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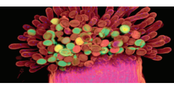

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RESEARCH PAPER

Karyotype evolution and phylogenetic analyses in the genus *Cardiospermum* L. (Paullinieae, Sapindaceae)

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Keywords

Chromosome banding; cytotaxonomy; fluorescence *in situ* hybridisation; phylogeny; polyploidy.

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ABSTRACT

***Cardiospermum* L. belongs to the Paullinieae tribe (Sapindaceae) and comprises 16 species. Of these, 12 species are present in South America and all occur in Brazil. *Cardiospermum* shows the most variable chromosome number of the tribe. Phylogenetic relationships within the genus *Cardiospermum*, especially with other species of the tribe, are poorly understood. This research focuses on characterisation of the karyotypic features of *Cardiospermum* using conventional cytogenetic methods, CMA/DAPI chromosome banding and fluorescence *in situ* hybridisation (FISH). To elucidate the phylogeny of the genus, the nuclear markers ITS1 and ITS2 were sequenced and analysed using maximum parsimony and Bayesian inference. *Cardiospermum* shows important diversity in basic numbers, with x = 7, 9, 10, 11 and 12. All species studied have metacentric and submetacentric chromosomes, some species have subtelocentric chromosomes, while telocentric chromosomes are absent. The interphase nuclei differentiate the *Cardiospermum* species into two groups. The CMA_3/DAPI chromosome banding revealed the presence of an AT-rich terminal region in *C. corindum*, *C. grandiflorum* and *C. urvilleoides*, whereas GC-rich regions were found in *C. grandiflorum*, *C. halicacabum* var. *halicacabum*, *C. halicacabum* var. *microcarpum*, *C. heringeri* and *C. integerrimum*. FISH revealed syntenic and non-syntenic distribution of the 18-5.8-26S and 5S rDNA. The syntenic distribution always occurred in the short arms of the same chromosome in all of the species. The phylogenetic relationships reveal, in part, the taxonomic arrangement of the genus *Cardiospermum*.**

INTRODUCTION

Cardiospermum L. comprises 16 species of pantropical distribution, all of which occur in the Americas (Ferrucci 2000a). In South America, 12 species are present, all occurring in Brazil: *C. anomalum* Cambess., *C. bahianum* Ferrucci and Urdampilleta, *C. corindum* L., *C. cristobaliae* Ferrucci and Urdampilleta, *C. grandiflorum* Sw., *C. halicacabum* L., *C. heringeri* Ferrucci, *C. integerrimum* Radlk., *C. oliveirae* Ferrucci, *C. procumbens* Radlk., *C. pterocarpum* Radlk. and *C. urvilleoides* (Radlk.) Ferrucci. *C. corundum*, *C. grandiflorum* and *C. halicacabum* are cosmopolitan species, considered invasive species in Africa and Oceania, damaging annual crops such as soybean (Johnston *et al.* 1979). The remaining nine species have a restricted distribution, with *C. bahianum*, *C. cristobaliae*, *C. integerrimum*, *C. heringeri*, *C. oliveirae*, *C. procumbens* and *C. urvilleoides* being endemic to Brazil.

Ferrucci (2000a) made a taxonomic treatment of the genus, and two new Brazilian species have recently been included in the genus (Ferrucci & Urdampilleta 2011a,b). *Cardiospermum* species are divided into three sections: *Cardiospermum* Radlk., *Carphospermum* Radlk. and *Ceratadenia* Radlk (Radlkofer 1878, 1931; Ferrucci 2000a). The diagnostic characters used to recognise the sections are morphology and number of floral nectariferous lobes, and seed features. The phylogenetic relationships among *Cardiospermum* and other Paullinieae genera are poorly understood. In phylogenetic analysis of the tribe Paullinieae, the *Cardiospermum–Urvillea* clade is differentiated from other genera of the tribe by derived characters such as herbaceous habit, reduced chromosome number, dry aril and membranous pericarp (Acevedo-Rodríguez 1993). However, the delimitation between *Cardiospermum* and *Urvillea* is not clearly defined.

Cardiospermum presents the highest diversity of chromosome number in the tribe Paullinieae; data on chromosome number are available for eight species of *Cardiospermum* and karyotypes have been described in four of them (Ferrucci 1989; Hemmer & Morawetz 1990). Four basic numbers, x = 7, 9, 10 and 11, were found in *Cardiospermum* (Ferrucci 1989); therefore, aneuploidy/disploidy has an important role in the karyotype evolution of this genus, as previously reported for *Urvillea* (Ferrucci 2000b). The distribution patterns of heterochromatin and nucleolar organising regions (NORs) were used to differentiate some genera and species in the Paullinieae tribe (Hemmer & Morawetz 1990; Nogueira *et al.* 1995; Urdampilleta *et al.* 2006, 2007, 2008). Hemmer & Morawetz (1990) studied the C-Giemsa and CMA/DAPI banding patterns of two species of *Serjania* and two of *Cardiospermum*. The results of this work

reflect the importance of the reduction in chromosome number and the accumulation of AT-rich heterochromatin as processes involved in karyotype evolution of Paullinieae.

The objective of the present study was to apply cytogenetic and DNA sequence-based methods to explore phylogenetic relationships among *Cardiospermum* species, using conventional cytogenetic techniques, CMA/DAPI banding, fluorescence *in situ* hybridisation (FISH) and sequence analysis of internal transcribed spacers (ITS1 and ITS2). The results obtained are compared with data from the literature to evaluate and understand evolutionary relationships among *Cardiospermum* species.

MATERIAL AND METHODS

Plant material

The *Cardiospermum* species studied in this work and collection data are detailed in Table 1. Voucher specimens were deposited at the herbaria of CTES (Herbarium of the Instituto de Botánica del Nordeste, Corrientes, Argentina), FUEL (Herbarium of the Universidade Estadual de Londrina, Paraná, Brazil) and UEC (Herbarium of the Universidade Estadual de Campinas, Brazil).

Chromosome preparation

Chromosomes were obtained from root meristems pretreated with 2 mM 8-hydroxyquinoline at 15 °C for 4–5 h, fixed in ethanol:acetic acid (3:1, v:v) for 24 h at room temperature and stored at −20 °C until use. Staining was performed using the HCl/Giemsa technique (Guerra 1983); after hydrolysis with 1N HCl (60 °C, 10 min), the root meristems were squashed in 45% acetic acid and stained in 2% Giemsa. Idiograms were prepared from chromosome measurements of five complete metaphases with a similar state of condensation using the MicroMeasure v. 3.3 software (available at http://www.colostate.edu/Depts/Biology/MicroMeasure). The nomenclature used for description of mitotic chromosome morphology is that proposed in Levan *et al.* (1964). Subsequently, the karyotypes were compared using the parameters: average chromosome length (ACL), total chromosome length (TCL) of the diploid genome, relationship between higher and lower chromosome (IR), average centromeric index (ACI) and asymmetry indices A_1/A_2 (Romero Zarco 1986), and CVCi and CVCl (Paszko 2006).

The slides for chromosome banding (CMA_3/DAPI) and FISH were prepared using roots previously digested with a solution of 4% cellulase and 40% pectinase at 37 °C for 2 h, and the chromosomes were squashed in 45% acetic acid. The coverslips were removed after freezing in liquid nitrogen and the slides were air-dried. For fluorescent banding, the aged slides were stained with $0.5\ mg \cdot ml^{-1}$ chromomycin A_3 (CMA_3) and $2\ mg \cdot ml^{-1}$ DAPI (4-6-diamino-2-phenylindole) (Schweizer 1976) and mounted in a 1:1 (v:v) glycerol/McIlvaine buffer pH 7.0 containing 2.5 mM $MgCl_2$.

Fluorescence *in situ* hybridisation (FISH)

The protocols for FISH followed the methodology of Schwarzacher & Heslop-Harrison (2000). The probe p*Ta*71 was used to map the 18S-5.8S-26S rDNA loci (Gerlach & Bedbrook 1979), labelled with biotin-14-dUTP by nick translation (Bionick; Invitrogen, Grand Island, NY, USA) and subsequently detected with avidin-FITC (Sigma, New York, NY, USA). For analysis of 5S rDNA loci, the probe was obtained by PCR amplification using primers RTPCR5S1 and RTPCR5S2 (Mathieu *et al.* 2003), with genomic DNA of *C. grandiflorum* as template. The 5S fragments were labelled with digoxigenin-11-dUTP (DIG Nick translation mix; Roche, Indianapolis, IN, USA) and detected with anti-DIG-rhodamine (Roche). The slides were mounted with antifade Vectashield (Vector Laboratories, Peterborough, UK) and photographs were taken using a BX51 microscope (Olympus, Center Valley, PA, USA) equipped with Evolution MT CCD system and Image ProPlus v. 6 software (Media Cybernetics, Inc., Bethesda, MD, USA) for digital image capture.

DNA isolation, amplification and ITS sequencing

Total genomic DNA was isolated from leaf tissue using the CTAB II protocol (Weising *et al.* 2005). The internal transcribed spacer region (ITS) was amplified with the PCR technique (Mullis & Faloona 1987) using ITS4/ITS5 (White *et al.* 1990) primer pair. PCR amplifications were performed using a MasterCycler (Eppendorf, Hamburg, Germany) in a total volume of 50 μl, containing 1.5 mM $MgCl_2$, 0.1 μM each primer, 0.2 mM dNTP and 1.25 U GoTaq DNA polymerase (Promega, Madison, WI, USA). PCR products were purified with a polyethylene glycol 8000 mixture (PEG 80010 20%, NaCl 2.5 M), and fluorescence sequencing was performed by Macrogen (Seoul, South Korea) with the same primers used for PCR amplification.

Alignment and phylogenetic analysis

Sequences were aligned with MAFFT 6 using the default options (Katoh & Toh 2008). Outgroup selection relied on the phylogenetic analysis of Harrington *et al.* (2005) and Buerki *et al.* (2009). *Paullinia pachycarpa* Benth. (Genebank EU720500.1), *Serjania glabrata* Kunth and *Urvillea triphylla* Radlk, all tribe Paullinieae, were included as outgroups for this work.

Phylogenetic reconstruction was performed using maximum parsimony (MP) criteria with the TNT 1.1 software (Goloboff *et al.* 2008). For construction of the parsimony tree, the traditional search engine was used with the tree bisection–reconnection (TBR) swapping algorithm, adding 10,000 random addition sequences (RAS) and saving 50 trees per RAS. The shortest equally most parsimonious trees were combined to produce a strict consensus tree. To assess support at each node, a bootstrap analysis was carried out with the same search algorithm used for constructing trees, with 10,000 RAS. Bremer support values (decay index; Bremer 1988) were obtained with suboptimal trees of 20 steps. In this study, nodes with bootstrap support of 50–74% are considered weakly supported, 75–89% as moderately supported and 90–100% as strongly supported.

To compare results from the maximum parsimony analysis with another analytical method, we used the Bayesian MCMC algorithm with MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003); the best-fitting model was the general time reversible (GTR) model with an alpha parameter for a gamma distribution.

Table 1. Species studied and karyotype characters: average chromosome length (ACL), total chromosome length (TCL) of the diploid genome, relationship between major and minor chromosomes (RI), average centromeric index (ACI), variation between larger and smaller chromosomes (CV) and A1/A2 (Romero Zarco 1986), and CVci/CVcl (Paszko 2006) asymmetry indices. Infrageneric classification according to Ferrucci (2000a).

			chromosome size (μm)					asymmetry indices				
species	2n	karyotype formulae	TCL	CV	ACL	RI	ACI	A1	A2	CVci	CVcl	collection data
section *Ceratadenia*												
C. grandiflorum	20	6 m + 2 sm + 2 st	31.0	1.9–1.1	1.5	1.8	37.6	0.37	0.18	25.8	17.9	Brazil, SP, Mogi-Guaçu, Firetti s/n (UEC). PR, Paiquerê, Urdampilleta *et al.* 213 (FUEL).
C. heringeri	14*	4 m + 1 sm + 2 st	44.5	4.2–2.2	3.2	2.1	35.8	0.39	0.23	37.7	22.7	Brazil, ES, Santa Teresa, Urdampilleta *et al.* 437 (UEC)
C. integerrimum	14	4 m + 1 sm + 2 st	38.9	3.5–2.0	2.8	1.6	36.2	0.38	0.18	35.4	18.3	Brazil, BA, Ubaitaba, Urdampilleta *et al.* 312 (UEC).
section *Cardiospermum*												
C. corindum	22	2 m + 2 sm + 7 st	17.8	1.2–0.6	0.8	2.1	27.3	0.61	0.26	29.5	25.7	Brazil, BA, Barra, Urdampilleta *et al.* 328 (UEC). Bolivia, Santa Cruz, Ferrucci *et al.* 2660 (CTES).
C. cristobaliae	24	5 m + 7 sm	72.6	4.3–1.9	3.0	1.9	34.2	0.46	0.25	22.7	24.7	Brazil, MG, Botumirim, Urdampilleta *et al.* 421 (UEC).
C. halicacabum												
var. *halicacabum*	22*	3 m + 7 sm + 1 st	17.9	1.0–0.7	0.8	1.5	33.1	0.49	0.12	19.1	12.0	Bolivia, Santa Cruz, Chiquitos, Ferrucci *et al.* 2525 (CTES)
var. *microcarpum*	22	4 m + 7 sm	23.6	1.5–08	1.1	2.1	36.7	0.40	0.21	19.4	20.6	Brazil, PR, 1º de Maio, Urdampilleta *et al.* 171. Argentina, Misiones, Urdampilleta 195 (FUEL)
C. oliveirae	20*	4 m + 5 sm + 1 st	18.5	1.0–0.8	0.9	1.5	30.4	0.55	0.12	23.2	12.3	Brazil, BA, Itaguaçu da Bahia, Urdampilleta *et al.* 337 e 338 (UEC).
C. procumbens	22*	5 m + 2 sm + 4 st	37.7	2.7–1.1	1.7	2.3	34.8	0.43	0.27	26.6	27.2	Brazil, MS, Sidrolandia, Urdampilleta *et al.* 322 (UEC).
C. pterocarpum	22	5 m + 4 sm + 2 st	30.3	2.1–1.0	1.4	2.2	35.1	0.42	0.23	29.6	23.2	Brazil, MS, Sidrolandia, Urdampilleta *et al.* 321 (UEC).
C. urvilleoides	24*	5 m + 7 sm	110.6	6.1–3.3	4.6	1.7	37.1	0.39	0.20	19.6	19.7	Brazil, MG, Itaobim, Urdampilleta *et al.* 425 (UEC).
section *Carphospermum*												
C. anomalum	18	7 m + 2 sm	16.0	1.1–0.8	0.9	1.4	40.2	0.31	0.10	18.9	10.0	Brazil, BA, Ibotirama, Urdampilleta *et al.* 330 (UEC).
C. bahianum	36	11 m + 7 sm	57.4	1.9–1.2	1.6	1.7	38.1	0.37	0.11	15.2	11.3	Brazil, BA, Rio de Contas, Urdampilleta *et al.* 389 (UEC).

*New chromosome counts.

Three Metropolis-coupled Markov chains with an incremental heating temperature of 0.2 were run for 20 million generations and sampled every 1000th generation. The analysis was repeated three times, starting from random trees. Convergence was accepted when standard deviations of split frequencies attained values below 0.01 and when the potential scale reduction factor index approached 1.0. After a burn-in period of 2×10^6 generations per run, a half-compatible ('halfcompat', *i.e.,* majority-rule consensus from MrBayes) consensus tree and its associated Bayesian posterior probability (BPP) was reconstructed using MrBayes v. 3.1.2 based on the 54,000 remaining trees.

RESULTS

Chromosome numbers

Chromosome numbers of the *Cardiospermum* species studied differed significantly, ranging from 2n = 14 to 2n = 36 (Table 1, Fig. 1). The results showed an important diversity in basic number, which varied among x = 7 (*C. heringeri* and *C. integerrimum*, 2n = 14), x = 9 (*C. anomalum*, 2n = 18 and *C. bahianum*, 2n = 36), x = 10 (*C. grandiflorum* and *C. oliveirae*, 2n = 20), x = 11 (*C. corindum*, *C. halicacabum* var. *halicacabum*, *C. halicacabum* var. *microcarpum*, *C. procumbens* and *C. pterocarpum*, 2n = 22) and x = 12 (*C. cristobaliae* and *C. urvilleoides*, 2n = 24). *C. bahianum* is the only polyploid known for the genus (2n = 4x = 36) and shares the basic number (x = 9) with *C. anomalum*. The new chromosome counts are provided in Table 1.

Karyology

The karyotype characters analysed, both in qualitative and quantitative terms, are summarised in Table 1 and Fig. 2. All the species studied possessed 'm' (metacentric) and 'sm' (submetacentric) chromosomes, and most species had 'st' (subtelocentric) chromosomes, whereas 't' (telocentric) chromosomes were absent. The karyotypes of *C. anomalum*, *C. halicacabum* var. *microcarpum* (Kunth) Blume, *C. cristobaliae* and *C. urvilleoides* did not show st chromosomes; by contrast, *C. corindum* exhibited a high number of st chromosomes. *C. anomalum*, *C. corindum*, *C. halicacabum* var. *halicacabum*, *C. halicacabum* var. *microcarpum* and *C. oliveirae* have small chromosomes of up to 1.5 µm, and TCL ranged from 16.0 to 23.6 µm. On the other hand, *C. bahianum*, *C. cristobaliae*, *C. grandiflorum*, *C. heringeri*, *C. integerrimum*, *C. procumbens*, *C. pterocarpum* and *C. urvilleoides* were recognised as having larger chromosomes, from 1.5 to 6.1 µm, and TCL of 30.3 to 110.6 µm (Table 1). *C. urvilleoides* had the largest chromosomes, over sixfold larger than *C. corindum* and *C. halicacabum* karyotypes (Table 1, Fig. 2).

The karyotypes of the species here studied were relatively asymmetric. *C. corindum* presented the karyotype with the highest asymmetry, in contrast to *C. anomalum*, whose karyotype was the most symmetrical. The analysis of symmetry did not allow us to group the species, whereas the dispersion patterns A_1/A_2 and CVci/CVcl showed some differences (Fig. 2), which allowed us to highlight some common features. Low values of karyotype asymmetry were observed in *C. anomalum* and *C. bahianum*, in contrast to *C. corindum*, which presented the highest values for these indices (Table 1, Fig. 2). Regarding A2 and CVcl (=A2 × 100), two groups were observed, one comprising species with low asymmetry, including *C. anomalum*, *C. bahianum*, *C. halicacabum* var. *halicacabum* and *C. oliveirae*, and the other containing the remaining species of higher asymmetry. The dispersion of A1 and CVci index values showed significant differences. The major differences were observed in *C. heringeri* and *C. integerrimum*, which had the highest CVci values, but one of the lowest A1 values among the species studied (Fig. 2).

Based on the structure of interphase nuclei, *Cardiospermum* species was divided into two groups: I and II (Fig. 3A–M). Group I, characterised by areticulate interphase nuclei, contains *C. anomalum*, *C. bahianum*, *C. corindum*, *C. halicacabum* var. *halicacabum*, *C. halicacabum* var. *microcarpum* and *C. oliveirae*. Within this group, chromocentres of *C. corindum* are large, densely stained and fewer in number, whereas the remaining species have smaller chromocentres and vary in number. Group II is characterised by having a semi-reticulate nucleus with highly condensed chromatin regions and contains *C. cristobaliae*, *C. grandiflorum*, *C. heringeri*, *C. integerrimum* and *C. urvilleoides*. In most species of group II a nucleus of similar size and a variable number of chromocentres was observed. However, two species, *C. procumbens* (Fig. 3J) and *C. pterocarpum* (Fig. 3K), had a nucleus of intermediate structure between the two groups. These groups were also recognised by prophase chromosome condensation after staining with HCl/Giemsa (Fig. 3N–Z). In species of group I, along with *C. pterocarpum* and *C. procumbens*, prophase chromosome condensation was always proximal, with highly condensed pericentromeric regions and decondensed terminal regions. Species of group II, particularly *C. cristobaliae*, *C. grandiflorum*, *C. heringeri*, *C. integerrimum* and *C. urvilleoides*, showed a terminal–interstitial condensation pattern, often displaying chromatin blocks condensed in terminal chromosome regions (Fig. 3).

In *C. corindum*, HCl/Giemsa staining gave a lower staining intensity in the six smaller chromosome pairs. The same pattern was observed in two chromosome pairs in *C. halicacabum* var. *microcarpum* and in some smaller pairs of *C. halicacabum* var. *halicacabum* (Fig. 1). The remaining species showed no difference in staining of mitotic chromosomes.

Chromosome banding pattern

Using fluorescence chromosome banding techniques (CMA_3/DAPI), AT-rich heterochromatin bands ($DAPI^+$) were observed in the terminal chromosomal regions of *C. corindum*, *C. grandiflorum* and *C. urvilleoides* (Fig. 4A, B and D, respectively). In the remaining species these AT-rich bands were not observed (Fig. 4E, G, I and K). GC-rich regions (CMA_3^+) were observed in *C. grandiflorum* (Fig. 4C), *C. halicacabum* var. *halicacabum* (Fig. 4F), *C. halicacabum* var. *microcarpum* and *C. integerrimum* (Fig. 4H), but CMA_3^+ sites in *C. procumbens* and *C. pterocarpum* were weakly stained (Fig. 4J and L). In *C. grandiflorum*, *C. halicacabum* var. *halicacabum* and *C. halicacabum* var. *microcarpum* CMA_3^+ bands in terminal regions of the short arm of two chromosome pairs were observed, but in *C. heringeri* and *C. integerrimum* the GC-rich regions were located in the pericentromeric regions of five chromosome pairs (Fig. 4H).

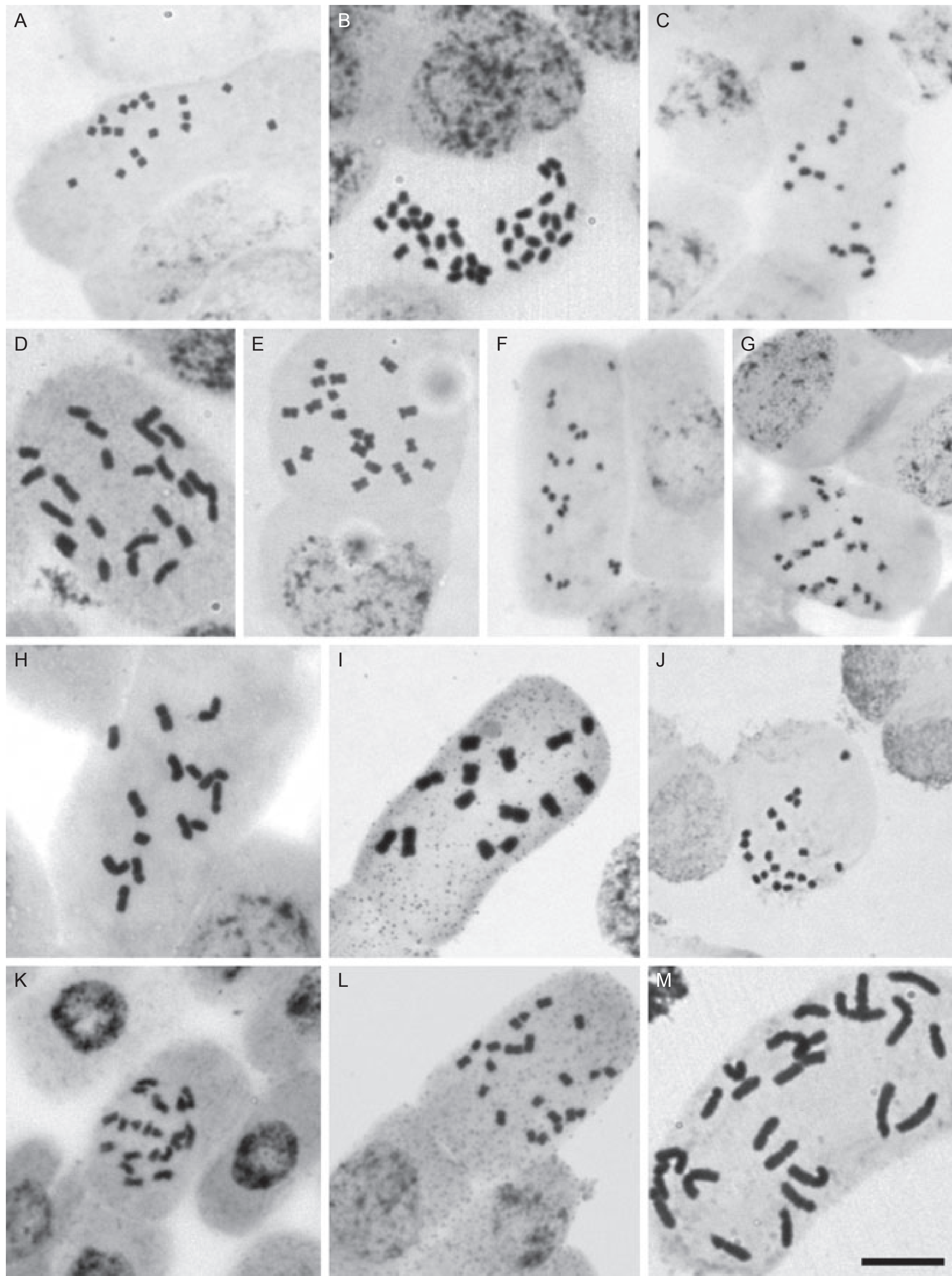

Fig. 1. Metaphase chromosome plates of *Cardiospermum* species stained with HCl-Giemsa. A: *C. anomalum* (2n = 18), B: *C. bahianum* (2n = 36), C: *C. corindum* (2n = 22), D: *C. cristobaliae* (2n = 24), E: *C. grandiflorum* (2n = 20), F: *C. halicacabum* var. *halicacabum* (2n = 22), G: *C. halicacabum* var. *microcarpum* (2n = 22), H: *C. heringeri* (2n = 14), I: *C. integerrimum* (2n = 14), J: *C. oliveirae* (2n = 20), K: *C. procumbens* (2n = 22), L: *C. pterocarpum* (2n = 22), M: *C. urvilleoides* (2n = 24). Bar: 10 μm.

Chromosome distribution of ribosomal DNA (18-5.8-26S and 5S)

The distribution of 18-5.8-26S and 5S rDNA varied among species of *Cardiospermum* (Fig. 5); two types of distribution pattern of 18-5.8-26S and 5S rDNA were observed. In the syntenic type, both 18-5.8-26S and 5S rDNA were mapped in the terminal regions of short arms of the same chromosome (Fig. 5A and B). This pattern was observed only in *C. anomalum* and *C. bahianum*; in both species, the 5S rDNA locus was always proximal to the terminal 18-5.8-26S rDNA locus. In *C. anomalum*, one pair of 18-5.8-26S rDNA signals and one

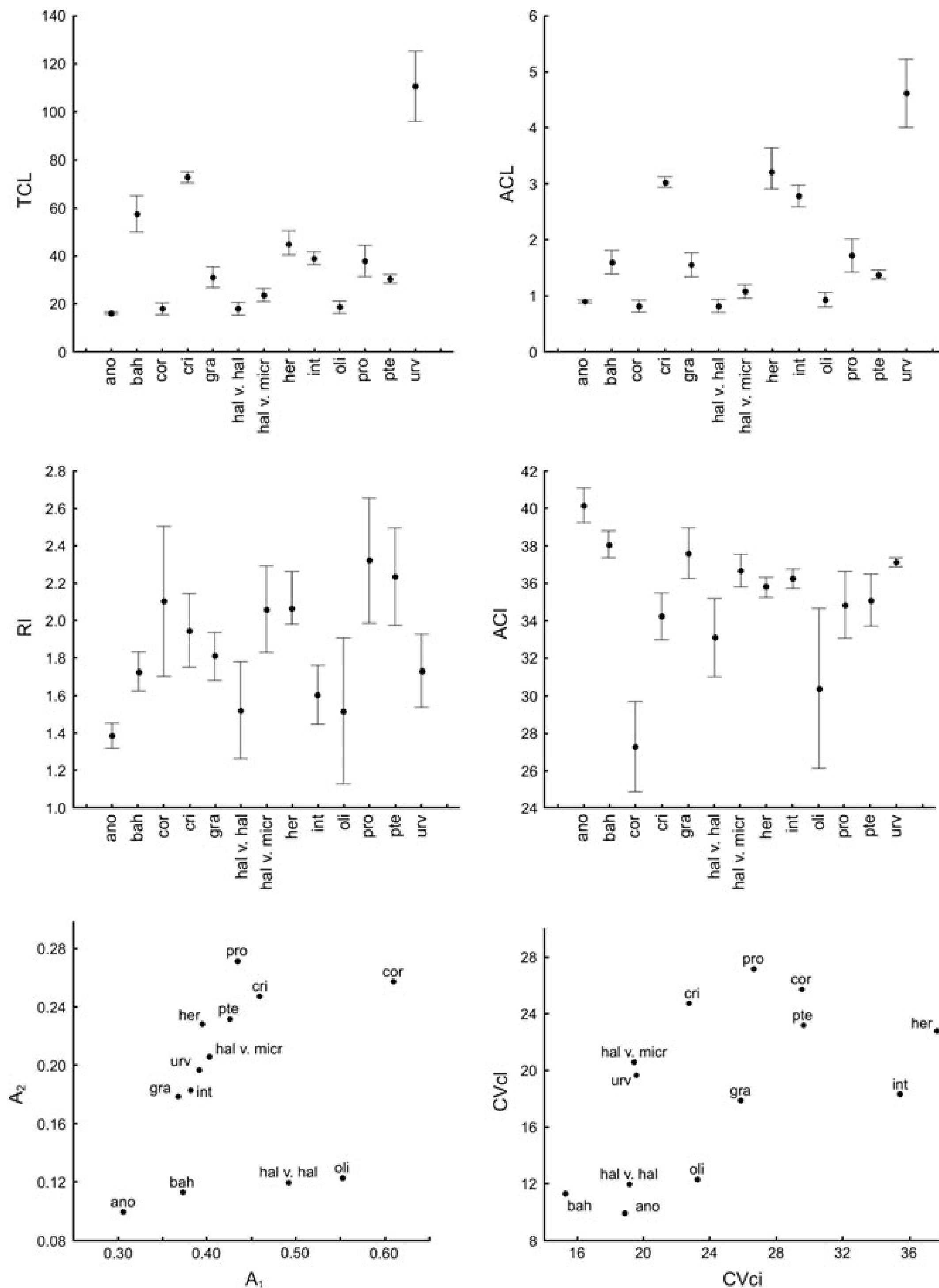

Fig. 2. Karyotype characters of *Cardiospermum*: total chromosome length (TCL), average chromosome length (ACL) of the diploid genome, relationship between major and minor chromosomes (RI), average centromeric index (ACI) and A1/A2, and CVcl CVci asymmetry indices. *C. anomalum* (ano), *C. bahianum* (bah), *C. corindum* (cor), *C. cristobaliae* (cri), *C. grandiflorum* (gra), *C. halicacabum* var. *halicacabum* (hal v. hal), *C. halicacabum* var. *microcarpum* (hal v. micr), *C. heringeri* (her), *C. integerrimum* (int), *C. oliveirae* (oli), *C. procumbens* (pro), *C. pterocarpum* (pte) and *C. urvilleoides* (urv).

pair of 5S rDNA were observed (Fig. 5A), whereas in *C. bahianum*, three pairs of 18-5.8-26S rDNA signals and two pairs of 5S rDNA were found (Fig. 5B). In the other species studied a non-syntenic-type pattern was observed, in which the 18-5.8-26S and 5S rDNA mapped in different chromosomes (Fig. 5C–L). In *C. corindum* three pairs of 18-5.8-26S rDNA loci were found (all in terminal regions) as well as three pairs of 5S sites (two terminal pairs on the short arm and one centromeric pair; Fig. 5C). *C. grandiflorum*, *C. halicacabum* and *C. oliveirae* had two pairs of 18-5.8-26S rDNA in terminal

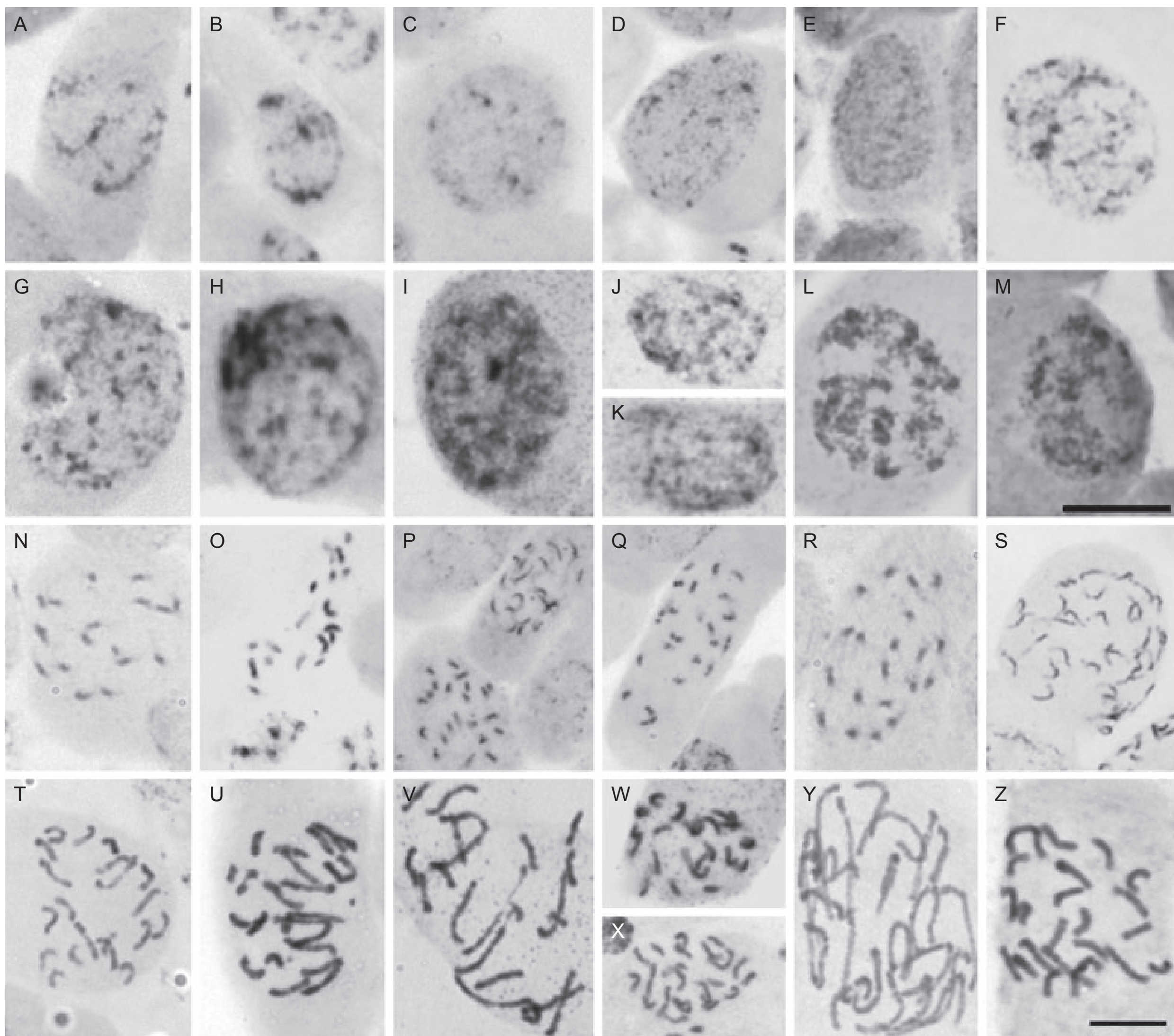

Fig. 3. A–M: variation of interphase nuclei types in *Cardiospermum* species stained with HCl-Giemsa (Bar: 10 μm). Group I, A: *C. anomalum*, B: *C. corindum*, C: *C. halicacabum* var. *halicacabum*, D: *C. halicacabum* var. *microcarpum*, E: *C. oliveirae*, F: *C. bahianum,* J: *C. procumbens*, K: *C. pterocarpum*. Group II, G: *C. grandiflorum*, H: *C. heringeri*, I: *C. integerrimum*, L: *C. urvilleoides*, M: *C. cristobaliae*. N–Z: variation of prophase condensation in *Cardiospermum* species stained with HCl-Giemsa (Bar: 10 μm). Group I, N: *C. anomalum*, O: *C. corindum*, P: *C. halicacabum* var. *halicacabum*, Q: *C. halicacabum* var. *microcarpum*, R: *C. oliveirae*, S: *C. bahianum*, W: *C. procumbens*, X: *C. pterocarpum*. Group II, T: *C. grandiflorum*, U: *C. heringeri*, V: *C. integerrimum*, Y: *C. urvilleoides*, Z: *C. cristobaliae*.

regions of the short arm and one pair of 5S rDNA in the interstitial-proximal region of the long arm (Fig. 5E, F, J and K). *C. cristobaliae*, *C. procumbens* and *C. pterocarpum* presented three pairs of rDNA 18-5.8-26S sites located in the terminal regions of the short arm and one pair of 5S rDNA loci in the pericentromeric region (Fig. 5D and I). In *C. grandiflorum*, a pair of 5S rDNA loci was heteromorphic (Fig. 5E). In *C. urvilleoides* three pairs of 5S rDNA loci were observed in terminal regions of short arms (Fig. 5L); in these species, the 18-5.8-26S rDNA sites were not detectable with the p*Ta*71 probe. *C. heringeri* and *C. integerrimum* exhibited the same rDNA distribution patterns and were differentiated from the other species studied as possessing 18-5.8-26S rDNA loci in the pericentromeric regions of five chromosome pairs and a single pair of chromosomes with 5S rDNA in interstitial regions of the long arm (Fig. 5G and H).

In general, GC-rich sites were observed as bands of CMA_3^+, which were directly associated with the 18-5.8-26S rDNA loci. *C. corindum* showed the 5S rDNA loci associated with AT-rich regions (Figs 4 and 5).

Phylogenetic analyses

In the *Cardiospermum* taxa studied, pair-wise sequence comparisons indicated sequence divergences (ITS1, 5.8S rDNA and ITS2) ranging from 3.58% to 26.9%. The sequence divergence value for ITS1 ranged from 4.33% to 21.36% and ITS2 from 5.91% to 24.85%. The divergence values between *Cardiospermum* taxa and outgroups was between 12.3% and 23.77%. When aligned, the sequences of whole ITS regions yielded a matrix of 768 positions, of which 263 were phylogenetically informative (34.24%). ITS1

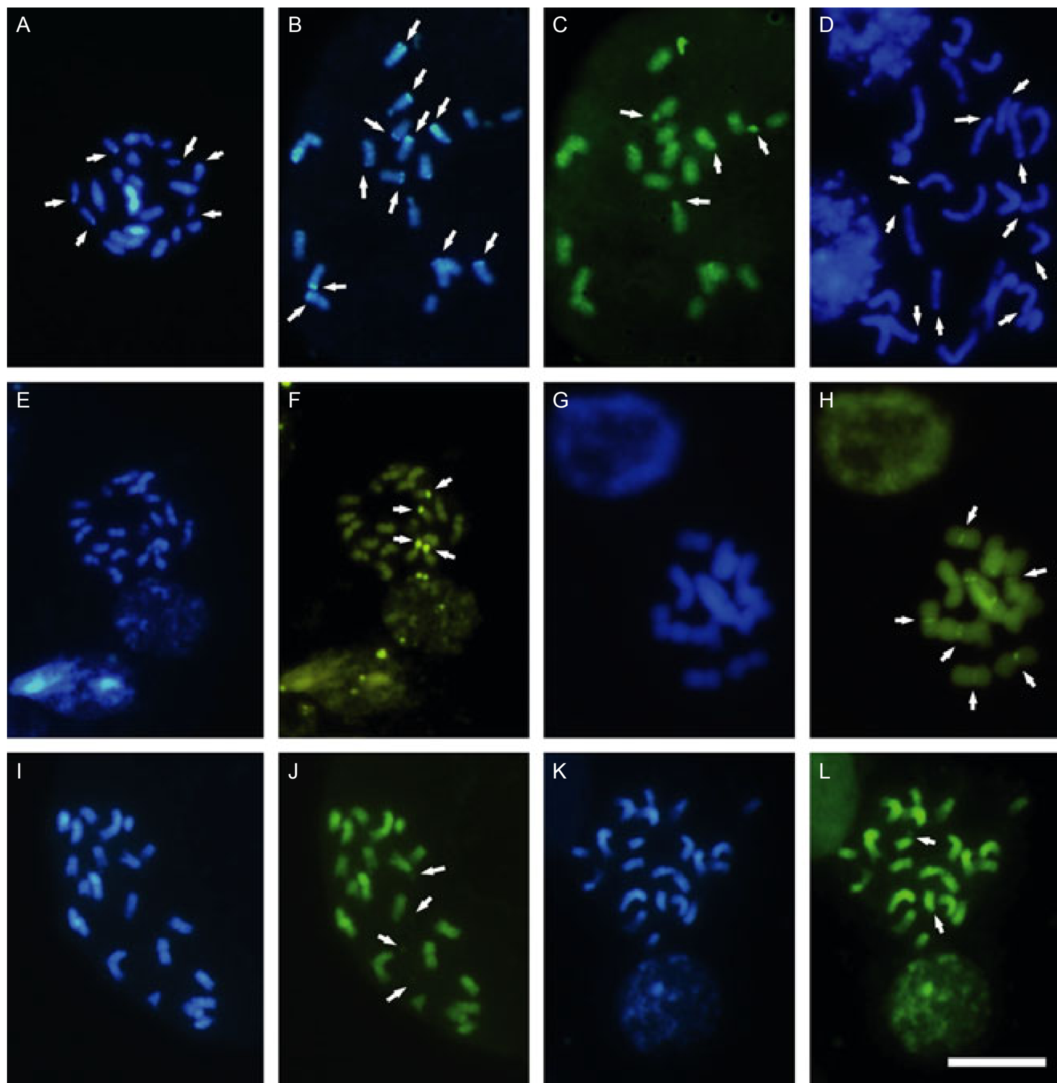

Fig. 4. Fluorescent chromosome banding (CMA_3/DAPI, green/blue) in *Cardiospermum*. AT-rich heterochromatin (CMA^0/$DAPI^+$, arrows) in terminal regions of *C. corindum* (A), *C. grandiflorum* (B, C) and *C. urvilleoides* (D). GC-rich heterochromatin (CMA_3^+/$DAPI^-$, arrows) in *C. grandiflorum* (B, C), *C. halicacabum* var. *halicacabum* (E, F), *C. integerrimum* (G, H), *C procumbens* (I, J) and *C. pterocarpum* (K, L). Bar: 10 μm.

and ITS2 contributed 50 and 118 informative characters, respectively.

Parsimony analysis (MP) of the ITS sequences yielded a unique most-parsimonious tree (length = 1234 steps; CI = 0.75, RI = 0.93). This phylogram shows high resolution, with strong bootstrap support throughout the tree, and four clades, A, B, C and D (Fig. 6A). Clade A shows strong bootstrap support between species belonging to the section *Ceratadenia* (*C. grandiflorum*, *C. heringeri* and *C. integerrimum*). Clade B is formed by a highly supported group of species of the *Carphospermum* section (*C. anomalum* and *C. bahianum*). Clades C and D include species of the section *Cardiopermum*, *C. procumbens*, *C. pterocarpum* and *C. oliveirae* in clade C, and *C. corindum* and *C. halicacabum* (two varieties) in clade D.

The phylogenetic tree inferred through the Bayesian approach has a similar topology to that inferred using MP, with nodes supported with a BPP (Bayesian posterior probability) mean of 0.95. The strict consensus tree with probability values is shown in Fig. 6B, with moderate to strong support throughout the tree. However, the resolution of some clades, especially those close to the root, was much higher with the Bayesian approach.

DISCUSSION

We studied 12 species of *Cardiospermum* from South America in this work, and report five new chromosome counts. These results confirm findings previously reported for the genus (Sugiura 1931; Diers 1961; Ferrucci 1981, 1989, 2000b; Hemmer & Morawetz 1990; Ferrucci & Urdampilleta 2011a,b) and offer new information in relation to karyotyping, banding patterns and distribution of repeated DNA sequences. All the karyotype data observed and their diversity were compared with the phylogenetic study.

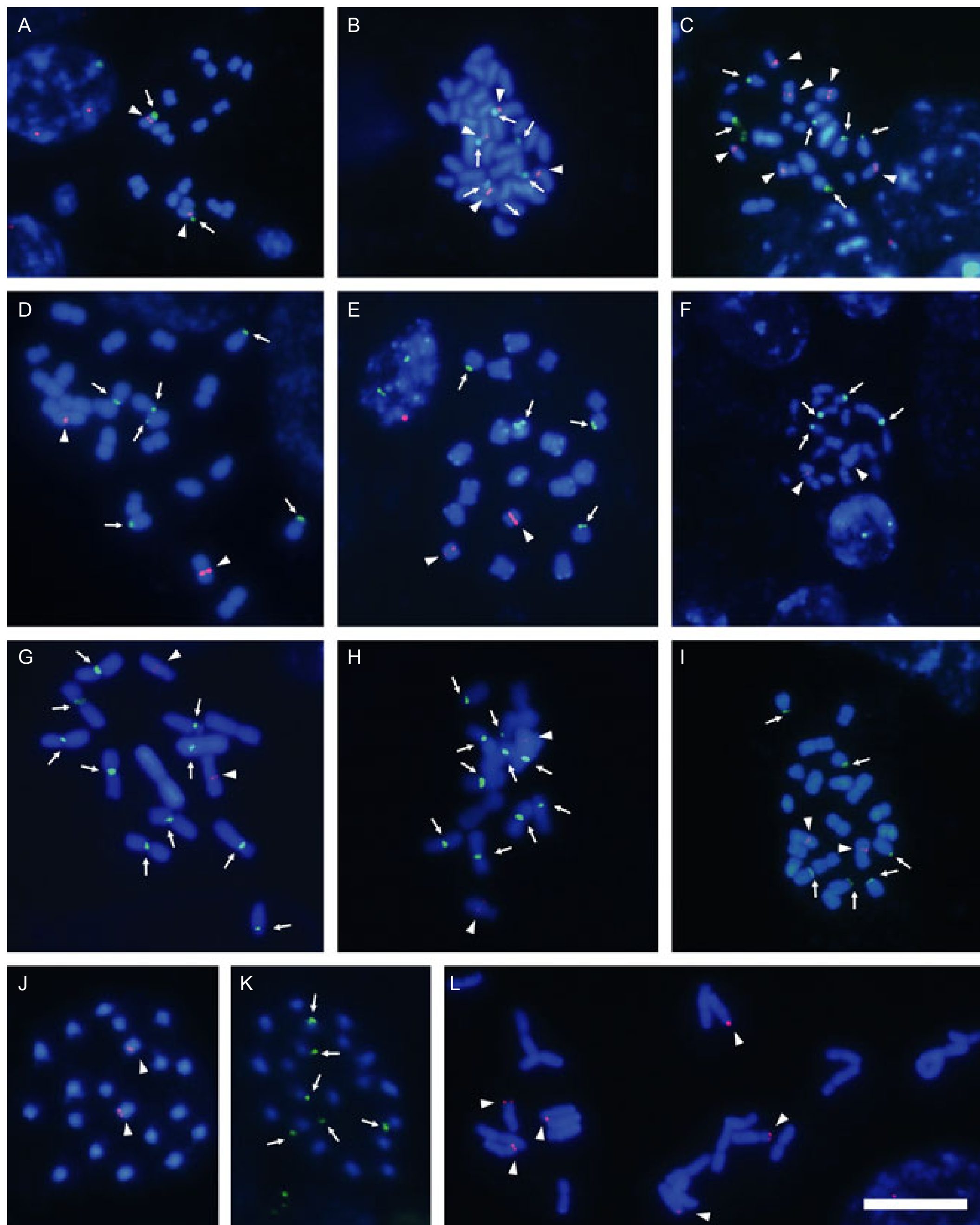

Fig. 5. Distribution of the 18-5.8-26S (green arrows) and 5S rDNA (red arrowheads) loci in *Cardiospermum*. A–B: Synteny of 18-5.8-26S and 5S rDNA; A: *C. anomalum*, B: *C. bahianum*. C–K: Non-synteny of 18-5.8-26S and 5S rDNA; C: *C. corindum*, D: *C. cristobaliae*, E: *C. grandiflorum*, F: *C. halicacabum* var. *halicacabum*, G: *C. heringeri*, H: *C. integerrimum*, I: *C. procumbens*, J–K: *C. oliveirae*, L: *C. urvilleoides*. K, FISH with only 18-5.8-26S rDNA probes. J and L, FISH with only 5S rDNA probes. Bar: 10 μm.

Chromosome numbers and systematics of *Cardiospermum*

This genus comprises 16 species, for which the chromosome number of 12 species is known. In this work, chromosome numbers of *C. halicacabum* var. *halicacabum* (2n = 22), *C. heringeri* (2n = 14), *C. oliveirae* (2n = 20), *C. procumbens* (2n = 22) and *C. urvilleoides* (2n = 24) are recorded for the first time. Chromosome numbers were confirmed for other species, that is *C. anomalum* (2n = 18; Ferrucci 2000a), *C. corindum* (2n = 22; Diers 1961), *C. halicacabum* (2n = 22; Sugiura 1931), *C. halicacabum* var. *microcarpum* (2n = 22; Ferrucci 1981), *C. integerrimum* (2n = 14) and *C. pterocarpum* (2n = 22; Ferrucci 1989). For *C. grandiflorum*, two chromosome numbers have been reported, 2n = 20 (Ferrucci 1989) and 2n = 22 (Dalgaard 1986; Paiva & Leitão 1989). A reduction in chromosome number is known for Paullinieae, which distinguishes it from other tribes of the family (Lombello & Forni-Martins 1998). The most frequent basic numbers in

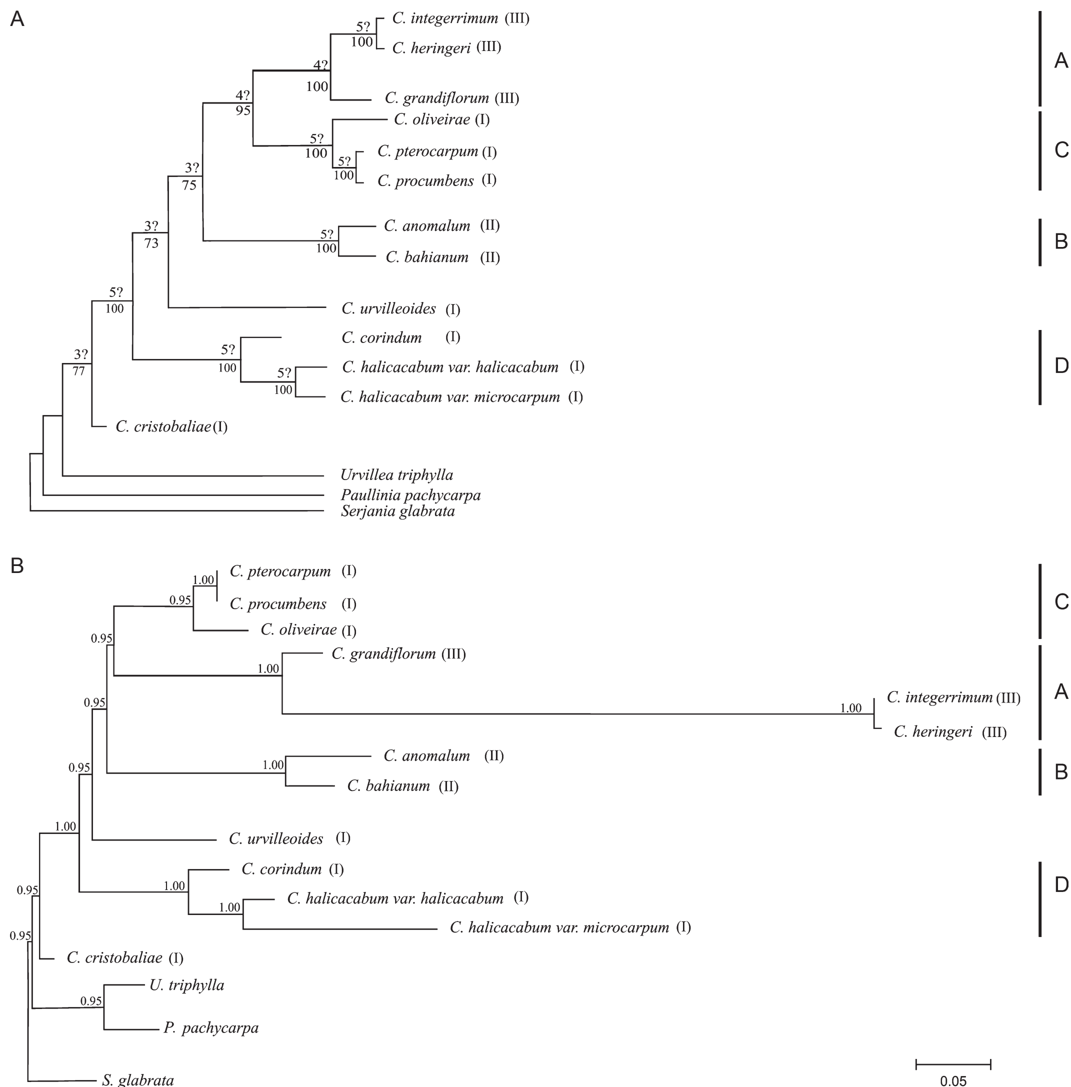

Fig. 6. A: maximum parsimony (MP) consensus tree inferred from ITS-1 and ITS-2 sequences. The Bremmer support and bootstrap values are provided above and below each node (respectively). B: Relationships inferred through Bayesian analysis with nodes supported with Bayesian posterior probability (BPP) mean of 0.95. Clade A represents the *Ceratadenia* section. Clade B was formed by a highly supported group of species of *Carphospermum* section. Clades C and D included species of section *Cardiospermum*.

woody tribes are x = 14, 15 and 16, whereas the most common basic number in Paullinieae is x = 12. The number x = 12 is conserved in *Houssayanthus* (Ferrucci 1981; Solís Neffa & Ferrucci 1998), *Paullinia* (Urdampilleta *et al.* 2007) and *Serjania*, and is present in *Urvillea* section *Stenelytron* (Ferrucci 2000b). In the present study, the basic number x = 12 was recorded for *C. urvilleoides*.

Among the genera of Paullinieae, *Cardiospermum* has the highest variation in basic number, ranging from x = 7 to x = 12, but x = 8 is currently absent. *Urvillea*, the genus most closely related to *Cardiospemum*, also presents variations in the basic number, with x = 11 and 12; this is associated with its infrageneric classification (Urdampilleta *et al.* 2006). However, in *Cardiospermum* this change in chromosome number shows a partial relationship to the subgeneric subdivision (Ferrucci 2000b).

Cardiospermum anomalum (2n = 18) and *C. bahianum* (2n = 36) belong to the *Carphospermum* section, and also share morphological characters, for examplb seed coat with paleaceous hairs (Ferrucci & Urdampilleta 2011a). This section is characterised by x = 9, which is an additional character that allows a natural infrageneric classification. Moreover, different basic numbers were found in sections *Ceratadenia* (x = 7 and 10) and *Cardiospermum* (x = 10, 11 and 12). *C. heringeri* and *C. integerrimum* are morphologically very similar, and both belong to *Ceratadenia*; this close relationship was confirmed for the chromosome number, with both species being 2n = 14. To date, within the family this chromosome number is unique to these two species, whereas the other species of the section, *C. grandiflorum*, has 2n = 20. In particular, in *C. heringeri* and *C. integerrimum* we suggest a significant reduction in chromosome number probably due to some dysploidy mechanism (see below). In section *Cardiospermum*, *C. cristobaliae* and *C. urvilleoides*, both with 2n = 24, are morphologically different from the remaining the species in this section.

Polyploidy in *Cardiospermum* is rare, whereas dysploidy has played a prominent role in karyotype evolution of the genus. *C. bahianum*, with 2n = 4x = 36, represents the first record of polyploidy in the genus. Polyploidy may arise independently in *Cardiospermum*, *Paullinia* (Urdampilleta 2009) and *Urvillea* (Urdampilleta *et al.* 2006); this could be a derived trait in the Paullinieae tribe (Acevedo-Rodríguez 1993). In Sapindaceae polyploidy is uncommon, with some records of polyploids in *Allophylus* L. and *Melicoccus* P. Browne (Ferrucci & Solís Neffa 1997).

Geographical distribution, cytogenetics and morphology of *Cardiospermum*

Considering the endemic species or those with restricted distribution (excluding *C. corindum*, *C. grandiflorum* and *C. halicacabum*, which are cosmopolitan), some associations among geographic distribution, chromosome number and morphology can be perceived (Fig. 7). Accordingly, all the species studied are found in the most important area of endemism for the genus (Coulleri & Ferrucci 2012). In particular, the species from south-central South America, *C. procumbens* and *C. pterocarpum*, have 2n = 22; these are unique species with a fleshy aril, a character always present in *Paullinia*. *C. procumbens* is endemic to Mato Grosso do Sul, whereas *C. pterocarpum* has a wider distribution, from Mato Grosso do Sul to northern Argentina (Corrientes), in the phytogeographic Chaco region.

Cardiospermum cristobaliae, *C. heringeri*, *C. integerrimum* and *C. urvilleoides* are characterised as having a large seed size (1–2 cm in diameter). *C. cristobaliae* and *C. urvilleoides* have 2n = 24, and *C. heringeri* and *C. integerrimum* have 2n = 14. *C. heringeri* and *C. integerrimum* are endemic to the Atlantic Forest in Espirito Santo and Bahia, respectively. Moreover, *C. cristobaliae* is endemic to rocky fields in northern Minas Gerais, while *C. urvilleoides* is endemic to a transition region between the Atlantic Forest and rocky fields of Minas Gerais and Bahia states.

The species with x = 9, *C. anomalum* (2n = 18) and *C. bahianum* (2n = 36), are distributed in the *caatinga* of northeast Brazil and are characterised by the presence of paleaceous hairs on the seed coat. Within the tribe, this trait is unique to these species of the *Carphospermum* section. In the *caatinga*, *C. oliveirae* (2n = 20) is also present and is characterised by a reduction in the basic number.

Karyomorphology and infrageneric classification of *Cardiospermum*

Hemmer & Morawetz (1990) described two nucleus types in *Cardiospermum*: the semi-reticulate type in *C. grandiflorum* and a trend to areticulate type in *C. halicacabum*. Our results confirm the subdivision of the genus into two species groups, one with areticulate nuclei (group I) and the other with semi-reticulate nuclei (group II). The highest density observed in interphase chromatin occurs through a high condensation of DNA. Nagl & Fusening (1979) suggest a relationship between the increase in chromatin condensation of interphase nuclei with an increment in DNA amount per nucleus, probably associated with an increment in heterochromatin amount (see below). Our results confirm the pattern of chromatin condensation in prophase reported in Hemmer & Morawetz (1990) for *C. grandiflorum* and *C. halicacabum*, showing significant differences between the studied *Cardiospermum* species. Clustering of the species resulting from the variation in patterns of prophase condensation (Group I = proximal and Group II = terminal-interstitial) are congruent with those resulting

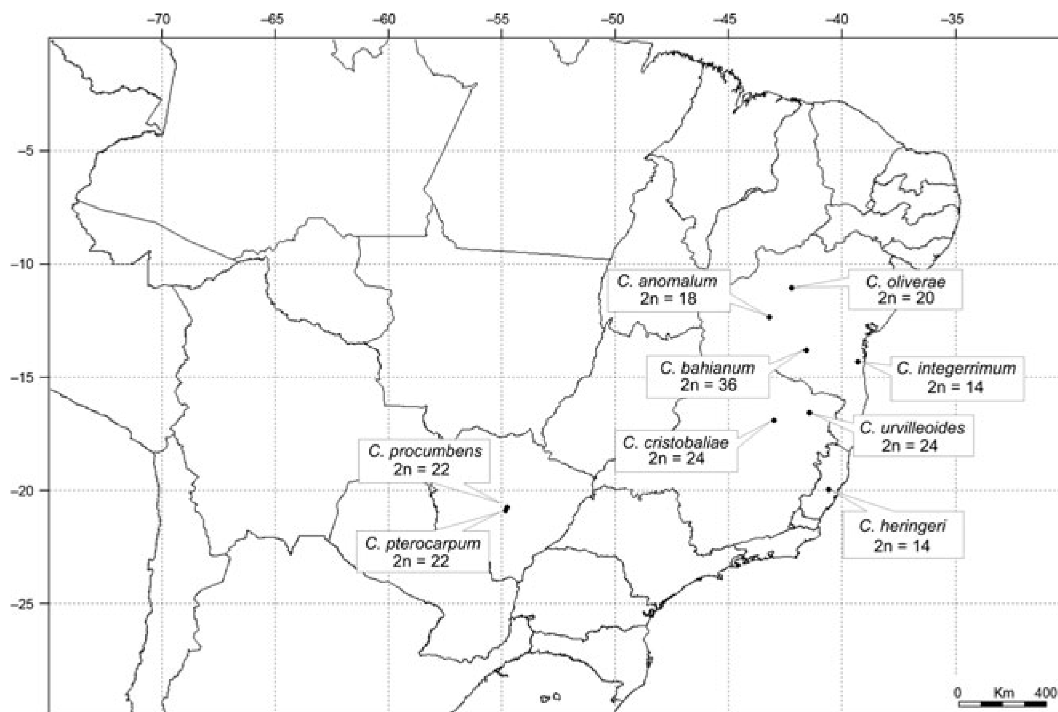

Fig. 7. Geographic distribution of chromosome number (2n) in studied *Cardiospermum* species with restricted distribution (excluding the invasive species: *C. corindum*, *C. grandiflorum* and *C. halicacabum*).

from interphase nucleus types. Thus, prophase condensation and nucleus type are two genetic traits that divide the species of *Cardiospermum* into two groups. Clusters I and II may be associated with chromosome size: species of group I have smaller chromosomes (0.6–1.9 μm) than species of group II (1–6 μm). *C. procumbens* and *C. pterocarpum* have characteristics of group I, such as areticulate interphase nucleus and proximal prophase condensation, but the chromosome size of these species places them at an intermediate position between groups. Clustering according to nucleus type and condensation chromatin pattern showed a relationship to the infrageneric classification of *Cardiospermum*. All species of *Carphospermum* and most species of *Cardiospermum* section were included in group I. Furthermore, the others species of *Cardiospermum* and all species of *Ceratadenia* were included in group II.

Lombello & Forni-Martins (1998) suggested that the reduction in chromosome number, as well as an increase in chromosome size, might distinguish the Paullinieae tribe from other tribes of Sapindaceae. This assumption is based on karyological studies in species of *Urvillea* and *Serjania*, which have chromosomes of up to 3–4 μm. However, the hypothesised increase in chromosome size for karyotype variation is not applicable to *Cardiospermum* species with small chromosomes (0.8–1.5 mm). The *Cardiospermum* chromosome morphology might derive from other species of the Paullinieae tribe that have larger chromosomes, in which a decrease in genome size might have an important role in evolution of the group. Hemmer & Morawetz (1990) interpreted the decrease in genome size of *C. halicacabum* as an adaptation to herbaceous habit, favouring a short life cycle. Weeds, such as *C. corindum* and *C. halicacabum*, often have common morphological characteristics associated with a decrease in the haploid genome size (TCL between 17.8 and 23.6 mm), as occurs in invasive species of the families Asteraceae, Brassicaceae, Fabaceae and Liliaceae (Ni & Guo 2005; Garcia *et al.* 2008). This criterion might be applied to invasive species (*C. corindum* and *C. halicacabum*), but not to non-invasive species such as *C. anomalum* and *C. oliveirae*, which have small genomes (TCL = 16.0 and 18.5 μm, respectively).

The *Cardiospermum* species studied were karyotypically highly variable. *C. anomalum* (2n = 18, 7 m + 2 sm) and *C. bahianum* (2n = 36, 11 m + 7 sm) are species with more symmetrical karyotypes, since they show little variation between chromosomes that are mostly metacentric. Variation in karyotype symmetry was not related to the separation into groups I and II observed for the interphase nuclei and prophase patterns of condensation. *C. corindum* was included in group I together with *C. anomalum* and *C. bahianum*; however, *C. corindum* has the most asymmetrical karyotype. In general, the asymmetry indices studied (A_1, A_2, CVcl, CVCI; Table 1) to differentiate some species do not support an infrageneric classification.

Banding patterns and repeated DNA

The terminal banding pattern of AT-rich heterochromatin ($DAPI^+$) was described in Hemmer & Morawetz (1990) for *C. grandiflorum*. In the present work, this pattern was observed in *C. corindum* and *C. urvilleoides*, and allowed us to differentiate these species from other species of the genus. However, the presence of these heterochromatin blocks is not related to the infrageneric arrangement. The AT-rich bands also occur in terminal regions in other species of the tribe; in *Urvillea* some species can be distinguished by the banding pattern (Urdampilleta *et al.* 2006, 2008). Thus, the accumulation of satellite DNA sequences forming AT-rich heterochromatic blocks might be an independent event in different species of the tribe Paullinieae.

The GC-rich sites (CMA_3^+) in plants are frequently associated with 18-5.8-26S rDNA (Guerra 2000). This association has been confirmed for several species in Paullinieae (Urdampilleta *et al.* 2008). Although the number and location of 18-5.8-26S rDNA sites varies, the location of NORs in the terminal region of the short arm is a conserved character in most species, except in *C. heringeri* and *C. integerrimum* (see below).

The synteny of ribosomal genes, co-location of 18-5.8-26S and 5S rDNA on the same chromosome, is an additional character that defines *Carphospermum*, which differentiates this section from the other two. Non-synteny is the rDNA distribution most frequent in *Cardiospermum*, a feature shared with some studied species of *Paullinia* (Urdampilleta *et al.* 2007), *Serjania* (Urdampilleta *et al.* 2012) and *Urvillea* (Urdampilleta *et al.* 2006). Thus, synteny of rDNA in *C. anomalum* and *C. bahianum* could be considered a derived character within *Cardiospermum*, and a direct marker of recent chromosomal rearrangements in the karyotype evolution of *Carphospermum*.

Reduction of chromosome number in *C. heringeri* and *C. integerrimum*

Cardiospermum heringeri and *C. integerrimum*, both belonging to *Ceratadenia*, are very closely related species, which is reflected both in morphological traits and karyological features. *C. heringeri* is endemic to mountains of west-central Espirito Santo and southeast Minas Gerais, whereas *C. integerrimum* is endemic to southeast Bahia. Sympatric distribution of this species has not been reported so far. *C. heringeri* and *C. integerrimum* have 2n = 14; this chromosome number is a unique record for the family, as well as for the Sapindales.

The distribution and number of 18-5.8-26S rDNA loci in pericentromeric regions is a chromosome marker that allows differentiation of *C. heringeri* and *C. integerrimum* from other species of *Cardiospermum*, as well as from the other Paullinieae species studied (Urdampilleta *et al.* 2006, 2007, 2012). This change in the genomic location and number of rDNA loci is direct evidence of structural chromosome rearrangements that led to modification of the karyotype in these species. Our results suggest that the karyotypes of *C. heringeri* and *C. integerrimum* could be derived from a chromosome number reduction associated with structural chromosome rearrangement, an evolutionary process called dysploidy, which leads to the differential localisation and increase in number of 18-5.8-26S rDNA loci.

Phylogeny, systematics and karyotype evolution in *Cardiospermum*

The phylogeny inferred from the ITS regions with both MP and Bayesian methods was relatively similar, suggesting a strong relationship between species that allows determination of probable directions of karyotype evolution of *Cardiospermum* (Fig. 8). The phylogeny obtained suggests a strong relationship in the monophyletic group that includes species of

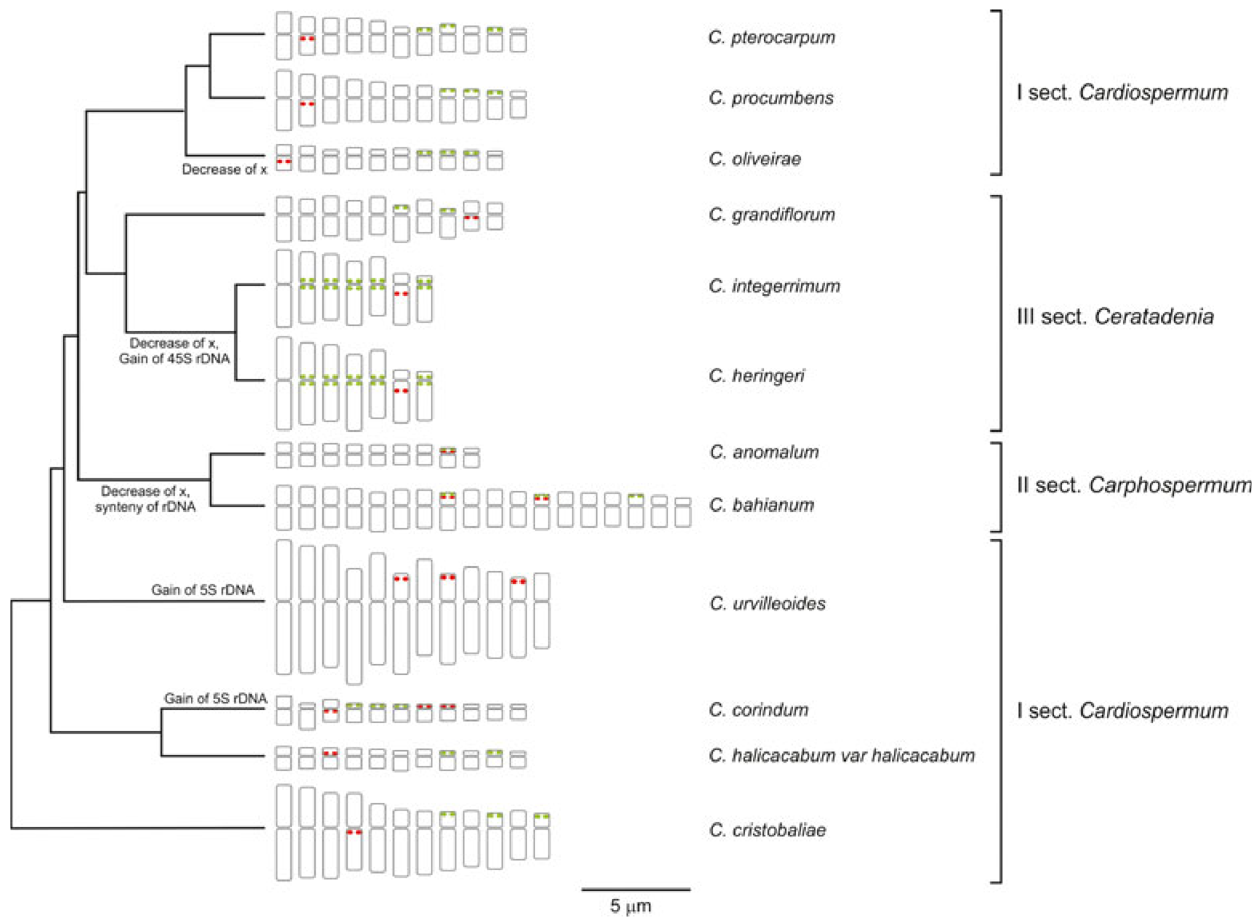

Fig. 8. Proposed phylogenetic relationships obtained from Bayesian inference of ITS-1 and ITS-2 sequences and chromosome evolution in *Cardiospermum*. In the ideograms, the distribution of the 18-5.8-26S (•) and 5S (•) rDNA are represented.

section *Ceratadenia* (clade A). The strong association between *C. heringeri* and *C. integerrimum* is supported by the difference in basic chromosome number and karyotype features analysed, possibly derived from a chromosome number reduction through dysploidy.

Clade B exclusively includes species of section *Carphospermum*; this close relationship is supported by morphological and karyotypical features. *C. anomalum* and *C. bahianum* are the only species of the genus having trichomes on the seed coat (Ferrucci & Urdampilleta 2011a). These species share two exclusive chromosomal characters, the basic number x = 9 and synteny for 18-5.8-26S and 5S rDNA (Fig. 8). These characters could be considered as derived through dysploidy and chromosome rearrangement.

Clade C includes *C. oliveirae*, *C. procumbens* and *C. pterocarpum*. The geographically close species *C. procumbens* and *C. pterocarpum* are characterised by their rhizomatous habit and seeds with a fleshy aril; however, the relationship of these species to *C. oliveirae* is not clear. Clade D includes the cosmopolitan species *C. corindum* and *C. halicacabum*, and is grouped with species of the genus *Cardiospermum*. The close relationship between *C. corindum* and *C. halicacabum* is supported in the karyotype features analysed in this work, as well as morphological similarities. The species *C. cristobaliae* and *C. urvilleoides* grouped in clade B belong to the *Cardiospermum* section according to Ferrucci & Urdampilleta (2011b), however, these species do not show a strong phylogenetic relationship with this section.

The high karyotypic diversity in *Cardiospermum* provides relevant information for understanding phylogenetic relationships among the Paullinieae. Molecular phylogeny suggests the occurrence of at least three independent events of chromosome reduction in the evolutionary history of *Cardiospermum*, which could lead to redistribution of rDNA, as synteny of rDNA in section *Carphospermum* and proximal distribution in section *Ceratadenia*. The main evolutionary mechanism in the variation of chromosome number appears to be dysploidy. Additionally, the amplification/deletion of repeated DNA might have an important role in karyotypic differentiation of species, mainly in the variations in genome size (J.P. Coulleri, J.D. Urdampilleta & M.S. Ferruci, unpublished observation).

In relation to infrageneric classification, *Carphospermum* and *Ceratadenia* sections are supported by a basic chromosome number of x = 9 and x = 7, respectively. Based on morphology and palynology studies, *Cardiospermum* is considered a genus with derived characters (Acevedo-Rodríguez 1993; Ferrucci & Anzótegui 1993; Van Der Ham & Tomlik 1994); our results confirm this and suggest that *Cardiospermum* has undergone a high rate of karyotype evolution in relation to other genera of Paullinieae, such as *Serjania* and *Houssayanthus*. The phylogenetic reconstruction, inferred from ITS regions, suggests divergence of *Ceratadenia* (only *C. heringeri* and *C. integerrimum*)

from *Cardiospermum–Carphospermum*, and close relationships among *Carphospermum* species. These results are consistent with various studies of morphological and cytogenetic traits. Thus, analysis of cytogenetic data combined with molecular and morphological phylogenetic studies improves our understanding of karyotype evolution in *Cardiospermum*, contributing to interpretation of evolutionary relationships among species and genera of Paullinieae.

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