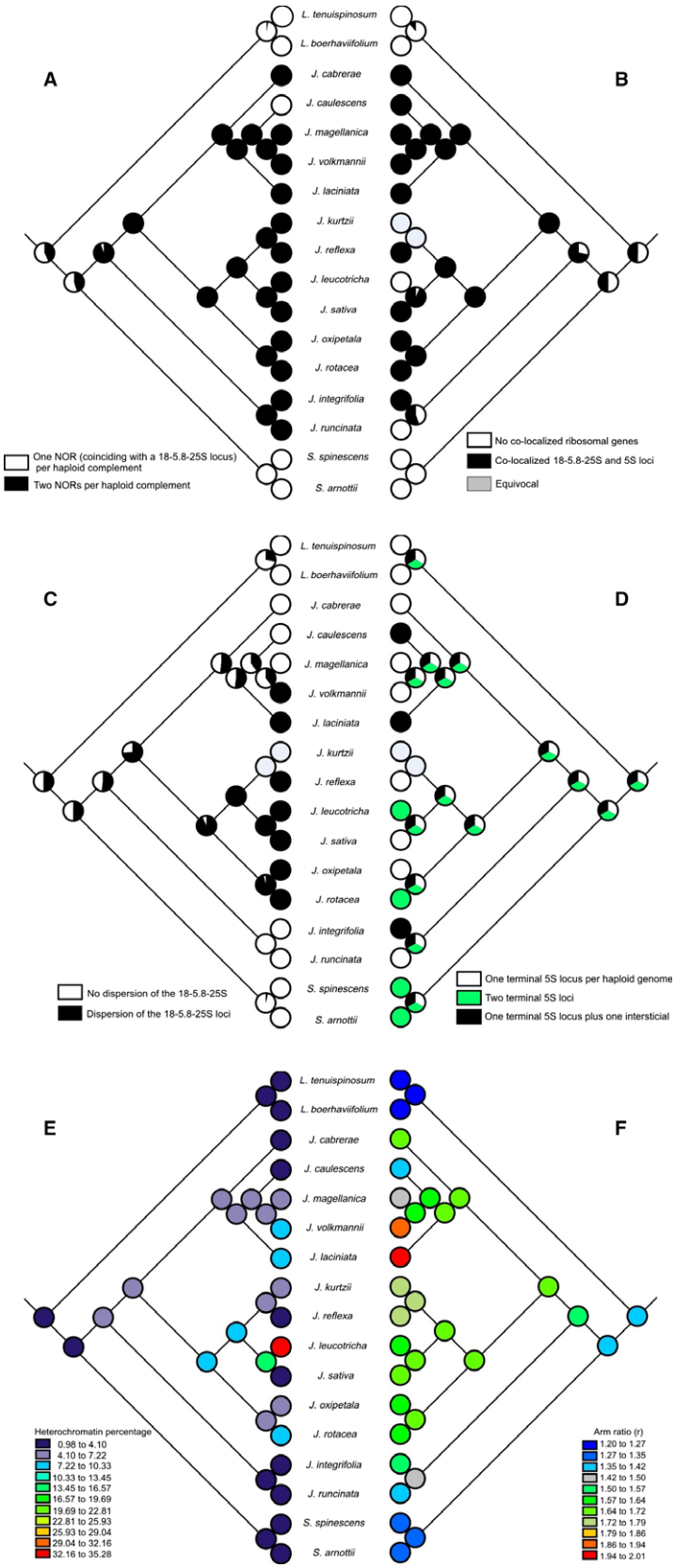
It has been observed that species with large chromosomes have greater numbers of heterochromatic bands than those with small chromosomes (Moscone *et al.*, 1996; Guerra, 2000; Chiarini *et al.*, 2014), and a correlation between karyotype length (and consequently chromosome size) and the number or length of heterochromatic bands has been reported in some families (e.g. Benko-Iseppon & Morawetz, 2000) and in some genera of Solanaceae (Pringle & Murray, 1993; Moscone *et al.*, 1996; Chiarini *et al.*, 2014). Certainly, *Jaborosa* has larger chromosomes and more heterochromatin than its closest related genera, *Lycium* and *Sclerophylax*. However, although there are examples of positive (Las Peñas, Bernardello & Kiesling, 2008) and negative (Stebbins, 1971) correlations between heterochromatin amount and chromosome asymmetry, such trends were not detected in the species considered here.

Ribosomal genes

In different plant families, the 18S-5.8S-26S sites may not only vary in their position (e.g. Raskina, Belyayev & Nevo, 2004; Altinkut *et al.*, 2006; Datson & Murray, 2006), but also in their transcriptional activity, the signals < 0.2 μm being regarded as inactive (Winterfeld & Röser, 2007). In most *Jaborosa* spp. examined so far, there are two main 18S-5.8S-26S sites per basic genome, although several species also had minor sites along the chromosome complement. This type of dispersion of 18S-5.8S-26S has been observed in other Solanaceae (Urdampilleta *et al.*, 2015). The presence of minor sites could represent the final stage of DNA loss from these loci, a well-established phenomenon, especially in polyploids (Kotseruba *et al.*, 2010; Chiarini *et al.*, 2014).

Concerning the 5S ribosomal genes, the occurrence of more than two signals in different positions, as detected in *Jaborosa*, has also been reported for other genera of Solanaceae, e.g. *Solanum* L. (Dong *et al.*, 2000), *Nicotiana* L. (Lim *et al.*, 2000; Kitamura *et al.*, 2001) and *Cestrum* L. (Urdampilleta *et al.*, 2015). A series of duplications and deletions in the different lineages could explain the current situation. Our results suggest that the 5S rDNA could be used as a marker of species and/or species groups, because its location and number of sites in *Jaborosa* spp. are not conserved.

Great variability in the number and position of the 18S-5.8S-26S and 5S loci has been reported among related species (Hasterok *et al.*, 2006; Heslop-Harrison & Schwarzacher, 2011; Lan & Albert, 2011; Chacón *et al.*, 2012; Morales *et al.*, 2012). Given that the rDNA family shows some features that are also associated with mobile elements (Li & Zhang, 2002; Stupar *et al.*, 2002), different types of transposable element have been postulated to be responsible for the rapid change in copy number and chromosomal location of rDNA in plant genomes (Dubcovsky & Dvorák, 1995; Mukai & Raina, 1999; Li & Zhang, 2002; Lönnig & Saedler, 2002; Li, Ru & Zhang, 2004; Raskina *et al.*, 2004; Altinkut *et al.*, 2006; Datson & Murray, 2006). The copy number of migrated rDNA repeats could then be amplified by unequal crossing over, to the extent that these new sites can be detected by FISH (Fukushima *et al.*, 2011). Such chromosomal phenomena could explain the situation found in *Jaborosa*, which always retained the diploid number $2n = 24$, but which varied in the number and location of rDNA. Indeed, closely related species with the same chromosome number can differ drastically in their rDNA, indicating rapid increase,


A
B
C
D
E
F
L. tenuispinosum
L. boerhaviifolium
J. cabrerae
J. caulescens
J. magellanica
J. volkmannii
J. laciniata
J. kurtzii
J. reflexa
J. leucotricha
J. sativa
J. oxipetala
J. rotacea
J. integrifolia
J. runcinata
S. spinescens
S. arnottii
One NOR (coinciding with a 18-5.8-25S locus) per haploid complement
Two NORs per haploid complement
No co-localized ribosomal genes
Co-localized 18-5.8-25S and 5S loci
Equivocal
No dispersion of the 18-5.8-25S
Dispersion of the 18-5.8-25S loci
One terminal 5S locus per haploid genome
Two terminal 5S loci
One terminal 5S locus plus one intersticial
Heterochromatin percentage
0.98 to 4.10
4.10 to 7.22
7.22 to 10.33
10.33 to 13.45
13.45 to 16.57
16.57 to 19.69
19.69 to 22.81
22.81 to 25.93
25.93 to 29.04
29.04 to 32.16
32.16 to 35.28
Arm ratio (r)
1.20 to 1.27
1.27 to 1.35
1.35 to 1.42
1.42 to 1.50
1.50 to 1.57
1.57 to 1.64
1.64 to 1.72
1.72 to 1.79
1.79 to 1.86
1.86 to 1.94
1.94 to 2.01

decrease or translocations of these genes. In Brassicaceae, for example, the number of rDNA sites can differ up to five-fold (Hasterok *et al.*, 2006), whereas, in *Alstroemeria* L. (Chacón *et al.*, 2012), the rDNA sites are polymorphic among and within species, with interstitial telomeric sites suggesting chromosome fusion. Inter- and intraspecific rDNA distribution can even occur without any modification of the chromosome morphology, again suggesting a noticeable incidence of transpositions in the diversification of rDNA distribution patterns (Fukushima *et al.*, 2011; Lan & Albert, 2011). The possible role of the Rabl orientation in determining the spread and accumulation of both types of rRNA gene has also been argued (Winterfeld & Röser, 2007).

Another remarkable phenomenon found in *Jaborosa* is the heteromorphism observed in the rDNA loci of some chromosome pairs. Similar polymorphisms have been reported in other plant families (Moreno *et al.*, 2015) and in *Solanum* (Chiarini *et al.*, 2014). Vaio *et al.* (2005), studying *Paspalum* L. (Poaceae), attributed the heterozygous karyotypes with the odd number of 18S-5.8-26S rDNA arrays to reproductive self-incompatibility. Indeed, *J. integrifolia*, one of the species that presents heteromorphic pairs, is the only one for which the reproductive system has been studied to date, and it is self-incompatible (Vesprini & Galetto, 2000). Further studies are needed to understand the causes underpinning these observations.

In higher eukaryotes, the 18S-5.8S-26S rDNA and 5S rDNA loci are transcribed by different RNA polymerases and are usually located on different chromosomes (Srivastava & Schlessinger, 1991). Co-localization of the 18S-5.8S-26S and 5S rDNA loci has commonly been reported in animals (Dobigny *et al.*, 2003), but is less frequent in plants (e.g. Garcia *et al.*, 2007; Chang *et al.*, 2009; Abd El-Twab & Kondo, 2012). The insertion of 5S rDNA into 18S-5.8S-26S rDNA is thought to be accidental and of unknown significance, which may be mediated by transposons or retrotransposons, as discussed above. In our study, the limited resolution of FISH and the high condensation of the metaphase chromosomes produced an overlap of 18S-5.8S-26S and 5S signals, which does not necessarily mean that they are at the same locus. Rather, it is more likely that they are in close proximity. Anyway, in *Jaborosa*, the presence of both rDNA signals on a single chromosome may be interpreted as evidence of chromosomal rearrangements and/or gene duplication.

Chromosome evolution

Jaborosa shows a distinctive evolutionary pathway at the chromosomal level when compared with its sister clades. For instance, most genera of Hyoscyameae are polyploids or have derived basic numbers ($2n$ = 28, 34, 42, 44, 48, 68, 72, 84, 96) originating by dysploidy, probably from a polyploid ancestor (Tu *et al.*, 2005), whereas *Lycium*, *Sclerophylax* and *Nolana* are mostly diploids with $2n$ = 24 (Chiarini & Barboza, 2008; Stiefkens *et al.*, 2009, 2010; Blanco *et al.*, 2012). Dispersal of Hyoscyameae from the New World to Eurasia in the early Miocene (Tu *et al.*, 2010) was accompanied by changes in the basic chromosome number, whereas, in *Jaborosa*, the full range of chromosomal changes detected here have occurred at the diploid level.

The available data suggest that the divergence of the common ancestor of *Jaborosa* involved an increase in chromosome size, with a slight increase in the amount of heterochromatin and chromosome asymmetry. Moreover, co-localization of the rDNA genes would be a new acquisition of the most recent common ancestor of *Jaborosa*, unlike the observations in *Lycium* and *Sclerophylax* (Chiarini & Barboza, 2008; Stiefkens *et al.*, 2010; this work). The heterochromatin percentage and chromosome asymmetry tend to increase at the same time in some species from the A_2 clade (*J. volkmannii* and *J. laciniata*), but this seems to be homoplasious and there is no evident correlation with any environmental factor, as there are species in the same clade that have a low heterochomatin percentage and inhabit either colder southern areas or warmer northern areas.

In *Jaborosa*, the possession of two pairs of 18S-5.8S-26S loci per basic genome seems to be the ancestral character state shared by most species, contrary to the situation in *Lycium* and *Sclerophylax*, which only have one pair. Only *J. caulescens* stands out for having lost one of the sites (estimated to have occurred at 1.63 Mya). The dispersion of minor 18S-5.8S-26S loci in *Jaborosa* is predicted to have occurred early in its diversification, whereas

Figure 5. Ancestral character state reconstruction of chromosome features in *Jaborosa* and allied taxa. A, Number of nucleolar organizer regions (NORs). B, Co-localization of rDNA genes. C, Dispersion of 18S-5.8S-26S. D, Number and position of 5S loci. E, Heterochromatin percentage. F, Arm ratio. A–D using maximum likelihood (ML, Mk1 model) for discrete character reconstruction, E and F using maximum parsimony for continuous character reconstruction, both implemented in Mesquite.

losses/translocations occurred independently in the more derived lineages. At the same time, the number and position of the 5S loci seem to be homoplasious, with some populations duplicating the 5S loci after species divergence.

The rDNA arrays may follow different evolutionary pathways. In some plant groups, 18S-5.8S-26S is more variable than 5S in terms of the number and location of sites (Książczyk, Taciak & Zwierzykowski, 2010), being the target of interlocus gene conversion, whereas 5S seems less vulnerable to this type of modification (Fulneček *et al.*, 2002). Conversely, in other plant groups, the array number of 5S rDNA is more variable than that of 18S-5.8S-26S rDNA, and the generation of a novel locus via transposition may be more frequent in the former than in the latter (Garrido *et al.*, 1994; Fukushima *et al.*, 2011; Lan & Albert, 2011). Alternatively, amplification or maintenance of the copy number in a novel array is more effective in 5S rDNA. In *Jaborosa*, contrasting evolutionary pathways of the organization of 5S and 18S-5.8S-26S rDNA sequences seem to have occurred, the first being more stable than the second. In a timespan of 3.7 Mya (the age of clade A), there was only one loss of an 18S-5.8S-26S site and a couple of dispersion events, whereas the number and position of the 5S site underwent several changes. This differential evolution of rDNA loci can offer some information regarding concerted evolution (Zhang & Sang, 1999; Li & Zhang, 2002). The terminal or near-terminal location of 18S-5.8S-26S rDNA may permit unequal crossing over without any deleterious recombination between the non-homologous chromosomes, which might facilitate the process of sequence homogenization (Zhang & Sang, 1999).

Chromosomes and geological events

The split of *Jaborosa* was estimated to have occurred during the late Miocene, when the Andes were still relatively low (400–2500 m) and the climate was warm and humid at southern latitudes (Hughes & Eastwood, 2006). These stable conditions would have allowed the accumulation of particular chromosomal characteristics over a broad timespan. The diversification in clades A_1 and A_2 was estimated to have taken place more recently, during the Pliocene–Pleistocene transition, correlating with the final uplift of the Andes to their current elevation (Hughes & Eastwood, 2006; Cosacov *et al.*, 2010). We suggest that the emergence of these new biomes led to colonization by *Jaborosa* spp. relatively recently, and was accompanied by chromosome and morphological diversification (Moré *et al.*, 2015; this work). In disturbed habitats, plants with different chromosome numbers or different karyotypes are loosely interconnected by occasional hybridization (Zohary & Plitmann, 1979; Samuel *et al.*, 2003). Colonization appears to be associated with reticulate evolution and the recombination of previously separated gene pools. The wide karyotypic variability observed in *Jaborosa* could be interpreted as a result of these relatively recent processes (Zohary & Plitmann, 1979; Samuel *et al.*, 2003). Several species in clade A grow in harsh microhabitats (e.g. sandy slopes > 3000 m), with individuals typically isolated or in widely separated populations comprising just a few individuals. It has also been noticed that dispersed repetitive sequences, such as retrotransposons, become more active under stress conditions, leading to an increase in the DNA amount (Raskina *et al.*, 2008; Ågren & Wright, 2011). In contrast, *Lycium* and *Sclerophylax*, which belong to much older clades that are established over wide areas (Pampas, Chaco and Monte phytogeographical provinces), and the L clade of *Jaborosa*, grow in more uniform, open areas, consistent with their karyotype uniformity. Lycieae are 4.90–5.11 Mya old (Miller *et al.*, 2011; Särkinen *et al.*, 2013) and their dispersal to the Old World was estimated to have taken place *c.* 3.64 Mya. Despite these recent events, African *Lycium* has a more conserved heterochromatin pattern, as demonstrated by Stiefkens *et al.* (2010), *Lycium* from Asia shows the usual symmetrical karyotypes (Sheidai, Narengi & Khatamsaz, 1999; Chen *et al.*, 2013), whereas European species have the same rDNA pattern as the South American species (L. Stiefkens, pers. comm.). In contrast, *Jaborosa*, which diversified at approximately the same time, shows profuse chromosome changes.

Overall, the data suggest that the genome structure in *Jaborosa* evolved quickly and dynamically, involving rearrangements (translocations, duplications, etc.), increases in the percentage of heterochromatin and chromosomal size, and changes in the number and position of both rDNA loci. However, despite all these changes often occurring independently in different lineages and at different rates, the number $2n = 24$ remained stable. It is noted that these contrasting evolutionary changes at the chromosome level have been accompanied by divergence or morphological differentiation of species. These observations contrast with the situation in the sister clade *Sclerophylax* and, especially, in *Lycium*, in which genomes seem to be more uniform and karyotypic orthoselection has been argued to have occurred (Bernardello, Stiefkens & Las Peñas, 2008; Stiefkens *et al.*, 2010), independently of the morphological variability observed in these two genera. In summary, the analysis of several chromosomal markers has clearly provided new insights

into the types and extents of chromosome changes that have taken place in different species during the evolution and morphological diversification in *Jaborosa*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Majority-rule consensus tree resulting from Bayesian analyses.
Table S1. Species and GenBank accession numbers for sequence data for the four plastid markers (*trnH-psbA*, *trnD-trnT*, *ndhF-rpl32*, *rpl32-trnL*).
Table S2. Matrix for ancestral character reconstruction.