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Chromosome diversity in species of the genus *Arachis*, revealed by FISH and CMA/DAPI banding, and inferences about their karyotype differentiation

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Abstract: The species of the genus *Arachis* (Leguminosae) are ordered into nine sections. The assignment of genome types in this genus has been based on cross-compatibility analysis and molecular cytogenetic studies. The latter has also allowed karyotypically establishing well-defined genomes and reassigning the genome of several species. However, most of these studies have been focused mainly on the sections Arachis and Rhizomatosae. To increase the knowledge about the chromosome diversity of the whole genus, here we performed a detailed karyotype characterization of representative species of most of the sections and genomes of *Arachis*. This characterization included chromosome morphology, CMA/DAPI chromosome banding, and chromosome marker localization (rDNA*loci* and one satDNA sequence) by fluorescent *in situ* hybridization (FISH). Based on the data obtained and other previously published data, we established the karyotype similarities by cluster analysis and defined eleven karyotype groups. The grouping was partly coincident with the traditional genome assignment, except for some groups and some individual species. Karyotype similarities among some genomes were also found. The main characteristics of each karyotype group of *Arachis* were summarized. Together, our results provide information that may be beneficial for future cytogenetic and evolutionary studies, and also contribute to the identification of interspecific hybrids.

Key words: Heterochromatin patterns, karyotype groups, molecular cytogenetic, rDNA-*loci*, satDNA.

INTRODUCTION

Arachis (Leguminosae) is a South American genus that comprises 82 species divided into nine taxonomic sections (Arachis, Caulorrhizae, Erectoides, Extranervosae, Heteranthae, Procumbentes, Rhizomatosae, Trierectoides and Triseminatae), established according to morphological characteristics, geographic distribution, and cross-compatibility (Krapovickas & Gregory 1994, Valls & Simpson 2005, 2017, Valls et al. 2013, Santana & Valls 2015). This genus includes important agronomic species, such as the cultivated peanut (*A. hypogaea* L., section Arachis), which is cultivated as an oilseed crop and as a direct source of human food, and the forage species *A. pintoi* Krapov. & WC Gregory (section Caulorrhizae) and *A. glabrata* Benth (section Rhizomatosae). Thus, carrying out studies to improve the knowledge of the germ plasm of this genus is important both taxonomically and agronomically.

Most *Arachis* species are diploid with x=10, only four are diploid with x=9, and five are

tetraploid with x=10. Initially, classical cytogenetic studies showed high chromosome homology among the species of the genus (Fernández & Krapovickas 1994, Lavia 1996, 2001, Lavia et al. 2009). Later, some molecular cytogenetic studies revealed greater karyotype variability (Seijo et al. 2004, Robledo et al. 2009, Robledo & Seijo 2008, 2010, Silvestri et al. 2015, Ortiz et al. 2017), but most of them were focused only on the species of the sections Arachis and Rhizomatosae. These karyotype characterizations, which were based on fluorescent *in situ* hybridization (FISH) of ribosomal genes and C-DAPI banding, together with previous crossing data, allowed establishing six different genomes for the species of the section Arachis and to define the R genome for the only diploid species of the section Rhizomatosae. In contrast, due to the lack of detailed karyotype studies on the remaining *Arachis* species, their genomes are still assigned based on the subgeneric divisions and interspecific crosses, as follows: Am (Heteranthae), C (Caulorrhizae), E (Trierectoides, Erectoides and Procumbentes), Ex (Extranervosae), T (Triseminatae) and R (Rhizomatosae) (Smartt & Stalker 1982). Pucciariello et al. (2013) established the number of ribosomal *loci* for the species with C genome, but did not determine the CMA/DAPI heterochromatin content and did not perform the physical mapping of the ribosomal *loci*. More recently, Ortiz et al. (2017) mapped these markers in the species of the section Rhizomatosae and a few species of the sections Erectoides and Procumbentes. All this indicates that the karyotype characterization and genome assignments of the genus are still incomplete and that, hence, the relationships between them are not yet well established.

Ribosomal *loci* and CMA/DAPI heterochromatin bands are helpful markers in comparative cytogenetic studies to infer evolutionary relationships between plant species (Acosta et al. 2016, Chalup et al. 2015, Do Nascimento et al. 2018, Yung et al. 2017). However, to perform more detailed karyotypes and so establish more precise chromosome homeologies within a group, it is sometimes necessary to increase the number of chromosomal markers. Satellite DNA (satDNA), which consists of repetitive units of variable length arranged in tandems of up to 100 Mbp, constitutes a significant part of plant nuclear genomes (Charlesworth et al. 1994, Schmidt & Heslop-Harrison 1998). SatDNA usually show particular chromosomal locations, being an important component of centromeric heterochromatin (Hudakova et al. 2001, Urdampilleta et al. 2009, Samoluk et al. 2016) and telomeric heterochromatin (Pich et al. 1996, Macas et al. 2000) and, less frequently, of interstitial heterochromatin (Mukai et al. 1993). Thus, the use of satDNA sequences as chromosome markers has become a useful tool to study major karyotype changes between closely related species and to make inferences about the evolution of genomes (Pich et al. 1996, Ugarkovic & Plohl 2002, Urdampilleta et al. 2009). Recently, Zhang et al. (2012) isolated several satDNA sequences from the *cot-1* fraction of *A. hypogaea*, which could be potentially used as probes to chromosome markers in *Arachis*. The analysis of the distribution of one of them, particularly the one called *clone 119* (NCBI: JQ673497), has shown a markedly different distribution between the two genomes of the allotetraploid *A. hypogaea* (Zhang et al. 2012, 2017). Thus, the analysis of the distribution of this satDNA in other species of *Arachis* would provide additional information about the karyotype variability within the genus, the karyotype characterization of genome types, and the evolutionary relationships among the genomes.

Based on all the above and to increase the genomic knowledge of genus *Arachis*, in the present study, we aimed to improve the karyotype characterization of the species belonging to the different sections of the genus *Arachis*, through the mapping of ribosomal genes by FISH in eight species, the CMA/DAPI banding in fourteen species, and the mapping of the satDNA *clone 119* by FISH in fifteen species. The data obtained and previous data were jointly analyzed to extend the karyotype characterization to most of the species of the genus, to establish chromosome homeologies among them, to determine the karyotype relationships within the genus and to comprehensively relate these relationships to the current genomic assignments.

MATERIALS AND METHODS

Plant material

Seeds and rhizomes of the *Arachis* species used in this study were obtained from the peanut germplasm collections of the Instituto Nacional de Tecnología Agropecuaria-Manfredi (Córdoba, Argentina), the Instituto de Botánica del Nordeste (Corrientes, Argentina) and the Centro Nacional de Recursos Genéticos e Biotecnologia (Embrapa Cenargen, Brasília, Brazil). The provenances and voucher specimens of the samples studied are cited in Table I.

CMA - DAPI staining

Double staining with the fluorochromes chromomycin A3 (CMA, Sigma Aldrich) and diamino-2-phenyl-indole (DAPI, Sigma Aldrich) was performed to reveal GC-rich and AT-rich heterochromatic regions respectively, according to Schweizer (1976) with minor modifications. The aged slides were double-stained at 37 °C with 0.5 mg/mL of CMA for 90 min, and subsequently with 2 µg/µL of DAPI for 30 min. After the staining of each fluorochrome, the slides were washed with distilled water. Finally, the slides were mounted with Vectashield medium (Vector Laboratories).

Probe labeling and fluorescent *in situ* hybridization (FISH)

The 5S and 45S rDNA *loci* were localized using the probes pA5S and pA18S-pA26S, respectively, isolated from genomic DNA of *A. hypogaea* (Robledo & Seijo 2008), and then labeled by PCR or nick translation technique with digoxigenin-11-dUTP (Roche Diagnostics) or biotin-11-dUTP (Sigma-Aldrich). The satDNA sequence *clone 119* was isolated by PCR from genomic DNA of *A. duranensis* K7988 by using primers designed by Zhang et al. (2012), and labeled by PCR with biotin-11-dUTP (Sigma-Aldrich). The *in situ* hybridizations of *clone 119* were combined with the probe pA5S with the objective of using this last chromosome marker as reference for mapping the *clone 119 loci* in relation to previously published positions of the 5S and 45S rDNA *loci*.

Pretreatment of slides and *in situ* hybridization were performed according to Seijo et al. (2004). The first set of antibodies consisted of anti-biotin produced in goat (Sigma-Aldrich) and monoclonal anti-digoxigenin conjugated to fluorescein isothiocyanate (FITC) produced in mouse (Sigma-Aldrich). The second set consisted of anti-goat conjugated to tetramethyl-rhodamineisothiocyanate (TRITC) produced in rabbit (Sigma-Aldrich) and anti-mouse conjugated to FITC produced in sheep (Sigma-Aldrich). Preparations were counterstained by mounting them with Vectashield medium (Vector Laboratories) containing 2 mg/mL of DAPI. Counterstaining with DAPI reveals a C-banding-like pattern with major heterochromatic bands fluorescing more intensely in *Arachis* species (Seijo et al. 2004).

Table I. List of the *Arachis* species karyotypically characterized, collection number and provenance and chromosome marker analyzed.

Section	Species	Collection number and Provenance	Chromosome marker
Arachis	*A. batizocoi* Krapov. & W.C. Gregory	KGBPScS 30079. Bolivia, Dept. Santa Cruz, Prov. Cordillera, Paja Colorada. (CTES)	CMA and DAPI bands, *clone 119 loci.*
	A. duranensis Krapov. & W.C. Gregory	K 7988. Argentina, Prov. Salta, Dept. General José de San Martín, Campo Durán. (CTES)	CMA and DAPI bands, *clone 119 loci.*
	A. glandulifera Stalker	SeSn 3263. Bolivia, Dept. Santa Cruz, San Ignacio, Prov. Velasco. (CTES).	CMA and DAPI bands, *clone 119 loci*.
	A. ipaënsis Krapov. & W.C. Gregory	KGBPScS. 30076. Bolivia, Dept. Tarija, Prov. Gran Chaco, Ipa. (CTES)	CMA and DAPI bands, *clone 119 loci.*
	A. praecox Krapov., W.C. Gregory & Valls	VSGr 6416. Brazil, St. MatoGrosso, Mun. Barra do Bugres. 71 km north of Cáceres, on the way to Barra do Bugres. (CTES, CEN)	CMA and DAPI bands, *clone 119 loci.*
	A. trinitensis Krapov. & W.C. Gregory.	WiCl 1117. Bolivia, Dpto. Beni, Prov. Cercado, Trinidad. Marzo 1990. (CTES)	CMA and DAPI bands, *clone 119 loci.*
Caulorrhizae	*A. pintoi* Krapov. & W.C. Greg	KGr 12787. Brazil, St. Bahia, Mun. Cruz das Almas. (CTES, CEN)	CMA and DAPI bands, *clone 119 loci,* 5S and 45S rDNA*loci.*
	A. repens Handro	GrKP 10538. Brazil, St. Minas Gerais, Mun. Sete Lagoas. (CEN, LIL)	CMA and DAPI bands, 5S and 45S rDNA*loci.*
Erectoides	*A. benthamii* Handro	NcMa 3101 (CEN). St. Mato Grosso do Sul, Campo Grande, BR-163. (CEN)	CMA and DAPI bands, 5S and 45S rDNA*loci.*
	A. paraguariensis Chodat & Hassl. subsp. paraguariensis	KGP 30109. Paraguay, Dept. Cordillera, 1 km N from NR 2. Ipacaraíto San Bernardino. (CTES)	*Clone 119 loci.*
	A. porphyrocalyx Valls & C.E. Simpson	VSPmWiSv 7303. Brazil, St. Minas Gerais, Mun. Uberaba. 19°58´S 47°47´W. (CTES)	*Clone 119 loci.*
Heteranthae	*A. dardani* Krapov. & W.C. Gregory	VSgSv 13400. Brazil, St. Alagoas, Mun. Porto Real do Colegio. 10°12´S 36°48´W. (CTES)	CMA and DAPI bands, 5S and 45S rDNA*loci.*
	A. pusilla Benth.	VPzVaW 13189. Brazil, St. Mato Grosso, Mun. San Francisco. 15°57´S 44°54´W.	CMA and DAPI bands, 5S and 45S rDNA*loci.*
	A. pusilla (A. Chev.) A.Chev.*	VFaPzSv 13107. Brazil, St. Minas Gerais, Mun. Januária. 15°29'S, 44°22´W. (CEN)	CMA and DAPI bands, *clone 119 loci,* 5S and 45S rDNA*loci.*
Procumbentes	*A. lignosa* (Chodat & Hassl.) Krapov. & W.C. Gregory	VOvSgSv13570. Brazil, St. Mato Grosso do Sul, Mun. Porto Murtinho. 21°31'S, 57°49'W. (CEN)	*Clone 119 loci.*
	A. matiensis Krapov., W.C. Gregory & C.E. Simpson.	SeSnGmRy 3719. Bolivia, Dept. Santa Cruz, Prov. Sandoval, San Matías. 16°21'S, 58°24'W. (CEN)	CMA and DAPI bands, *clone 119 loci*, 5S and 45S rDNA*loci.*
	A. vallsii Krapov. & W.C. Gregory	VRGeSv 7635. Brazil, St. Mato Grosso do Sul, Mun. Miranda. 20°07´S, 56°42´W. (CEN).	CMA and DAPI bands, 5S and 18S–26S rDNA*loci.*
Rhizomatosae	*A. burkartii* Handro	SeLaSn 2839. Argentina, Prov. Misiones, Dept. Apóstoles, Azara. 28º 5`S, 55º41´W. (CTES)	*Clone 119 loci.*
	A. nitida Valls, Krapov. & C.E. Simpson	SvPzHn 3785. Paraguay, Dept. Amambay, 21 km W of Bella Vista. 22º 18´S, 56º 31´W. (CTES).	*Clone 119 loci.*
Triseminatae	*A. triseminata* Krapov. & W.C. Gregory	W144. Brazil, St. Bahia, Mun. Iuiú. 14° 39´S 43°29´O. (CEN)	CMA and DAPI bands, *clone 119loci,* 5S and 45S rDNA*loci.*

*** Before *A. sylvestris*; the synonymization with *A. pusilla* has been recently discussed (see Santana & Valls 2015).**

Abbreviations: Collectors: B = DJ Banks; Cl = D Claure; Fa = L Faraco de Freitas; G =WC Gregory; Ge = MAN Gerin; Gm = M Grabiele; Gr = A Gripp; Hn = R Heyn; K = A Krapovickas; La = GI Lavia; Ma = LAZ Machado; Nc = NMS Costa; Ov = JC Oliveira; P = J Pietrarelli; Pm = RN Pittman; Pz = EA Pizarro; R = VR Rao; Ry = W Reynoso; S = CE Simpson; Sc = A Schinini; Se = JG Seijo; Sg = AK Singh; Sn = VG SolísNeffa; Sv = GP Silva; Va = SES. Valente; V = JF Valls; W = WL Werneck; Wi= D. Williams. Dept.: Department; Mun: Municipality, town; Prov.: Provence; St.: State.

Fluorescence microscopy and image acquisition

Chromosomes were viewed with a Leica DMRX fluorescence microscope (Leica) and digitally photographed with a computer-assisted Leica DC350 digital camera system. Red, green, and blue images were captured in black and white by using the respective filters for TRITC, FITC, CMA and DAPI excitations. Digital images were processed with Photoshop, version 7.0 (Adobe, San Jose, CA, USA).

Karyotype morphometry

For karyotype determination, we used three to six individuals per species and four metaphase plates per individual. Chromosome measurements were made using the MICROMEASURE software version 3.3 (Reeves & Tear 2000). Karyotype description is based on the nomenclature by Levan et al. (1964). Chromosomes were classified in categories according to the centromeric index (CI = short arm x 100/total length of the chromosome): metacentric (*m*) when CI = 50–37.5, submetacentric (*sm*) when CI = 37.5–25, and subtelocentric (*st*) when CI = 25-12.5. SAT chromosomes were classified on the basis of the satellite relative size and position of the centromere (Fernández & Krapovickas 1994). Data from homologous chromosomes were combined to mean values, first between chromosomes in the same metaphase and subsequently between chromosomes in different metaphases of the same species, and then the total karyotype length was obtained by summing the average length of each chromosome pair. The mean chromosome length was calculated by dividing the karyotype length by the haploid number of chromosomes of the species. Chromosome bands and rDNA *loci* were mapped using the index di = dx 100/a, where d = distance of *loci* center from the centromere and a = length of the corresponding chromosome arm, according to Greilhuber & Speta (1976). The karyotype asymmetry indices were estimated using the intrachromosomal (A_1) and interchromosomal (A_2) indexes by Romero Zarco (1986). Mean values for each species were represented as haploid complements in the ideograms. Chromosomes were ordered first by morphology and then by decreasing size. Some chromosomes within each ideogram were re-ordered according to tentative homeologies on the basis of the current nomenclature proposed for other *Arachis* species (Seijo et al. 2004, Robledo et al. 2009, Robledo & Seijo 2010, Ortiz et al. 2017).

Clustering analysis

To establish species groups sharing greater karyotype similarities, we made a cluster analysis from a matrix of 15 different cytogenetic characters for 46 species of the genus *Arachis*. The matrix was constructed on the basis of previous cytogenetic data and the data here published, coded either as binary or multistate in the case of qualitative chromosomal characteristics or as continuous characters in the case of quantitative variables (Supplementary Material – Table SI). The chromosome characteristics considered were: basic number (x), total karyotype length by chromosome set, chromosome mean length, intrachromosomal asymmetry index (A_1), interchromosomal asymmetry index (A_2), total percentage of DAPI heterochromatin, presence/absence of centromeric DAPI bands, type of centromeric DAPI bands (conspicuous, tiny, or absent), percentage of chromosomes with centromeric DAPI bands, presence/absence of distal DAPI bands, presence/absence of centromeric CMA bands, percentage of chromosomes carrying the 5S *loci*, position of the 5S rDNA *loci* on pair #3 (long arm vs. short arm), presence/absence of

A chromosomes, and co-localization of 5S and 45S rDNA *loci* on pair #10. The number of 45S rDNA *loci* was not included in the analysis due to its high variability among species of the same section, and/or inclusive to the same genome. The INFOSTAT software version 2015 (Di Rienzo et al. 2015) was used to standardize the matrix, to calculate the average distance, and to generate a phenogram. The distances between the species were estimated by applying the coefficient of dissimilarity of Gower, and clustering was performed using the unweighted pair-group method (UPGMA). Phenogram distortion was measured by computing the cophenetic correlation coefficient (*r*).

RESULTS

Karyotype morphometry

The eight species analyzed had symmetrical karyotypes mainly composed of metacentric chromosomes with lengths ranging from 1.37 to 4.07 µm (Table II). All species had a haploid karyotype formula of 9*m* + 1*sm*, except *A. triseminata* (section Triseminatae) with 10*m*. The haploid karyotype length varied between 17.51 µm in *A. triseminata* and 34.82 µm in *A. matiensis* (section Procumbentes), whereas the mean chromosome length varied between 1.91 µm in *A. dardani* (section Heteranthae) and 3.63 µm in *A. matiensis*. The lowest centromeric index was 41.70 and corresponded to *A. repens* (section Caulorrhizae), whereas the highest was 44.00 and corresponded to *A. triseminata*. The indexes of intrachromosomal asymmetry (A_1) ranged from 0.15 in *A. benthamii* (section Erectoides) to 0.27 in *A. repens*, while the interchromosomal asymmetry (A_2) ranged from 0.09 in *A. vallsii* (section Procumbentes) to 0.20 in *A. pusilla* (section Heteranthae). All species showed only one pair of secondary constrictions located on pair #10. In *A. benthamii* and *A. matiensis*, the secondary constrictions were in the short arms, while in the rest they were in the long arms. Generally, the constrictions were extended at early metaphase, and the satellites remained far from the corresponding proximal segments of the chromosome arms (Figures 1 and 2).

CMA and DAPI heterochromatin distribution

The analysis of $CMA^+/DAPI^-$ (CMA bands) and $DAPI^+/CMA^-$ (DAPI bands) heterochromatin distribution in the karyotypes of fourteen species of *Arachis* revealed a great heterogeneity, but some peculiarities were common among some of them.

The karyotypes of *A. batizocoi*, *A. duranensis*, *A. praecox*, *A. trinitensis* (section Arachis), *A. benthamii* (section Erectoides), *A. matiensis* and *A. vallsii* (section Procumbentes) were characterized by the presence of centromeric DAPI bands in 7 to 10 chromosome pairs and only one pair of CMA bands (Figures 2 and 3). In all these species, the CMA bands were associated with the secondary constrictions. In the first three species of the section Arachis (i.e. *A. batizocoi*, *A. duranensis*, *A. praecox*) and in *A. vallsii*, DAPI heterochromatin was displayed as conspicuous bands, whereas in *A. trinitensis*, *A. benthamii* and *A. matiensis*, it appeared as tiny bands. Additionally, *A. matiensis* had distal DAPI bands on the short arms of pair #2 and in only one chromosome of pair #7, while *A. praecox* had interstitial bands on the long arms of pair #3. The total amount of CMA heterochromatin per haploid complement varied from 1.98% of the total karyotype length in *A. batizocoi* to 3.05% in *A. trinitensis*, while the total amount of DAPI heterochromatin varied from 7.68% in *A. trinitensis* to 21.41% in *A. praecox* (Table III).

Similar to the preceding species, *A. pintoi* and *A. repens* (section Caulorrhizae) had one pair of CMA bands associated with the secondary constrictions (Figures 2 and 3). In

Table II. Karyotype features of species belonging to different sections of *Arachis*.

Species	Karyotype formulae	KL, µm (SE)	MCL, µm (SE)	Size range, µm	CI (SE)	Asymmetry Index	
						A_1	A_2
Section Arachis							
A genome *(A. duranensis)*	9m+1sm	29.44 (3.39)	2.87 (0.10)	2.10 – 3.38	44.30 (0.05)	0.20	0.13
B genome *(A. ipaënsis)*	9m+1sm	30.38 (1.90)	3.03 (0.09)	2.50 - 3.49	42.80 (0.01)	0.22	0.08
D genome *(A. glandulifera)*	5m+2sm+3st	37.38(2.23)	3.94 (0.10)	3.54 - 4.44	35.95 (0.01)	0.42	0.08
F genome *(A. trinitensis)*	9m+1sm	27.75 (3.52)	2.78 (0.09)	2.16 - 3.37	41.60 (0.01)	0.19	0.09
G genome *(A. praecox)*	8m+1sm	29.89 (1.63)	3.28 (0.03)	2.50 - 3.80	43.01 (0.01)	0.25	0.12
K genome *(A. batizocoi)*	8m+2sm	35.30 (1.80)	3.52 (0.11)	4.12 - 2.48	40.30 (0.05)	0.32	0.13
Section Caulorrhizae							
A. pintoi	9m+1sm	21.64 (1.19)	2.16 (0.05)	1.75 - 2.52	42.18 (0.01)	0.26	0.11
A. repens	9m+1sm	22.45 (2.89)	2.24 (0.14)	1.75 - 2.63	41.70 (0.03)	0.27	0.12
Section Erectoides							
A. benthamii	9m+1sm	30.84 (1.96)	3.08 (0.09)	2.61 - 3.53	43.44 (0.01)	0.15	0.10
Section Heteranthae							
A. dardani	9m+1sm	18.51 (4.46)	1.91 (0.20)	1.37 - 2.20	43.23 (0.01)	0.21	0.16
*A. pusilla*13189	9m+1sm	20.75 (3.54)	2.10 (0.16)	1.78 - 2.67	43.90 (0.01)	0.20	0.17
*A. pusilla*13107	9m+1sm	19.61 (2.60)	2.05 (0.12)	1.60 - 2.67	42.25 (0.02)	0.25	0.20
Section Procumbentes							
A. matiensis	9m+1sm	34.82 (2.87)	3.63 (0.12)	2.91 - 4.07	43.50 (0.04)	0.23	0.10
A. vallsii	9m+1sm	28.32 (1.90)	2.83 (0.09)	2.65 -3.25	43.28 (0.02)	0.22	0.09
Section Triseminatae							
A. triseminata	10m	17.51 (1.20)		1.57 - 2.15		0.18	0.10

Abbreviations: The values are corresponded by haploid complement. TKL= Total Karyotype Length; MCL= Median Chromosome Length; CI= Centromeric Index; A_1= intrachromosomal asymmetry index; A_2=interchromosomal asymmetry index; SE= Standard Error.

contrast, they had tiny centromeric DAPI bands in four chromosome pairs. In *A. pintoi*, the total amount of CMA and DAPI heterochromatin was 2.25% and 2.78% of the total karyotype length respectively, whereas in *A. repens* it was 2.27% and 2.93%, respectively (Table III).

Arachis ipaënsis (section Arachis), *A. dardani* (section Heteranthae) and *A. triseminata* (section Triseminatae) had karyotypes completely devoid of DAPI heterochromatin and one to three pairs of CMA bands (Figure 3 and Table III). In the three species, one of the pairs of CMA bands was associated with the secondary constrictions, while the others were centromeric and were only detected in one chromosome pair for *A. dardani* and in two for *A. triseminata*. The total amount of CMA heterochromatin varied from 2.98% of the total karyotype length in *A. ipaënsis* to 9.24% in *A. triseminata*.

The remaining two species (i.e. *A. glandulifera* and *A. pusilla*) had particular heterochromatin distribution patterns. *Arachis glandulifera* (section Arachis) displayed five pairs of CMA bands and seven pairs of DAPI bands (Figure 3). Both the CMA and DAPI bands were heterogeneous in size. All CMA bands were

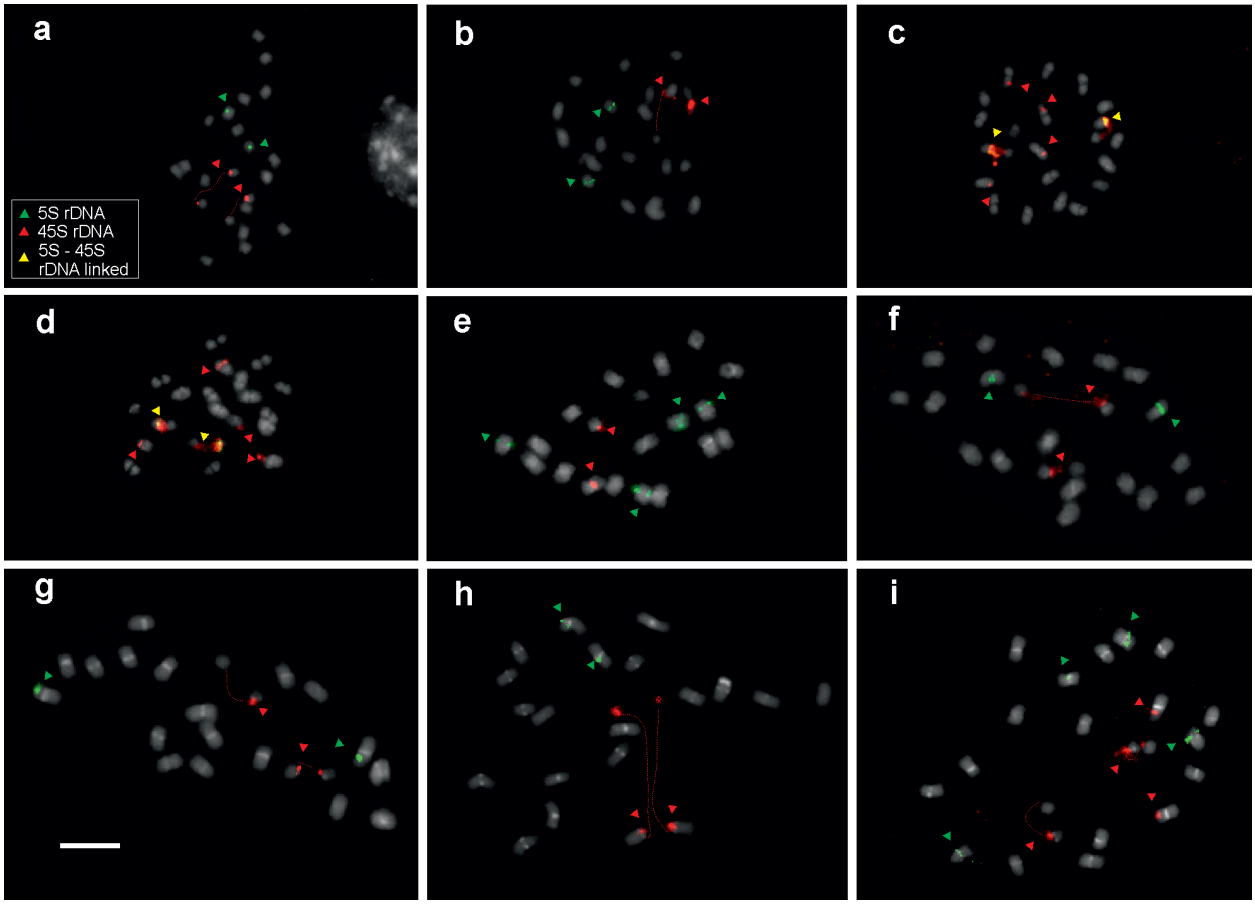

Figure 1. Somatic metaphases of *Arachis* species belonging to different sections after double fluorescent *in situ* hybridization (FISH) with ribosomal markers. The 5S rDNA*loci* are illustrated by the green signals and the 18S–26S rDNA*loci* by the red signals. DAPI counterstaining in gray is highlighting the heterochromatin bands. The arrows illustrate the homologous position of rDNA signals. a- *A. triseminata* (Triseminatae); b- *A. dardani* (Heteranthae); c- *A. pusilla*VFaPzSv 13107 (Heteranthae); d- *A. pusilla* V13189 (Heteranthae); e- *A. pintoi* (Caulorrhizae); f- *A. repens* (Caulorrhizae); g- A. *benthamii* (Erectoides); h- *A. matiensis* (Procumbentes); i- *A. vallsii* (Procumbentes). Scale bar 5 µm.

interstitial, and one pair of them was associated with the secondary constrictions, whereas all DAPI bands were located in the centromeres, except two pairs that were interstitial. The total amount of CMA and DAPI heterochromatin was of 4.87% and 8.11% of the total karyotype length, respectively (Table III). *Arachis pusilla* (section Heteranthae) had centromeric CMA bands in all chromosome pairs and lacked any type of DAPI bands (Figures 2 and 3). The total amount of CMA heterochromatin was 27.62% of the total karyotype length in the accession *A. pusilla* 13189 and 30.77% in *A. pusilla* 13107, but the band size ranged from faint bands to conspicuous blocks (Table III).

Chromosomal mapping of rDNA *loci*

The eight species analyzed had one pair of 5S rDNA *loci* proximally located on the metacentric pair #3, except *A. pusilla*, in which it was located on the submetacentric pair #10 (Figures 1 and 2). In *A. pintoi*, *A. repens*, *A. vallsii* and *A. triseminata*, these *loci* were on the short arms, while in *A. benthamii*, *A. dardani*, *A. pusilla* and *A. matiensis* these *loci* were on the long arms. Regardless of this, all 5S rDNA *loci* covered from one fourth

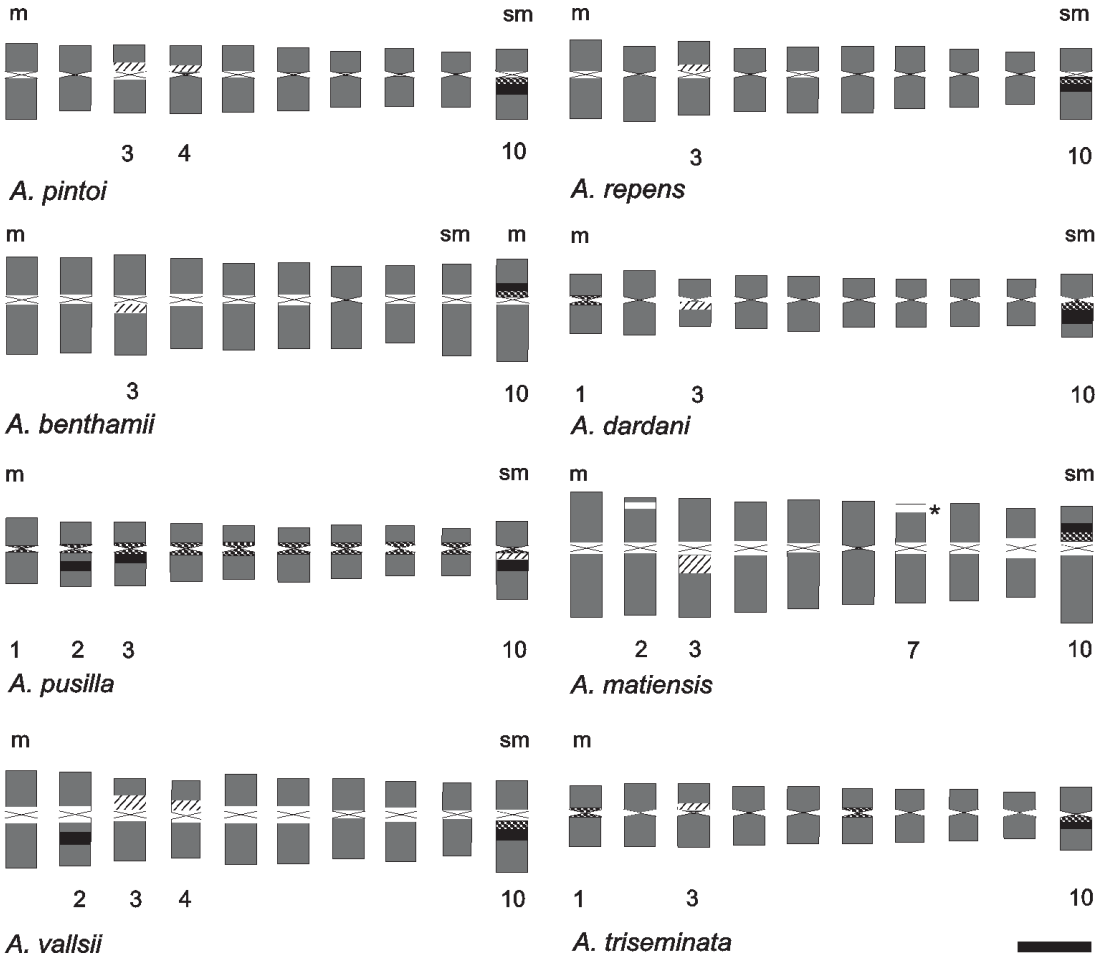

Figure 2. Idiograms of *Arachis* species, showing the distribution of 5S (striped) and 18S-26S rDNA*loci* (black shaded), DAPI heterochromatic bands (white shaded), and CMA heterochromatic bands (point bands). The chromosomes are ordered by morphology according to decreasing size, but some chromosomes were re-arranged according to tentative homologies between the karyotypes. *band observed in only one homologous chromosome. m= metacentric, sm= submetacentric. Scale bar 2 µm.

to one third of the arm length. Additionally, *A. pintoi* and *A. vallsii* had one pair of proximal *loci*on the short arms of the metacentric pair #4. In both species, the signals of these additional *loci* were smaller and fainter than the *loci* on pair #3 (Figure 1).

Similarly, all the species had one pair of 45S rDNA *loci* on the long arms of pair #10, except in *A. benthamii* and *A. matiensis*, in which it was located on the short arms (Figures 1 and 2). In all species, these *loci* co-located with the only secondary constrictions detected and covered one third to half of the arm length. Additionally,

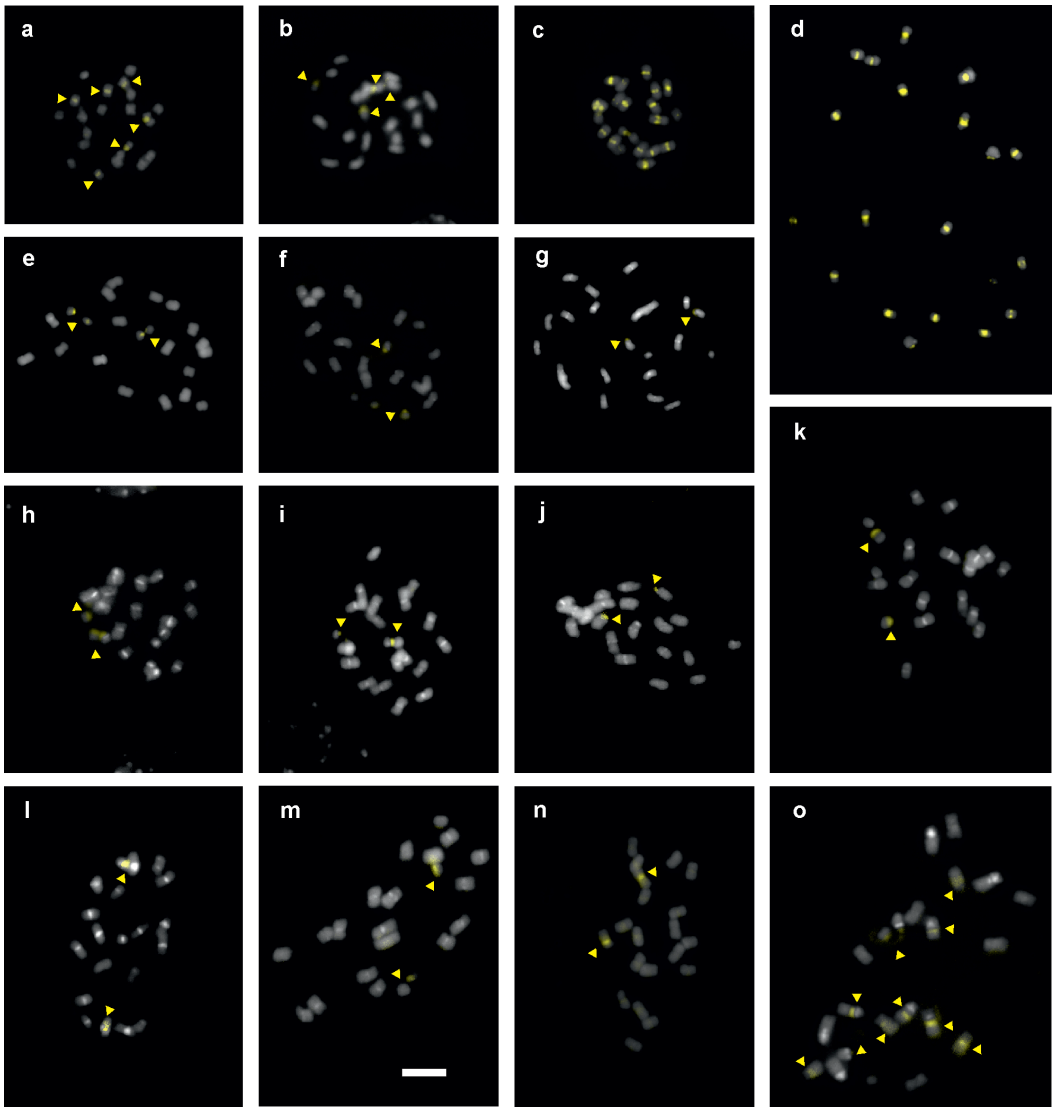

Figure 3. Somatic metaphases of *Arachis* species belonging to different sections after CMA/DAPI banding technique. The yellow signals correspond to CMA heterochromatin bands and the white signals to DAPI heterochromatin bands. a- *A. triseminata* (Triseminatae); b- *A. dardani* (Heteranthae); c- *A. pusilla* V13189 (Heteranthae); d- *A. pusilla*VFaPzSv 13107 (Heteranthae); e- *A. pintoi* (Caulorrhizae); f- *A. repens* (Caulorrhizae); g- *A. benthamii* (Erectoides); h- *A. duranensis* (Arachis, A genome); i- *A. vallsii* (Procumbentes); j- *A. matiensis* (Procumbentes); k- *A. praecox* (Arachis, G genome); l- *A. batozocoi* (Arachis, K genome); m- *A. trinitensis* (Arachis, F genome); n- *A. ipaënsis* (Arachis, B genome); o- *A. glandulifera* (Arachis, D genome). The yellow arrows point some weak CMA bands. Scale bar 5 µm.

Table III. CMA/ DAPI banding features of species belonging different sections of *Arachis*.

Species	CMA		DAPI			
	%	NB	%	NB cen	NB di	pattern
Section Arachis						
A genome *(A. duranensis)*	2.51	1	18.77	10	0	1
B genome *(A. ipaënsis)*	2.98	1	0	0	0	5
D genome *(A. glandulifera)*	4.87	5	8.11	7	2	4
F genome *(A. trinitensis)*	3.05	1	7.68	7	0	2
K genome *(A. batizocoi)*	1.98	1	16.33	9	0	1
Section Caulorrhizae						
A. pintoi	2.25	1	2.78	4	0	3
A. repens	2.27	1	2.93	4	0	3
Section Erectoides						
A. benthamii	2.15	1	9.18	9	0	2
Section Heteranthae						
A. dardani	8.56	2	0	0	0	5
*A. pusilla*13189	30.77	10	0	0	0	5
*A. pusilla*13107	27.62	10	0	0	0	5
Section Procumbentes						
A. matiensis	2.90	1	8.90	9	2*	2
A. vallsii	2.69	1	14.52	10	0	1
Section Triseminatae						
A. triseminata	9.24	3	0	0	0	5

Abbreviations: NB=Number of bands; NB cen= Number of centromeric bands; NB di= Number of distal bands; %= Percentage on the complement; pattern= pattern of type of DAPI bands (see Discussion)*band observed in only one homologous chromosome.

A. pusilla and *A. vallsii* had one second pair of interstitial *loci* on the long arms of the metacentric pair #2, and *A. pusilla* had a third pair in proximal position on the long arms of the metacentric pair #3. In both species, the signals of these additional *loci* were smaller and fainter than those on pair #10 (Figure 1). In *A. pusilla*, one pair of 45S rDNA *loci* and the only pair of 5S rDNA *loci* detected were linked on the long arms of pair #10.

Chromosome mapping of satDNA *clone 119*

The localization of satDNA *clone 119* in the karyotype of 15 *Arachis* species revealed a great variability in the presence, number and position of the hybridization sites (Figure 4) (Supplementary Material – Figure S1). However, some peculiarities were common among some of them. Three species, *A. pintoi*, *A. pusilla* and *A. triseminata* were characterized by having metaphases devoid of hybridization sites. Almost all the remaining species had one pair of proximal sites on the short arms of pair #3, except *A. paraguariensis*, *A. porphyrocalyx* (section Erectoides), *A. lignosa*, *A. matiensis* (section Procumbentes), *A. burkartii* and *A. nitida* (section Rhizomatosae), which had the proximal sites on the long arms (Figure S1). In the case of *A. nitida*, the number of sites detected was four due to their polyploidy level (4x), but all were located in the same position on chromosomes with identical morphology. In all species, this pair of satDNA *clone 119* sites was linked on adjacent position to the 5S rDNA *loci*, except in *A. glandulifera* (section Arachis), in which it was linked in different arms. In *A. paraguariensis*, *A. lignosa*, *A. matiensis*, *A. burkartii*and *A. nitida*, these were the only hybridization sites detected. In contrast, *A. porphyrocalyx* and the five species of section Arachis (*A. duranensis*, *A. ipaënsis*, *A. glandulifera*, *A. trinitensis* and *A. batizocoi*) had two to seven pairs of additional sites. Although there was no common pattern in the distribution of additional sites, the chromosome pairs harboring them mainly corresponded to that of pairs #5 and #9, and less frequently to that of pairs #1, #2, #6, #7 and #8 (Figure S1). All additional sites were located on the centromeres, or at least in a position proximal to them. Particularly, pairs #4 and #10 showed no hybridization sites in any of these species.

Arachis praecox (2n = 18) was differentiated by having, in addition to the proximal sites in the short arms of pair # 3, a dispersed distribution pattern of satDNA *clone 119* signals along six pairs of chromosomes.

Chromosome markers

The chromosome markers detected together with the chromosome morphometry were useful to identify chromosome pairs on some karyotypes. Thus, the mapping of 5S and 45S rDNA *loci* and DAPI/CMA banding allowed the individualization of four pairs in *A. matiensis*, *A. vallsii* and *A. triseminata*, three in *A. dardani* and *A. pintoi*, and two in the remaining species (Figure 2), whereas the hybridization sites of *clone 119*, the5S rDNA *loci* and DAPI banding allowed the individualization of three pairs in *A. porphyrocalyx*, five in *A. batizocoi* and all pairs of the complement in *A. glandulifera* (Figure S1).

The combination of chromosome markers was also helpful to evaluate putative chromosome homeologies. Thus, the tentative homeology of pair #3 harboring 5S rDNA *loci* was confirmed by the additional hybridization of satDNA *clone 119*, except in *A. pintoi*, *A. pusilla* and *A. triseminata*, in which satDNA *clone 119* was not detected. In all species, pair #3 corresponded to metacentric chromosomes (of medium size mainly) harboring one pair of proximal satDNA *clone 119* sites and one pair of 5S rDNA *loci* more distal on the same arms, except in *A. glandulifera*, where these markers are harbored by different

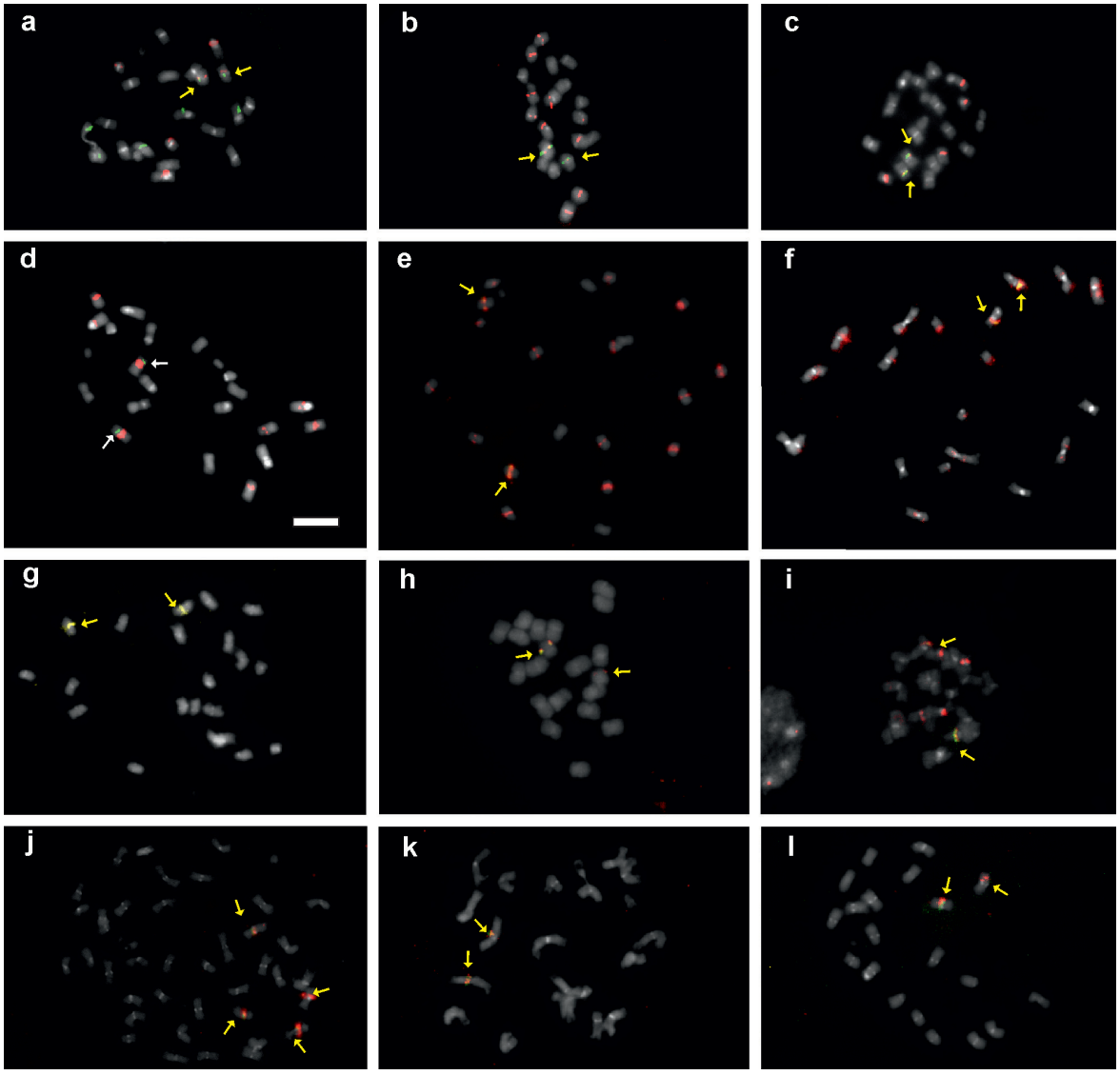

Figure 4. Somatic metaphases in representative species of different sections of *Arachis* after double fluorescent *in situ* hybridization (FISH) with clone 119 and 5S rDNA markers. The clone 119 *loci* are illustrated by the red signals and the 5S rDNA*loci* by the green signals. DAPI counterstaining in gray is highlighting the heterochromatin bands. The yellow arrows illustrate the position where both markers are linked signals in chromosome #3, whereas white arrows show when both markers are located on different positions. a- *A. batizocoi* (Arachis, K genome); b- *A. benensis* (Arachis, F genome); c- *A. duranensis* (Arachis, A genome); d- *A. glandulifera* (Arachis, D genome); e- *A. ipaënsis* (Arachis, B genome); f- *A. praecox* (Arachis, G genome); g- *A. lignosa* (Procumbentes) ; h. *A. burkartii*(Rhizomatosae); i- *A. porphyrocalyx* (Erectoides); j- *A. nitida* (Rhizomatosae); k- *A. paraguariensisparaguariensis* (Erectoides); l- *A. matiensis* (Procumbentes). Scale bar 5 µm.

arms of a submetacentric pair. Similarly, pairs #5 and #9 of *A. duranensis*, *A. batizocoi*, and *A. porphyrocalyx* are homeologous by harboring the only two additional sites of satDNA *clone 119*. In the three species, pair #5 corresponded to metacentric chromosomes of medium-high size, while pair #9 corresponded to the smallest metacentric chromosomes of the karyotype, except in *A. batizocoi*, in which it corresponded to a submetacentric pair.

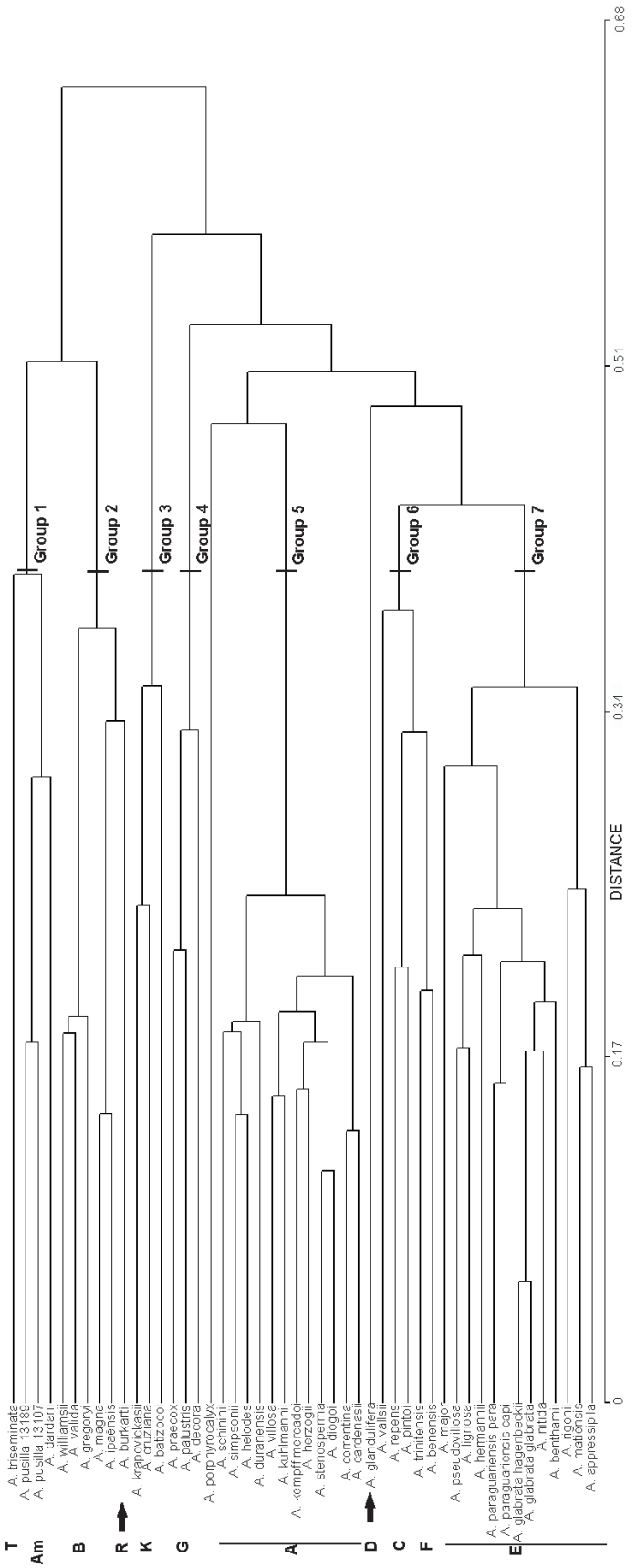

Figure 5. Dendrogram representing the relationship among the karyotypes of 45 *Arachis* species from the sections Arachis, Caulorrhizae, Erectoides, Heteranthae, Procumbentes, Rhizomatosae and Triseminatae. Letters are the designations of genome type. Clustering was performed using unweighted pair-group linkage type, with the Gower distance method. Cophenetic correlation coefficient (*r*) 0.92.

Clustering analyses by karyotype similarities

In the dendrogram obtained, the taxa were grouped in two large clusters by the presence or absence of centromeric DAPI bands (Figure 5). Within these clusters, excepting for *A. glandulifera* and *A. porphyrocalyx*, all taxa were grouped in seven groups supported by values of Gower dissimilarity coefficient lower than 0.40, as described below.

Group 1 is formed by *A. pusilla*, *A. dardani* (section Heteranthae) and *A. triseminata* (section Triseminatae), grouped by the presence of centromeric CMA bands additional to those associated with the secondary constrictions and by having the shortest chromosomes. *Arachis triseminata* is less related to the rest by having the most symmetrical karyotype and by the location of 5S rDNA *loci* on the short arms of pair #3.

Group 2 is formed by five species of the section Arachis (*A. williamsii*, *A. valida*, *A. gregoryi*, *A. magna* and *A. ipaënsis*) and the only diploid species of the section Rhizomatosae (*A. burkartii*). These species are grouped by the absence of centromeric CMA bands additional to those associated with the secondary constrictions, and by having longer chromosomes than the previous group. Within this group, *A. burkartii* appears less related to the rest by the interstitial location of the 5S rDNA *loci* on the long arms of pair #3 and by having a higher interchromosomal asymmetry.

Group 3 is formed by *A. batizocoi*, *A. cruziana* and *A. krapovickasii* (section Arachis). These species are grouped by having three pairs of 5S rDNA *loci*, one pair of 5S and 45S rDNA *loci* linked on the long arms of pair #10, conspicuous centromeric DAPI bands in only nine chromosomes, and high intra-chromosomal asymmetry values and low interchromosomal asymmetry values.

Group 4 is formed by *A. decora*, *A. palustris* and *A. praecox* (section Arachis). These species are grouped by having a basic number x = 9, conspicuous centromeric DAPI bands in all or almost all the chromosomes, the highest DAPI heterochromatin percentages and high interchromosomal asymmetry values.

Group 5 is formed by 12 species of the section Arachis, grouped by having A chromosomes, conspicuous centromeric DAPI bands in all or almost all the chromosomes and high interchromosomal asymmetry values.

Group 6 is formed by *A. vallsii* (section Procumbentes), *A. pintoi*, *A. repens* (section Caulorrhizae), *A. benensis* and *A. trinitensis* (section Arachis). These species are grouped by having small to medium karyotype lengths and low interchromosomal asymmetry values. Within this group, *A. vallsii* appears less related by having conspicuous centromeric DAPI bands in all the chromosomes and by having higher DAPI heterochromatin percentage, whereas *A. benensis* and *A. trinitensis* appear more closely related by having tiny centromeric DAPI bands in more than half of the chromosomes, and *A. pintoi* and *A. repens* by having tiny centromeric DAPI bands in less than half of the chromosomes and interchromosomal asymmetry values higher than the previous species.

Finally, group 7 is formed by the 13 taxa from sections Erectoides, Procumbentes (except *A. vallsii*) and Rhizomatosae (except *A. burkartii*). These species are grouped by having small dot-like centromeric DAPI bands in all or almost all the chromosomes, by the location of 5S rDNA *loci* on the long arms of the chromosomes of pair #3, and low interchromosomal asymmetry values.

Arachis porphyrocalyx and *A. glandulifera* were not included in any group because they have particular chromosome characteristics.

DISCUSSION

The present work constitutes the most comprehensive karyotype analysis of the genus *Arachis* since the initial studies carried out by Fernández & Krapovickas (1994). It comprised a comparative analysis by morphology, fluorescent banding and chromosome markers revealed by FISH of the karyotypes of 45 *Arachis* species corresponding to almost all sections.

Karyotype morphology

Like the rest of the species of the genus, the species analyzed showed symmetric karyotypes with predominance of metacentric chromosomes. The karyotype formula of 9m + 1sm was common to almost all of them. Similarly, all karyotypes were composed of small chromosomes according to the categories proposed by Lima de Faria (1980). Our results on the morphometric analysis of metaphase chromosomes counterstained with DAPI (after FISH treatment) are the first karyotype data for *A. benthamii*, *A. matiensis* and *A. triseminata*, while those for *A. dardani*, *A. pusilla*, *A. pintoi*, *A. repens* and *A. vallsii* are in agreement in most of the details with previous reports by classical and molecular techniques (Fernández & Krapovickas 1994, Lavia 2001, Silva et al. 2010, Pucciariello et al. 2013).

In terms of the number and size of chromosomes, and karyotype formulae, the data for the eight species analyzed support the high conservation of the karyotype structure in the genus. Similarly, the high symmetry of their karyotypes is also a conserved feature among *Arachis* species. According to Stebbins (1971), in groups of phylogenetically related species with mainly symmetrical karyotypes, like the genus *Arachis*, an increase in asymmetry could be interpreted as a derived character. Coinciding with this, *A. triseminata* (section Triseminatae) and *A. benthamii* (section Erectoides), which belong to sections considered as primitive according to Krapovickas & Gregory (1994), showed the least asymmetric karyotypes, whereas *A. pintoi* and *A. repens* (section Caulorrhizae), which belong to one of the sections considered most derived, showed the most asymmetric karyotypes. Even in a comparative analysis of the karyotype lengths among most of the *Arachis* species, the small karyotypes corresponded to species of sections considered primitive and the large karyotypes corresponded to species of sections considered derived. Within this range, and coinciding with the increase in karyotype asymmetry and the evolutionary position of the sections, *A. triseminata* has the smallest karyotype of the genus, while *A. dardani*, *A. pusilla*, *A. pintoi* and *A. repens*, in this order, have larger karyotypes. In contrast, *A. benthamii*, *A. matiensis* and *A. vallsii* have karyotype lengths up to 1.5 times larger than the species with more asymmetric karyotypes (particularly *A. pintoi* and *A. repens*) and, according to Krapovickas & Gregory (1994), belong to a derived section. Increases in the karyotype length of this type have also been observed in other species of the sections Procumbentes and Erectoides (Ortiz et al. 2017). For example, *A. appressipila* and *A. major* (sections Procumbentes and Erectoides, respectively) have less asymmetric karyotypes than *A. pintoi* and *A. repens* but 1.8 times larger. These discrepancies could be explained by the fact that the three species with large karyotypes (*A. benthamii*, *A. matiensis* and *A. vallsii*), as the rest of species of the sections Procumbentes and Erectoides, have DAPI bands that span up to 14.52% of the length of their karyotypes, whereas the species of the sections Triseminatae, Heteranthae and Caulorrhizae lack or have low amount (less than 2.93% of the length of karyotype) of this type of heterochromatin. Moreover, the DAPI heterochromatin in species

of the sections Procumbentes and Erectoides is distributed as centromeric bands with equal size in both arms in all or almost all the chromosomes; therefore, the increases in the chromosome lengths are similar in both chromosome arms and do not affect the karyotype symmetry. So, the conservation of a karyotype structure composed mostly of metacentric chromosomes but with differences in the karyotype lengths suggests that the variations in chromosome lengths rather than the asymmetry were the main changes occurred during their differentiation. On the other hand, the discrepancies between the evolutionary position of these sections (primitive vs. derived) and the values of asymmetry and length of the karyotypes may be due to the fact that the section Erectoides is considered as basal whereas the section Caulorrhizae is considered as derived. In this sense, on the basis of morphology and crossability, Krapovickas & Gregory (1994) suggested that the sections Triseminatae, Trierectoides, Erectoides, Extranervosae and Heteranthae are the most primitive, while the sections Procumbentes, Caulorrhizae, Rhizomatosae and Arachis have a more recent origin. However, these authors highlighted that the species of the section Erectoides had lower degree of intersectional isolation than those of the remaining primitive sections. From a karyotype approach, the species of the section Erectoides share more chromosome features with the derived sections than with the primitive ones. The length of the chromosomes of the species of the section Erectoides is similar to that of those of the species of the sections Procumbentes, Rhizomatosae and Arachis; therefore, they have a range of karyotype length that overlaps with that observed in them. Moreover, and in relation with the increase in chromosome size, all or almost all their chromosomes have DAPI bands like those harbored by the chromosomes of the derived sections. In contrast, the two species of the section Caulorrhizae have small chromosomes, the lengths of their karyotypes are in the middle of the range and closer to those of the species of primitive sections, and the DAPI heterochromatin forms tiny bands only in four chromosome pairs. A possible derived position of the section Erectoides within the genus is supported by several molecular phylogenies, and their species form a single clade together with the species of the sections Procumbentes, Rhizomatosae and Trierectoides, which are also closely related to the clade of species of the section Arachis (Bechara et al. 2010, Friend et al. 2010, Wang et al. 2010). Likewise, in those phylogenies, the two species of the section Caulorrhizae form a clade with an intermediate position between the most basal and the most derived sections.

CMA heterochromatin distribution

The occurrence of GC-rich heterochromatin (CMA bands) adjacent to or interspersed with nucleolus organizing regions (NORs) has been extensively described for many plant species (Guerra 2000, Hou et al. 2004), and the *Arachis* species are not the exception. Thus, one pair of CMA bands corresponding to the only NORs occurs in all species here analyzed. However, a greater number of CMA bands, which co-located with non-active 45S rDNA *loci*, have been reported in other *Arachis* species (Ortiz et al. 2017, Samoluk et al. 2019). Considering the diversity in the number of 45S rDNA *loci* among *Arachis* species, the patterns of CMA banding observed do not reflect those of the 45S rDNA *loci*. In most species, the number of 45S rDNA *loci* exceeds that of the CMA bands detected. This behavior of CMA in relation to 45S rDNA *loci* could be due to the higher sensitivity of FISH to reveal 45S rDNA *loci* in comparison with CMA

banding. Supporting this, CMA-neutro small 45S rDNA *loci* have been reported for some *Cestrum* species (Nunes Fregonezi et al. 2006).

Arachis triseminata (section Triseminatae), *A. dardani*, *A. pusilla* (section Heteranthae), and *A. glandulifera* (section Arachis) were differentiated from the rest by having CMA bands additional to those associated with NORs. In these species, CMA bands have different sizes; but, while the bands in the first three species are all centromeric, in *A. glandulifera* they are located in different positions. This is the first report of CMA banding for *A. triseminata*, while the band patterns observed for *A. dardani* and *A. pusilla* are in agreement with those published by Silva et al. (2010), and the pattern of *A. glandulifera* with that detected by Samoluk et al. (2019). These last authors demonstrated that, in *A. glandulifera*, each CMA band corresponds to one 45S rDNA *locus*. In contrast, we observed that all additional CMA bands in *A. triseminata*, *A. dardani* and *A. pusilla* do not co-locate with any 45S rDNA *loci*. CMA bands not associated with 45S rDNA *loci* have been reported in some other groups of species, as *Solanum* (Moyetta et al. 2017), Cactaceae (Castro et al. 2016), *Cestrum* (Nunes Fregonezi et al. 2006), *Citrus* (Barros e Silva et al. 2010) and *Capsicum* (Grabiele et al. 2018); and the nature of the sequences that composed those CMA bands has been analyzed in some cases. In x=12 species of *Capsicum*, additional CMA bands not associated with 45S rDNA *loci* are composed mainly of a mega satellite derived from a 45S rDNA sequence; by contrast, in x=13 species of *Capsicum*, the additional CMA bands do not hybridize with the probes from this mega satellite. Similarly, in some *Citrus* species, most CMA bands co-locate with 45S rDNA and/or satDNA CsSat sequences (CsSat is a satellite DNA sequence from *Citrus*, with a high GC content), but other bands do not hybridize with any of the probes from these sequences. So, it is clear that the CMA heterochromatin condition is not related to a specific sequence, but to sequences with GC contents, distribution and chromatin conformation that are particular. Several satellite sequences have been isolated and characterized for *Arachis* genomes, all from species of the section Arachis (Zhang et al. 2012, 2017, Samoluk et al. 2016), and, until now, none of them have been associated with CMA bands. Therefore, we cannot discard that the additional CMA bands of *A. triseminata*, *A. dardani* and *A. pusilla* are composed of satellite sequences derived from the 45S rDNA sequence. This is because the probes here used to reveal the 45S rDNA *loci* cover only the 18S and 26S rDNA genes, the intergenic spacer (IGS) region was not represented and some satellite sequences derived from this region have been reported for several species of Solanaceae (Stupar et al. 2002, Lim et al. 2004, Jo et al. 2009).

Among the three species with additional centromeric CMA bands not associated with 45S rDNA *loci*, *A. pusilla* is the only one that has bands of variable size along all the chromosome complement. This heterochromatin distribution pattern is not strange because it is the same as that shown by DAPI heterochromatin in most *Arachis* species (Robledo et al. 2009, Robledo & Seijo 2010, Silva et al. 2010, Silvestri et al. 2015, Ortiz et al. 2017). In fact, generalized proximal bands are the most common distribution pattern of the heterochromatin in angiosperms with small chromosomes (Guerra 2000). However, so far, the GC-rich composition is a feature only of *A. pusilla* within the genus *Arachis*. In relation to this particularity, *A. pusilla* has the longest chromosome length and the highest interchromosomal index among the species with CMA bands not associated with 45S rDNA *loci*. Therefore, the differential accumulation of some GC-rich sequences, revealed in the form of CMA bands of different size, could have been

involved in the karyotype differentiation of *A. pusilla*.

Patterns of DAPI heterochromatin distribution

Among angiosperms, there is a notable variability in heterochromatin distribution patterns, but drastic or discontinuous changes are not common within groups of related species (Galasso et al. 1996, Guerra 2000, Nunes Fregonezi et al. 2006). In the genus *Arachis*, the heterochromatin distribution on the karyotypes is conserved. In all the species with DAPI heterochromatin, this is predominantly located in the proximal regions of both chromosome arms. However, differences in the number of bands and percentage of heterochromatin among different species groups allow establishing distinct distribution patterns. Thus, taking into account our results and previously published data about DAPI banding (Seijo et al. 2004, Robledo et al. 2009, Robledo & Seijo 2010, Silvestri et al. 2015, Ortiz et al. 2017), five patterns of heterochromatin distribution can be established:

Pattern 1 corresponds to a generalized (bands occurring in at least 70% of the chromosomes of the karyotype) and homogeneous distribution of conspicuous bands, which is observed as centromeric blocks of similar size in all or almost all the chromosomes, except pairs #9 and #10, which have longer bands in the A and K genomes. This pattern coincides with the distribution described in the species with A, G, and K genomes of the section Arachis (Robledo et al. 2009, Robledo & Seijo 2010, Silvestri et al. 2015), as well as with *A. porphyrocalyx* of the section Erectoides (Silvestri et al. 2017) and *A. vallsii* of the section Procumbentes.

Pattern 2 corresponds to a generalized and homogeneous distribution of tiny bands, which are observed as centromeric blocksin the form of "dots" in species of the sections Erectoides and Procumbentes and in 4x species of the section Rhizomatosae (Ortiz et al. 2017) or in the form of bands in the species with F genome of the section Arachis (Robledo & Seijo 2010).

Pattern 3 corresponds to a homogeneous but restrained (bands occurring in some chromosomes of the karyotype) distribution of tiny bands that is observed in less than half of the chromosome pairs. This pattern is particular of species of the section Caulorrhizae.

Pattern 4 corresponds to a restrained and notably heterogeneous distribution, which is observed as conspicuous bands of very dissimilar size at pericentromeric and interstitial positions in more than half of the chromosome pairs, and without heterochromatin bands in the remaining chromosome pairs. This pattern is particular of *A. glandulifera* of the section Arachis (Robledo & Seijo 2008).

Finally, *pattern 5* corresponds to karyotypes devoid of centromeric DAPI bands, and is observed in the species of the sections Heteranthae and Triseminatae, the species with B genome of the section Arachis (Robledo & Seijo 2010), and the only 2x species of the section Rhizomatosae, i.e. *A. burkartii* (Ortiz et al. 2017).

Distribution patterns of ribosomal DNA

In the genus *Arachis*, the 5S and 45S rDNA *loci* are commonly located on different chromosome pairs (Raina & Mukai 1999, Seijo et al. 2004, Robledo et al. 2009, Robledo & Seijo 2010, Lavia et al. 2011, Silvestri et al. 2015, Ortiz et al. 2017). Coinciding with this behavior, the number of *loci* per karyotype is not correlated. The number of 5S and 45S rDNA *loci* range from one to three pairs and from one to five, respectively; but almost all species have only one pair of 5S rDNA *loci*, while one, two and three pairs of 45S rDNA *loci* are present in a similar frequency. Thus, the number of rDNA *loci* detected in the

eight species here analyzed is in agreement with that reported for other *Arachis* species. Likewise, in seven of them, the rDNA *loci* were on chromosomes with morphology similar to that of those harboring them in the rest of the genus, and in the expected positions. Besides, the location of additional 5S rDNA *loci* in *A. pintoi* and *A. vallsii* is in agreement with the distribution pattern observed within the genus, although the presence of more than one pair of these *loci* is not the rule. In contrast, the location of the 5S rDNA *loci* of *A. pusilla* is not common. Until now, 5S rDNA *loci* on the long arms of the submetacentric chromosomes of pair #10 and linked to one pair of 45S rDNA *loci* have been observed only in the species with K genome of the section Arachis (Robledo & Seijo 2010) and in *A. stenophylla* of the section Erectoides (Du et al. 2016). However, in K genome species, these 5S rDNA *loci* are additional to those shared by all *Arachis* species located on pair #3, whereas in *A. stenophylla*, the 5S rDNA *loci* are located more distal than 45S rDNA *loci*, conversely to the proximal position observed in *A. pusilla*. Thus, the location of the only pair of 5S rDNA *loci* of *A. pusilla* is a particularity of this species.

This is the first report on the mapping of 5S and 45S rDNA *loci* in *A. benthamii*, *A. dardani* and *A. vallsii*, while the number and location of 5S and 45S rDNA *loci* observed for *A. pusilla*, *A. matiensis* and *A. repens* are coincident with previously reported data (Raina & Mukai 1999, Pucciariello et al. 2013). In contrast, our results differ from those observed by Raina & Mukai (1999) for *A. triseminata*, where an additional pair for each rDNA *loci* has been observed. The difference in the number of 45S rDNA *loci* may be attributed to the fact that we did not detect a pair described by those authors as "minute-size", whereas the difference in the number of 5S rDNA *loci* may be attributed to the fact that they recognized the hybridization signals as constituted by two "tandemly located" *loci*. For *A. pintoi*, we detected the same number of rDNA *loci* as Raina & Mukai (1999) and as that previously observed (Lavia et al. 2011). However, both in this work and in Lavia et al. (2011), the two pairs of 5S rDNA *loci* were found to be located in proximal position on the short arms of different chromosome pairs, while in Raina & Mukai (1999) the two pairs of *loci* were found to be "tandemly located" on the same chromosome arm. Until now, a tandem arrangement of rDNA *loci* has been reported only for 45S rDNA *loci* in *A. valida* and *A. williamsii* of the section Arachis (Robledo & Seijo 2010) and in *A. burkartii* of the section Rhizomatosae (Ortiz et al. 2017), whereby the distribution of 5S rDNA *loci* in *A. triseminata* and *A. pintoi* should be reconsidered.

The variations in the distribution pattern of rDNA *loci* in groups of closely related species, commonly named "repatterning", have been explained via structural rearrangement events such as translocations, inversions, duplications and deletions. All these events commonly result in structural changes of karyotype. Contrarily, the transposition of rDNA genes mediated by the activity of transposable elements may be equally important in the "repatterning" of rDNA *loci* (Raskina et al. 2004a, Datson & Murray 2006) without affecting the karyotype structure. In addition, *in situ* amplification of pre-existing minor rDNA arrays via unequal crossing over or transposition can also lead to the formation of novel rDNA *loci* (Datson & Murray 2006, Eickbush & Eickbush 2007, Lan & Albert 2011, Yung et al. 2017). Independently of the event type involved in the distribution pattern changes of rDNA *loci*, "repatterning" has been associated with the speciation of several species and is considered as an integral part of this process (Raskina et al. 2004b, Baum & Feldman 2010, Lan & Albert 2011). Previous studies in the section Arachis have related changes in the number of 45S rDNA *loci*

to the differentiation of karyotype groups within the A genome species (Robledo et al. 2009), to the diversification of species assigned to the same genome (Robledo & Seijo 2010), and even to the differentiation between the two subspecies of *A. paraguariensis* (Ortiz et al. 2017). In this study, we showed that "repatterning" could also be associated with the amplification of the number of 5S rDNA *loci*, as is the differentiation of karyotypes for *A. pintoi* and *A. repens* of the section Caulorrhizae. We also showed that "repatterning" events can occur by changes in the location of rDNA *loci*, as is the case of the 5S rDNA *loci* in *A. pusilla* and *A. dardani*, which can be parsimoniously explained as the result of a single event of chromosomal translocation. Recently, by using telomeric markers and repetitive and single-copy oligonucleotides as probes, Du et al. (2016, 2018) demonstrated structural rearrangements among the karyotypes of *Arachis* species. Therefore, the "repatterning" events in *Arachis* species could have occurred not only by amplification of the number of *loci* but also by chromosomal rearrangements.

Sites of linked 5S and 45S rDNA *loci*

The location of 5S and 45S rDNA *loci* on the same chromosome or even in adjacent positions on the same arm has been observed in many plant groups (Garcia et al. 2009, Modin et al. 2007, Li et al. 2016). Roa & Guerra (2015) found that the frequency of linked rDNA *loci* is directly influenced by the representation of these *loci* in the karyotypes, so the frequency is much lower in karyotypes with single rDNA *loci* than in those with multiple *loci*. Our results and those previously reported about the location of rDNA *loci* in *Arachis* species partially support this hypothesis. All species with linked 5S and 45S rDNA *loci*, either on the same chromosome (*A. burkartii*, *A. ipaensis*) or in adjacent positions on the same arm (*A. pusilla*, *A. magna*, *A. benensis* and the three species with K genome), have a high number of ribosomal *loci*. However, only 29.63% of the diploid species of *Arachis* with multiple sites presented linked *loci*. Therefore, although the presence of multiple *loci* contributes to the occurrence of linked *loci*, in the genus *Arachis*, other factors could be involved in their frequency. In this sense, our results showed that most species with multiple sites have large chromosomes and only a few have small chromosomes, but also that only 21% of the species with large chromosomes and almost all the species with small chromosomes present linked *loci*. These data suggest that another factor that could increase the occurrence of linked *loci* is the limited space of the chromosome.

Satellite DNA *clone 119*

The mapping of *clone 119* on the karyotypes of representative species of the different sections and genomes showed that this sequence is not equally represented within the genus *Arachis*. Its absence in the more basal sections suggests that it was originated in an ancestor common to the sections Arachis, Erectoides, Procumbentes and Rhizomatosae. At the same time, the presence of multiple additional sites in species of the section Arachis suggests that the dispersion of this satDNA occurred later or during the differentiation of the genomes of this section, and supports that proposed by Seijo et al. (2017) that the genomic fraction of satDNA may have been one of the most dynamic in the karyotype evolution of the genus. In this sense, the localizations of *clone 119* and 5S rDNA *loci* on opposite chromosomal arms of pair #3 in *A. glandulifera* suggest a chromosome rearrangement as a pericentromeric inversion or an intrachromosomal translocation. This hypothesis could also explain the different chromosome morphology between pair #3 in

A. glandulifera (sm) with respect to pair #3 of the rest of the species (m). Similarly, the high number of sites observed in *A. porphyrocalyx* supports that proposed in Silvestri et al. (2017) that this species does not share the same genome as the remaining species of the section Erectoides, but is more similar to those of the section Arachis.

In many groups of species, the satDNA sequences associated with the heterochromatin are shared only among closely related species, or are species specific or even chromosome specific (Ceccarelli et al. 2010). The fact that *clone 119* hybridized in some DAPI bands, but not in all, revealed that there are differences in the representation and composition of the satellite sequences that form these bands in *Arachis* species, as suggested by Samoluk et al. (2016). Moreover, the hybridization of *clone 119* on DAPI+ and DAPI-neutro of centromeric regions suggests that this is a common sequence for this chromosome region, independently of the chromatin conformation and the nucleotide composition of this region.

Congruence among karyotype groups, taxonomic position and genome assignment of *Arachis* species

The establishment of the genomes in the genus *Arachis* has been traditionally based on cross-compatibility assays added to morphological and chromosome features (Smartt et al. 1978, Gregory & Gregory 1979, Singh & Moss 1982, 1984, Singh 1986, Stalker 1991, Fernández & Krapovickas 1994). Since the first genome designation was based on compatibility crossing, and incompatibilities may occur due to single genes, cytoplasmic effects, or other biological factors, chromosome similarities may be found between species that are assigned to different genomes. Therefore, here we intend to discuss the karyotype characteristics of the genomes.

Our chromosome analyses of basic morphology as the chromosome length and asymmetry indexes, the amount and distribution of DAPI and CMA heterochromatin, and the number and position of 5S rDNA sites enabled us to propose eleven karyotype groups. Some of these groups have been described before whereas others are here described for the first time. Below we summarize the main characteristics that differentiate each karyotype group.

Karyotype group 1: This group has karyotypes with very small chromosomes devoid of DAPI bands (pattern type 5). It includes species of the sections Heteranthae and Triseminatae, with Am and T genome respectively. Since similarities between the Am and T genomes are mainly based on the lack of DAPI heterochromatin and the chromosome size, we assigned them the same karyotype group. Another characteristic was the centromeric CMA bands, but these were not observed in *A. interrupta* or *A. veigae* of the section Heteranthae (Silva et al. 2010); therefore, we cannot include it as a particularity of this karyotype group. On the other hand, these species also share their natural distribution in the northeast of Brazil, which is different from the other sections of the genus. However, *A. triseminata* is genetically isolated from the rest of the species because no successful crossings with any members of other sections have been obtained, including those with *A. dardani* (Am genome) (Gregory & Gregory 1979, Krapovickas & Gregory 1994). Besides, in several molecular analyses, *A. triseminata* was not closely related to Heteranthae species (Hoshino et al. 2006, Bechara et al. 2010, Friend et al. 2010). The diversification between these two genomes could be by gene differentiation and/or cannot be reflected by the chromosome markers used. Therefore, although the cytogenetic data suggest the same karyotype group for these species, the

reproductive isolation and the phylogenetic relationships support the conservation of different genomes for each taxonomic section.

Karyotype group 2: This group has karyotypes with medium to long chromosomes devoid of centromeric DAPI bands (pattern type 5). It includes all species with B genome of the section Arachis and *A. burkartii* with R genome of the section Rhizomatosae. Like the first group, this group includes species with two different genomes, but, in this case, the genetic distance between both genomes would seem to be greater. *Arachis burkartii* is reproductively isolated from all species of the genus, including the B genome species. In addition, their natural geographical distribution areas are distant from each other, and the taxonomic and phylogenetic relationships have shown that they are not close (Krapovickas & Gregory 1994, Bechara et al. 2010, Friend et al. 2010). Therefore, although they have common chromosomal characteristics, their genome assignments are conserved.

Karyotype group 3: This group has karyotypes with long chromosomes, conspicuous centromeric DAPI bands (pattern type 1), three pairs of 5S rDNA *loci*, and one 5S and one 45S rDNA *loci* linked on the long arms of pair #10. It includes *A. batizocoi*, *A. cruziana* and *A. krapovickasii* assigned to the K genome (Robledo & Seijo 2010).

Karyotype group 4: this group consists of the species x=9 with conspicuous centromeric DAPI bands (pattern type 1). It includes *A. decora*, *A. palustris* and *A. praecox* assigned to the G genome (Silvestri et al. 2015).

Karyotype group 5: This karyotype group consists of the species x=10 that possess the chromosome pair "A" and conspicuous centromeric DAPI bands (pattern type 1). It includes all species assigned to the A genome (Robledo et al. 2009).

Karyotype group 6: This karyotype group has small to medium chromosomes with tiny centromeric DAPI bands in more than half of the chromosome pairs (pattern type 2). It includes *A. benensis* and *A. trinitensis* assigned to the F genome (Robledo & Seijo 2010).

Karyotype group 7: This group has karyotypes with small to medium chromosomes and tiny centromeric DAPI bands in less than half of the chromosome pairs (pattern type 3). It includes *A. pintoi* and *A. repens* assigned to the C genome of the section Caulorrhizae.

In the cluster analysis, the species of karyotype groups 6 and 7 were grouped together; however, we propose that they should be considered as different karyotypes because they show distinct banding DAPI pattern. This is supported by the fact that no successful crossings between F genome species of the sections Arachis and Caulorrhizae species have been reported (Krapovickas 1973, Krapovickas & Gregory 1994), and by the fact that neither the geographical distribution nor the phylogenies show relations between the F genome and Caulorrhizae species (Krapovickas & Gregory 1994, Friend et al. 2010).

The cytogenetic mapping here performed, as well as morphological and molecular data (Krapovickas & Gregory 1994, Gimenes et al. 2000, Bechara et al. 2010, Friend et al. 2010), confirm the high similarities between the two species of the section Caulorrhizae. In relation to the crossing data, the hybrids between these species have 86.8% pollen fertility, which is a high value in interspecific hybrids (Gregory & Gregory 1979). Besides, the pairing and segregation of chromosomes on meiosis and the pollen formation are normal (Pucciariello et al. 2013). Therefore, we consider that these species share the same karyotype group and also the same genome type.

Karyotype group 8: This karyotype group has long chromosomes with small dot-like centromeric DAPI bands (pattern type 2) and the 5S rDNA *loci* located on the long arms of pair #3. It includes species of the sections Erectoides and Procumbentes assigned to the E genome (except *A. vallsii* and *A. porphyrocalyx*), and the 4x species of the section Rhizomatosae.

The E genome has been assigned to the sections Erectoides, Procumbentes and Trierectoides due to reproductive affinity (Smartt & Stalker 1982). Previous chromosome data and molecular phylogenies confirm the close relationships between these species (Bechara et al. 2010, Friend et al. 2010). Moreover, the 4x species of the section Rhizomatosae appear close to the E genome species in molecular phylogeny (Bechara et al. 2010), share chromosome characteristics (Ortiz et al. 2017) and produce F1 hybrids with diploid species of the sections Procumbentes and Erectoides (Krapovickas & Gregory 1994). All these data support the fact that these species belong to the same karyotype group.

Finally, three species were considered as monotypic karyotype groups:

Karyotype group 9: *Arachis glandulifera* (section Arachis) has a karyotype with long chromosomes, heterogeneous centromeric DAPI bands (pattern type 4), the highest intrachromosomal asymmetry index, and interstitial CMA bands associated with 45S rDNA *loci*. It has been assigned to the D genome (Robledo & Seijo 2008),

Karyotype group 10: *Arachis porphyrocalyx* (section Erectoides) has x=9, the chromosome pair "A", conspicuous centromeric DAPI bands (pattern type 1), a high intrachromosomal asymmetry index and the 5S rDNA *loci* on the long arms of pair #3 (Silvestri et al. 2017).

Karyotype group 11: *Arachis vallsii* (section Procumbentes) has a karyotype with small to medium chromosomes, conspicuous centromeric DAPI bands (pattern type 1) and two pairs of 5S rDNA *loci*.

The first monotypic karyotype group is in agreement with its genomic assignment, whereas the other two have chromosome features that are different from those of the remaining species of the sections Procumbentes and Erectoides, all assigned to the E genome. It has been reported that *A. porphyrocalyx* has more similarities to the A genome species than to the E genome ones (Silvestri et al. 2017). In addition, our results showed that *A. porphyrocalyx* is the only diploid species not belonging to the section Arachis that has more than one pair of sites of satDNA *clone 119*. However, there are no interspecific crossing data involving this species and those of section Arachis. In the cluster analysis, *A. vallsii* grouped with species of the C and F genomes, but shared the pattern of DAPI bands with the A, G and K genomes of section Arachis. In addition, great reproductive affinity between *A. vallsii* and different species of the section Arachis has been revealed (Wondracek-Lüdke et al. 2015). Finally, neither of the last two species were included in the molecular phylogenies of the genus, so new analysis including them could help to review the genome constitution of these species.

In summary, in the present study, we provided detailed karyotypes of eight species belonging to five sections of *Arachis* through the mapping of ribosomal genes by FISH and the DAPI banding after FISH. In addition, we extended the karyotype characterization to 14 species belonging to seven sections by CMA/DAPI banding and to 15 species belonging to seven sections by the localization of satDNA *clone 119* by FISH. Comparative analysis of the data here obtained and previously published data revealed great chromosome variability within the genus *Arachis*. However, we were able to recognize five patterns of

DAPI heterochromatin distribution and several chromosome homeologies, and thus established 11 karyotype groups which may contribute to better understanding the karyotype affinities among *Arachis* species. Most karyotype groups were in agreement with the genome assignments of their species, while the assignments of the 4x species of the section Rhizomatosae, *A. porphyrocalyx* and *A. vallsii* need to be reviewed. The data of chromosome marker distribution and chromosome homologies here provided will be useful to perform evolutionary studies and to identify interspecific hybrids.

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SUPPLEMENTARY MATERIAL

Table SI.
Figure S1.

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