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REGULAR PAPER

Cytogenetic evidences on the evolutionary relationships between the tetraploids of the section *Rhizomatosae* and related diploid species (*Arachis*, Leguminosae)

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Abstract *Rhizomatosae* is a taxonomic section of the South American genus *Arachis*, whose diagnostic character is the presence of rhizomes in all its species. This section is of particular evolutionary interest because it has three polyploid (*A. pseudovillosa, A. nitida* and *A. glabrata*, $2n=4x=40$) and only one diploid (*A. burkartii*, $2n=2x=20$) species. The phylogenetic relationships of these species as well as the polyploidy nature and the origin of the tetraploids are still controversial. The present study provides an exhaustive analysis of the karyotypes of all rhizomatous species and six closely related diploid species of the sections *Erectoides* and *Procumbentes* by cytogenetic mapping of DAPI/CMA heterochromatin bands and 5S and 18–26S rDNA loci. Chromosome banding showed variation in the DAPI heterochromatin distribution pattern, which, together with the number and distribution of rDNA loci, allowed the characterization of all species studied here. The bulk of chromosomal markers suggest that the three rhizomatous tetraploid species constitute a natural group and may have at least one common diploid ancestor. The cytogenetic data of the diploid species analyzed evidenced that the only rhizomatous diploid species—*A. burkartii*—has a karyotype pattern different from those of the rhizomatous tetraploids, showing that it is not likely the genome donor of the tetraploids and the non-monophyletic nature of the section *Rhizomatosae*. Thus, the tetraploid species should be excluded from the R genome, which should remain exclusively for *A. burkartii*. Instead, the karyotype features of these tetraploids are compatible with those of different species of the sections *Erectoides* and *Procumbentes* (E genome species), suggesting the hypothesis of multiple origins of these tetraploids. In addition, the polyploid nature and the group of diploid species closer to the tetraploids are discussed.

Keywords *Arachis* · Evolutionary relationships · Karyotype · Heterochromatin · rDNA loci · *Rhizomatosae*

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Introduction

The genus *Arachis* (Leguminosae) is native to South America and comprises 81 formally recognized species (Krapovickas and Gregory 1994; Santana and Valls 2015; Valls and Simpson 2005; Valls et al. 2013). These species are mostly autogamous, with geocarpic fruits, and have two basic chromosome numbers, $x=9$ and $x=10$ (Fernández and Krapovickas 1994; Krapovickas and Gregory 1994; Lavia et al. 2008; Peñaloza and Valls 2005). These entities have been assembled into nine taxonomic sections: *Arachis, Caulorrhizae, Erectoides, Extranervosae, Heteranthae, Procumbentes, Rhizomatosae, Trierectoides*, and *Triseminatae* (Krapovickas and Gregory 1994; Santana and Valls 2015; Valls and Simpson 2005; Valls et al. 2013).

According to cross compatibility assays and detailed cytogenetic analyses, the section *Arachis* has six different genomes (A, B, D, F, G and K) (Smartt et al. 1978; Stalker 1991; Robledo and Seijo 2008, 2010; Robledo et al. 2009; Silvestri et al. 2015). The other species of the genus are still

assigned to other six genomes based on the infrageneric division (Smartt and Stalker 1982): Am (*Heteranthae*), C (*Caulorrhizae*), E (*Trierectoides, Erectoides* and *Procumbentes*), Ex (*Extranervosae*), T (*Triseminatae*) and R (*Rhizomatosae*). However, a better characterization of these genomes and the species within them is needed.

The section *Rhizomatosae* is currently defined based exclusively on morphological features, mainly by the presence of rhizomes (Krapovickas and Gregory 1994; Valls and Simpson 2005). This section is a very interesting group, because it is represented by three polyploid (*A. pseudovillosa, A. nitida* and *A. glabrata*, $2n = 4x = 40$) and only one diploid (*A. burkartii*, $2n = 2x = 20$) species (Gregory et al. 1973; Fernández and Krapovickas 1994; Peñaloza and Valls 2005). On the basis of leaf morphology and geographic distribution, two varieties of *A. glabrata*, var. *glabrata* and var. *hagenbeckii*, have been recognized (Krapovickas and Gregory 1994). This species, commonly known as "rhizoma peanut", is the most agronomically important *Rhizomatosae* species because it produces high-quality forage and is used as cover crop and ornamental turf (Prine 1964, 1972; Rouse et al. 2004). Several cultivars, like 'Florigraze', 'Arb', 'Arbrook' and 'Latitude 34' in the USA (Muir et al. 2010; Prine et al. 1981, 1986) and, 'Prine' in Australia (Bowman et al. 1998) have been developed and are commercialized as forage.

From a cytogenetic point of view, the polyploid nature of the rhizomatous tetraploid species has been a controversial issue; while some authors have proposed that *A. glabrata* is autopolyploid (Singh and Simpson 1994), others have suggested that it may be allopolyploid (Gregory and Gregory 1979; Jahnavi and Murty 1985; Raman 1981). More recently, chromosome associations in meiosis of *A. glabrata* and *A. nitida* (Ortiz et al. 2011) have shown a high degree of homology among the four chromosome sets of the former but partial homology among those of the latter. On this basis, it has been suggested that these species might be autotetraploids or segmental allotetraploids with different degrees of diploidization.

Under the hypothesis of monophyly of the section *Rhizomatosae, A. burkartii* might be at least one of the parental species of the rhizomatous polyploids. However, several molecular analyses have shown that *A. burkartii* and the tetraploids are not closely related species (Angelici et al. 2008; Bechara et al. 2010; Friend et al. 2010; Gimenes et al. 2002). Crosses involving the tetraploids of the section *Rhizomatosae* do not produce F_1 hybrids with the diploid *A. burkartii* (Krapovickas and Gregory 1994), but produce F_1 hybrids with diploid species of other sections such as *Erectoides* (*A. hermannii, A. paraguariensis, A. major* and *A. benthamii*), *Procumbentes* (*A. appressipila* and *A. rigonii*) and *Arachis* (*A. batizocoi, A. duranensis* and *A. stenosperma*). Moreover, the tetraploid species are distributed from the Mato Grosso state in Brazil to the north of Argentina, while the diploid *A. burkartii* grows from the north of Argentina to the north of Uruguay and the south of Brazil (Krapovickas and Gregory 1994; Valls and Simpson 2005), having a narrow contact area in the northeast of Argentina. All this information has raised doubts about the role of *A. burkartii* in the origin of the rhizomatous tetraploids and suggests multiple origins of the tetraploids, including genome donors from different taxonomic sections.

Karyotype characterizations have played an important role in the understanding of the genomic affinities within the genus *Arachis* (Seijo et al. 2017). The analysis of the complements by banding techniques and by mapping rDNA loci by fluorescent *in situ* hybridization (FISH) has provided useful information to characterize the six genomes (A, B, D, F, G and K) of the section *Arachis* (Robledo and Seijo 2008, 2010; Robledo et al. 2009, 2010; Silvestri et al. 2015), to identify the most probable diploid progenitors of *A. hypogaea* (Seijo et al. 2004), and to demonstrate the autopolyploid ($3x$) origin of the tropical forage *A. pintoi* (Lavia et al. 2011). In addition, the analysis of chromosome homeologies has allowed suggesting evolutionary relationships among species of the section *Arachis*.

In this context, we conducted a detailed cytogenetic study by analyzing the distribution patterns of heterochromatin and rDNA loci localization in all the rhizomatous species and six diploid species of the sections *Erectoides* and *Procumbentes*, in order to: (1) describe chromosomal markers that may contribute to the genome characterization of the species of the section *Rhizomatosae*, (2) cast light on the evolutionary relationships among the species of the section *Rhizomatosae* and (3) infer the nature of the three tetraploid species and their probable diploid genome donors. The data obtained are discussed considering the karyotype information available for other *Arachis* species.

Materials and methods

Plant material

The provenances and the collection numbers of the species analyzed are cited in Table 1. Seeds and rhizomes for this study were obtained from 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, Brasilia, Brazil). The diploid species analyzed here were selected among those reported to produce hybrids with the rhizomatous tetraploids (Stalker 1985; Krapovickas and Gregory 1994). The geographic distribution of each of the species analyzed is shown in Fig. 1.

Table 1 List of the *Arachis* species studied, including their provenance and collection number

Section and species	Provenance[a] and collection number[b]
Sect. *Rhizomatosae* Krapov. and W.C. Greg.	
A. burkartii Handro	Argentina, Prov. Misiones, Dept. Apóstoles, Azara, 28º05′26.6″ S, 55º41′25.4″ W. Se., La., So. 2839
	Argentina, Prov. Corrientes, Dept. Mercedes, PR 123, km 155.5. Se., So. 2865
	Argentina, Prov. Corrientes, Dept. Monte Caseros, 30º11′31.5″ S, 57º41′47″ W. Se., So. 2872
A. glabrata Benth var. *glabrata*	Argentina, Prov. Corrientes. Dept. Ituzaingó, PR 34 to San Carlos, 27º31′11.4″ S, 56º04′09″ W. La., Or., Cll., Vg. 121
	Argentina, Prov. Corrientes, Dept. Concepción, NR 117, km 28. Se., So. 2876
	Argentina, Prov. Misiones, Dept. Candelaria, road to Profundidad, 5 km SW from NR 12. Se., La., So. 2842
A. glabrata Benth. var. *hagenbeckii* (Harms ex Kuntze) F. J. Herm.	Paraguay, Dept. Paraguari, 2 km N from Caapucú. K., G., P., Sc. 30107
A. nitida Valls, Krapov. and C.E. Simpson	Paraguay, Dept. Amambay, 21 km W from Bella Vista. Sv., Pz., Hn. 3785
A. pseudovillosa (Chodat and Hassl.) Krapov. and W.C. Greg.	Paraguay, Dept. Amambay, Finca Elvira, 20 km NW from Pedro Juan Caballero. G., K. 10559
Sect. *Erectoides* Krapov. and W.C. Greg.	
A. hermannii Krapov. and W.C. Greg.	Brazil, St. MS, Mun. Coxim, 1.5 km W from BR-163, 18°32′S, 54°45′W. V., R., Ge., Sv. 7560
A. major Krapov. and W. C. Greg.	Brazil, St. MS, Mun. Rio Negro, 19°24′09″ S, 55°14′29″ W. V., Po., Bi. 9468
	Brazil, St. MS, Mun. Aquidauana, BR-262, 20°20′ S, 56°06′ W. V., R., Ge., Sv. 7632
A. paraguariensis Chodat and Hassl. subsp. *Paraguariensis*	Paraguay, Dept. Cordillera, 1 km N from NR 2, road Ipacarai to San Bernardino. K., G., P. 30109
A. paraguariensis subsp. *capibariensis* Krapov. and W.C. Greg.	Brazil, St. MS, Mun. Porto Murtinho, road to Jardim. H., L., K., He. 565/566
Sect. *Procumbentes* Krapov. and W.C. Greg	
A. appressipila Krapov. and W.C. Greg.	Brazil, St. MS, Corumbá. G., K., P. 9993
A. lignosa (Chodat and Hassl.) Krapov. and W.C. Greg.	Brazil, St. MS, Porto Murtinho. V., Rc., Sg., Sv. 13,570
A. rigonii Krapov. and W.C. Greg.	Bolivia, Dept. Santa Cruz, Santa Cruz de las Sierra. G., K., P. 10034

[a]*NR* National route, *PR* Provincial route, *BR* Brazilian route, *Prov* province, *St* State, *Dept* department, *Mun* Municipality

[b]Collectors: Bi, L. Bianchetti; Cll, M. Collavino; G, W.C. Gregory; Ge, M.A.N. Gerin; H, R.O. Hammons; He, V. Hemsy; Hn, R. Heyn; K, A. Krapovickas; L, W.R. Langford; La, G.I. Lavia; Or, A.M. Ortiz; P, J.R. Pietrarelli; Po, A. Pott; Pz, E. Pizarro, R, V.R. Rao; Rc, R.C. Oliveira; Sc, A. Schinini; Se, J.G. Seijo; Sg, A.K. Singh; So, V. Solís Neffa; Sv, G.P. Silva; V, J.F.M. Valls; Vg, A. Vega

Chromosome preparations

All plants were grown under greenhouse conditions. Healthy root tips (5–20 mm long) of seedlings and rhizomes were pretreated with 2 mM 8-hydroxyquinoline for 3 h at room temperature (Fernández and Krapovickas 1994) and fixed and stored in absolute ethanol:glacial acetic acid (3:1) at −20 °C. Root apices were digested in 1% (w/v) cellulose (from *Trichoderma viride*; Onozuka R-10, Serva) plus 10% (v/v) pectinase dissolved in 40% glycerol (from *Aspergillus niger*, Sigma–Aldrich) in 0.01 M citrate buffer, pH 4.8, at 37 °C for 60 min. Subsequently, the meristematic cells were removed from the root tip and squashed in 45% (v/v) aqueous acetic acid. Coverslips were removed with CO_2 and the slides were air dried, aged for 1–2 days at room temperature, and then kept at −20 °C until use.

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 room temperature with 0.5 mg/mL of CMA for 60 min and subsequently with 2 µg/µL of DAPI for 30 min. After that, the slides were mounted with Vectashield medium (Vector Laboratories).

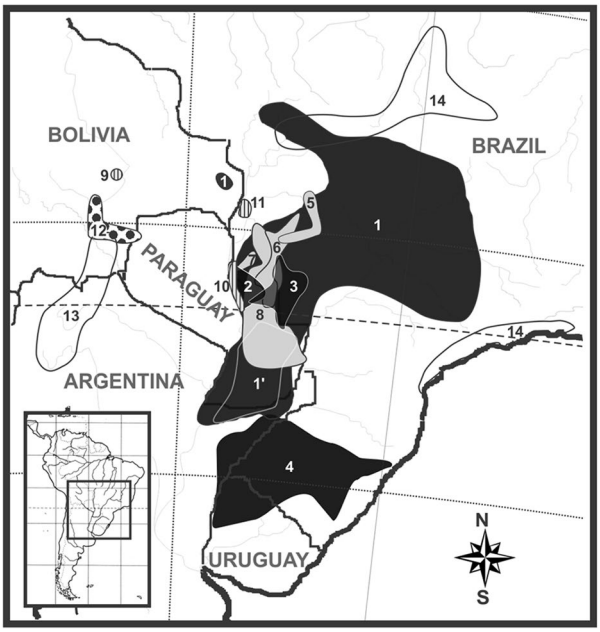


Fig. 1 Geographic distribution of the *Arachis* species studied. *1 A. glabrata* var. *glabrata, 1′ A. glabrata* var. *hagenbeckii, 2 A. nitida, 3 A. pseudovillosa, 4 A. burkartii, 5 A. hermannii, 6 A. major, 7 A. paraguariensis* subsp. *capibariensis, 8 A. paraguariensis* subsp. *paraguariensis, 9 A. rigonii, 10 A. lignosa, 11 A. appressipila, 12 A. batizocoi, 13 A. duranensis, 14 A. stenosperma*. Only the distribution of the species of each section analyzed here are represented in the map: *black shaded: Rhizomatosae* species, *gray shaded: Erectoides* species, striped: *Procumbentes* species, *dotted*: K genome species, and *black solid line*: A genome species

Probe labeling and fluorescent in situ hybridization

The 5S rDNA and 18S–26S rDNA loci were simultaneously localized using the probes pA5S, pA18S and pA26S isolated from genomic DNA of *A. hypogaea* (Robledo and Seijo 2008) and labeled by nick translation with digoxigenin-11-dUTP (Roche) or biotin-11-dUTP (Sigma Aldrich). Pretreatment of slides, chromosome and probe denaturation, conditions for the *in situ* hybridization (hybridization mixes containing DNA probes at a concentration of 2.5–3.5 ng/μL, with a stringency to allow sequences with 80–85% identity to remain hybridized), post-hybridization washing, blocking and indirect detection with fluorochrome-conjugated antibodies were performed according to Moscone et al. (1996). The first set of antibodies consisted of anti-biotin produced in goat and monoclonal anti-digoxigenin conjugated to fluorescein isothiocyanate (FITC) produced in mouse, and the second set consisted of anti-goat conjugated to tetramethyl-rhodamine isothiocyanate (TRITC) produced in rabbit and anti-mouse conjugated to FITC produced in sheep (all from Sigma–Aldrich). Preparations were counterstained and mounted with Vectashield medium containing 2 mg/mL of DAPI. Counterstaining with DAPI revealed a C-banding-like pattern, with major heterochromatic bands fluorescing more intensively (cf. Seijo et al. 2004).

Image capturing and chromosome analysis

Red, green, yellow and blue images were captured in black and white using appropriate filters for TRITC, FITC, CMA, and DAPI excitation, respectively. Digital images were combined and processed using Photoshop software, version 12.0 (Adobe), only with functions that applied equally to the whole image.

Chromosome measurements were carried out after FISH treatment on at least eight metaphases from different individuals using the free version of the MicroMeasure 3.3 program (Reeves 2001). The centromeric index (ci = short arm length ×100/chromosome length) was used to classify the chromosomes as metacentric (*m*, ci = 50–37.51) or submetacentric (*sm*, ci = 37.50–25.01), according to Levan et al. (1964). Satellite (SAT) chromosomes were classified according to Fernández and Krapovickas (1994) and Lavia (2000). 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 and Speta (1976).

Data from homologous chromosomes (determined by length, centromeric index, and patterns of bands and rDNA loci) were combined to obtain mean values, first between chromosomes in the same metaphase and subsequently among different metaphases of the same species. The intrachromosomal and interchromosomal asymmetry indices were determined using the A_1 and A_2 indices respectively (Romero-Zarco 1986). Mean karyotype values for each species were represented as haploid complements in the idiograms. Chromosomes were ordered primarily by morphology and then by decreasing length. Some chromosomes within each idiogram were re-ordered according to tentative homeologies on the basis of the current nomenclature proposed for other *Arachis* species (Robledo et al. 2009; Robledo and Seijo 2010; Seijo et al. 2004). In the tetraploid species, the tentative homeologous/homologous chromosomes were identified with the same number.

A cluster analysis of the chromosome data was carried out to examine the karyotype similarity among the rhizomatous tetraploids and the diploid species of the sections *Rhizomatosae, Erectoides, Procumbentes* and *Arachis*. Additional data for three species of the section *Arachis, A. batizocoi, A. duranensis* and *A. stenosperma*, were extracted from Robledo et al. (2009) and Robledo and Seijo (2010). A matrix of 15 operational taxonomic units (OTUs) × 18 chromosome variables was constructed. The chromosome variables considered were: the mean chromosome

length (ML), centromeric index (CI), intrachromosomal asymmetry index (A_1), interchromosomal asymmetry index (A_2), percentage of *m* and *sm* chromosomes, type of SAT chromosomes, percentage of centromeric $DAPI^+$ heterochromatin per complement, percentage of chromosomes with centromeric $DAPI^+$ heterochromatic bands, percentage of chromosomes with subtelomeric $DAPI^+$ heterochromatic bands, pattern of DAPI band distribution (absent, conspicuous and dot-like bands), percentage of chromosomes carrying the 5S and 18–26S rDNA loci, position (interstitial vs. proximal) of the 5S rDNA locus on pair #3 and the 18–26S rDNA locus located on the SAT chromosomes, co-localization of the 18–26S and 5S rDNA loci on the same chromosome pair (presence vs. absence) as well as the presence of two 18–26S rDNA loci on the SAT chromosomes and the presence of the A9 chromosomes (the characteristic small pair of the A genome). The InfoStat program version 2015 (Di Rienzo et al. 2015) was used to standardize the matrix, to calculate the average taxonomic distance, and to generate a phenogram. Clustering was performed using the unweighted pair-group method (UPGMA). Phenogram distortion was measured by computing the cophenetic correlation coefficient (*r*).

Results

The general karyotype features, the number of heterochromatic bands and the number of the 5S and 18S–26S sites detected for ten *Arachis* species are listed in Table 2. Representative somatic metaphases for each species are shown in Figs. 2 and 3 and the consensus idiograms are illustrated in Fig. 4. The interspecific relationships inferred from their karyotypes are shown in Fig. 5.

General karyotype features

The three tetraploid species of the section *Rhizomatosae* had their complements mainly constituted by metacentric (*m*) chromosomes of similar size. Among them, *A. pseudovillosa* had a haploid karyotype formula composed of 20 *m*, whereas *A. nitida* and both varieties of *A. glabrata* had 19 $m+1$ *sm*. The mean chromosome length ranged from 2.59 µm (*A. nitida*) to 3.19 µm (*A. pseudovillosa*), and the total haploid karyotype length ranged from 51.67 to 63.91 µm in the same species. The A_1 index ranged from 0.12 (*A. pseudovillosa*) to 0.17 (*A. nitida*), while A_2 index ranged from 0.11 (both varieties of *A. glabrata* and *A. pseudovillosa*) to 0.13 (*A. nitida*). As a whole, the karyotype of *A. pseudovillosa* was the most symmetric among the rhizomatous tetraploid species. One pair of SAT chromosomes type 3 A was detected in all tetraploids and corresponded to two of the four metacentric chromosomes #10.

Arachis burkartii had a haploid karyotype formula composed of 10 *m*, with a mean chromosome length of 2.96 µm and a total haploid karyotype length of 29.62 µm. The A_1 and A_2 asymmetry indices were 0.17 and 0.15, respectively. The SAT chromosomes were type 8 and corresponded to the metacentric pair #10.

The diploid species of the sections *Erectoides* and *Procumbentes* had karyotypes composed of chromosomes with similar size and the formulae were 8 $m+2$ *sm* or 9 $m+1$ *sm*. The mean chromosome length ranged from 2.80 µm (*A. paraguariensis subsp. paraguariensis*) to 4.09 µm (*A. major*), and the total haploid karyotype length ranged from 28.05 µm to 40.94 µm in the same species. The A_1 index ranged from 0.15 (*A. rigonii*) to 0.24 (*A. appressipila*), while the A_2 index ranged from 0.08 (*A. paraguariensis* subsp. *capibariensis*) to 0.14 (*A. major*). All diploid species had one pair of SAT chromosomes (pair #10), which was submetacentric and type 9 in all species of the section *Procumbentes*, but metacentric and with different morphology in the section *Erectoides* (Table 2).

Heterochromatin distribution

In all the species analyzed, the DAPI direct staining showed a pattern of heterochromatin similar to that detected after FISH. Two different patterns of $DAPI^+$ heterochromatin were observed among *Rhizomatosae* species with different ploidy levels. The karyotypes of the tetraploids had small dot-like $DAPI^+/CMA^-$ bands (AT-rich heterochromatin) in the centromeres of all the chromosomes (Fig. 2). These heterochromatic bands were of similar size and the total amount of heterochromatin ranged from 8.07% (*A. glabrata* var. *hagenbeckii*) to 12.10% (*A. pseudovillosa*) of the karyotype length (Table 2). The karyotype of *A. burkartii* was completely devoid of detectable heterochromatin (Fig. 2).

Diploid *Erectoides* and *Procumbentes* species showed centromeric dot-like $DAPI^+/CMA^-$ bands of similar size in all or almost all the chromosomes of the complement (Figs. 3, 4). The total amount of heterochromatin ranged from 7.12% (*A. major*) to 12.85% (*A. rigonii*) of the karyotype length (Table 2), and in general the species of *Erectoides* had the lowest amounts of heterochromatin. The heterochromatin was restricted to the centromeres in all the species except in *A. rigonii* (Fig. 3k) and *A. appressipila* (Fig. 3m) of the section *Procumbentes*, which presented one pair of small subtelomeric $DAPI^+/CMA^-$ bands on the short arms of metacentric pair #7.

All rhizomatous tetraploid species had one proximal $CMA^+/DAPI^-$ band (GC-rich heterochromatin) on the four chromosomes #10 (Fig. 2). *Arachis nitida* had an additional proximal $CMA^+/DAPI^-$ band on two chromosomes #2 (Fig. 2e). In all cases, the $CMA^+/DAPI^-$ bands on chromosomes #10 were localized in the secondary constrictions.

Table 2 Karyotype features of the *Arachis* species belonging to the sections *Rhizomatosae, Erectoides* and *Procumbentes*

Section and species	2n	General karyotype features							Heterochromatin[a]						Number and localization of rDNA sites[b]	
		A9 Pair	Karyotype formula	SAT	TCL, µm (SE)	ML, µm (SE)	Size range, µm	Centromeric index (SE)	Asymmetry indexes		$DAPI^+CMA^-$/C-$DAPI^+$		CMA^+DAPI-/C-$DAPI^-$		18–26S	5S[c]
									A_1	A_2	% (SE)	NCB	% (SE)	NCB		
Sect. *Rhizomatosae*																
A. glabrata var. *glabrata*	40	–	19 *m* + 1 *sm*	3A	55.76 (1.64)	2.79 (0.08)	2.15–3.40	46.04 (0.19)	0.15	0.11	8.31 (0.32)	20	1.39 (0.10)	2	2 (10, 10)	2 (3, 3)
A. glabrata var. *hagenbeckii*	40	–	19 *m* + 1 *sm*	3A	55.98 (1.22)	2.80 (0.06)	2.15–3.36	45.77 (0.15)	0.14	0.11	8.07 (0.23)	20	1.29 (0.05)	2	2 (10, 10)	2 (3, 3)
A. nitida	40	–	19 *m* + 1 *sm*	3A	51.67 (1.58)	2.59 (0.08)	1.89–3.22	45.40 (0.20)	0.17	0.13	9.03 (0.38)	20	1.99 (0.12)	3	3 (2, 10, 10)	2 (3, 3)
A. pseudovillosa	40	–	20 *m*	3A	63.91 (1.30)	3.19 (0.10)	2.70–3.69	46.78 (0.30)	0.12	0.11	12.10 (0.41)	20	2.48 (0.30)	3	3 (2*, 10, 10)	2 (3, 3)
A. burkartii	20	–	10 *m*	8	29.62 (0.53)	2.96 (0.05)	2.03–3.63	45.15 (0.33)	0.17	0.15	0	0	0.80 (0.07)	1	4 (3^+, 4, 10^)	1 (3)
Sect. *Erectoides*																
A. hermannii	20	–	8 *m* + 2 *sm*	2	36.06 (0.93)	3.60 (0.09)	2.91–4.42	44.53 (0.16)	0.18	0.11	9.40 (0.24)	9	1.78 (0.08)	1	1 (10)	1 (3)
A. major	20	–	8 *m* + 2 *sm*	2	40.94 (0.69)	4.09 (0.06)	3.09–4.86	43.76 (0.62)	0.21	0.14	7.12 (0.20)	9	1.44 (0.08)	1	1 (10)	1 (3)
A. paraguariensis subsp. *capibariensis*	20	–	9 *m* + 1 *sm*	3A	28.88 (0.72)	2.89 (0.07)	2.46–3.34	43.90 (0.36)	0.20	0.08	7.89 (0.16)	8	1.57 (0.19)	1	1 (10)	1 (3)
A. paraguariensis subsp. *paraguariensis*	20	–	8 *m* + 2 *sm*	4	28.05 (0.65)	2.80 (0.07)	2.43–3.27	42.94 (0.35)	0.23	0.10	8.83 (0.28)	8	1.47 (0.06)	1	5 (1^, 2, 4, 10)	1 (3)
Sect. *Procumbentes*																
A. lignosa	20	–	8 *m* + 2 *sm*	9	32.70 (0.83)	3.27 (0.08)	2.40–3.83	43.86 (0.55)	0.21	0.12	12.35 (0.30)	10	1.83 (0.19)	1	1 (10)	1 (3)
A. rigonii	20	–	8 *m* + 2 *sm*	9	30.15 (0.93)	3.01 (0.09)	2.33–3.50	45.56 (0.42)	0.15	0.11	12.85 (0.24)	10	1.76 (0.15)	1	1 (10)	1 (3)
A. appressipila	20	–	9 *m* + 1 *sm*	9	36.16 (0.89)	3.61 (0.08)	2.86–4.22	42.19 (0.34)	0.24	0.11	11.89 (0.30)	10	2.32 (0.22)	2	2 (9, 10)	1 (3)
Sect. *Arachis*[d]																
A. duranensis	20	1	9 *m* + 1 *sm*	–	28.95 (0.15)	2.89 (0.15)	–	44.00	0.21	0.15	14.59 (0.08)	10	–	–	2 (2, 10)	1 (3)

Table 2 (continued)

Section and species	2n	General karyotype features						Heterochromatin[a]						Number and localization of rDNA sites[b]	
		A9 Pair	Karyotype formula	SAT	TCL, µm (SE)	ML, µm (SE)	Size range, µm	Centromeric index (SE)	Asymmetry indexes		$DAPI^+CMA^-/C\text{-}DAPI^+$		$CMA^+DAPI\text{-}/C\text{-}DAPI^-$		18–26S	5S[c]
									A_1	A_2	% (SE)	NCB	% (SE)	NCB		
A. stenosperma	20	1	9 *m*+1 *sm*	–	35.69 (0.14)	3.56 (0.15)	–	45.00	0.19	0.15	13.21 (0.14)	10	–	–	3 (2, 7, 10)	1 (3)
A. batizocoi	20	–	7 *m*+3 *sm*	–	36.15 (0.12)	3.64 (0.08)	–	41.00	0.29	0.08	11.63 (0.18)	9	–	–	2 (4, 10)	3 (3, 8, 10)

Data correspond to the haploid complement except chromosome number

SAT SAT chromosome type, *TCL* total chromosome length, *ML* mean length by chromosome, size range corresponding to mean values expressed in µm with standard error (SE), A_1 intrachromosomal asymmetry index, A_2 interchromosomal asymmetry index, *m* metacentric, *sm* submetacentric

[a]Mean heterochromatin content expressed in % of the total karyotype length and in parentheses standard error (SE). *NCB* number of chromosomes with bands

[b]Number in parentheses are designations of the chromosome pairs bearing ribosomal sites. *Sites observed in only one homologous chromosome (heteromorphic locus), ^two 18-26S rDNA sites, [+]co-localization of 5S rDNA and 18-26S rDNA sites

[c]Chromosome pairs without DAPI bands are underlined

[d]Data are extracted from Robledo et al. (2009) and Robledo and Seijo (2010)

All the diploid species analyzed had $CMA^+/DAPI^-$ bands on the proximal (sect. *Erectoides*) or interstitial secondary constrictions of the short (sect. *Procumbentes*) and long arms (sect. *Rhizomatosae*) of pair #10 (Figs. 2, 3, 4). Additionally, *A. appressipila* also displayed interstitial $CMA^+/DAPI^-$ bands on the short arms of submetacentric pair #9 (Fig. 3m).

Chromosomal mapping of rDNA loci by FISH

The number of 5S rDNA sites per haploid complement in the *Rhizomatosae* species was conserved: two pairs were observed in the tetraploids and one pair in the diploid species (Table 2). However, in the tetraploids, the sites were proximally localized on the long arms of pair #3 (Figs. 2, 4), while in *A. burkartii* the sites were interstitial (Fig. 2h).

In contrast to the conserved 5S rDNA sites, the number and localization of the 18–26S rDNA sites were more variable. Among the *Rhizomatosae* species, all the tetraploids had one 18–26S rDNA site proximally localized on the four chromosomes #10 (Figs. 2, 4). *Arachis nitida* had additional 18–26S rDNA sites on the short arms of two (out of four) chromosomes #2 (Fig. 2f), while *A. pseudovillosa* in only one (out of four) chromosome #2 (Fig. 2d). *Arachis burkartii* displayed four pairs of 18–26S rDNA sites: the largest and brightest ones were interstitially localized in the secondary constriction of pair #10, while the other three were detected in the proximal regions of the short arms of pairs #3 and #4 and in the long arms of pair #10 (Figs. 2h, 4).

All the species of the sections *Erectoides* and *Procumbentes* had one pair of 18–26S rDNA sites localized in the secondary constrictions of pair #10 (Figs. 3, 4). *Arachis paraguariensis* subsp. *paraguariensis* displayed four additional pairs of 18–26S rDNA sites: two of them proximally localized on both arms of pair #1, one on the short arms of pair #2, and the other one interstitially localized on the long arms of pair #4 (Figs. 2h, 4). *Arachis appressipila* also had one pair of interstitial sites on the short arms of pair #9 (Figs. 2n, 4).

All 18–26S rDNA sites co-localized with $CMA^+/DAPI^-$ bands, except the heteromorphic 18–26S rDNA locus localized on one chromosome #2 of *A. pseudovillosa* (Fig. 2c, d), and the sites of pairs #3, #4, and #10 (the proximal ones) in *A. burkartii* (Fig. 2g, h) and pairs #1, #2, #3 in *A. paraguariensis* subsp. *paraguariensis* (Fig. 3g, h).

Karyotype similarities

For the hierarchical cluster analysis using 18 chromosome features, the karyotype data of three species of the section *Arachis* were included (Fig. 5). *Arachis batizocoi* (genome

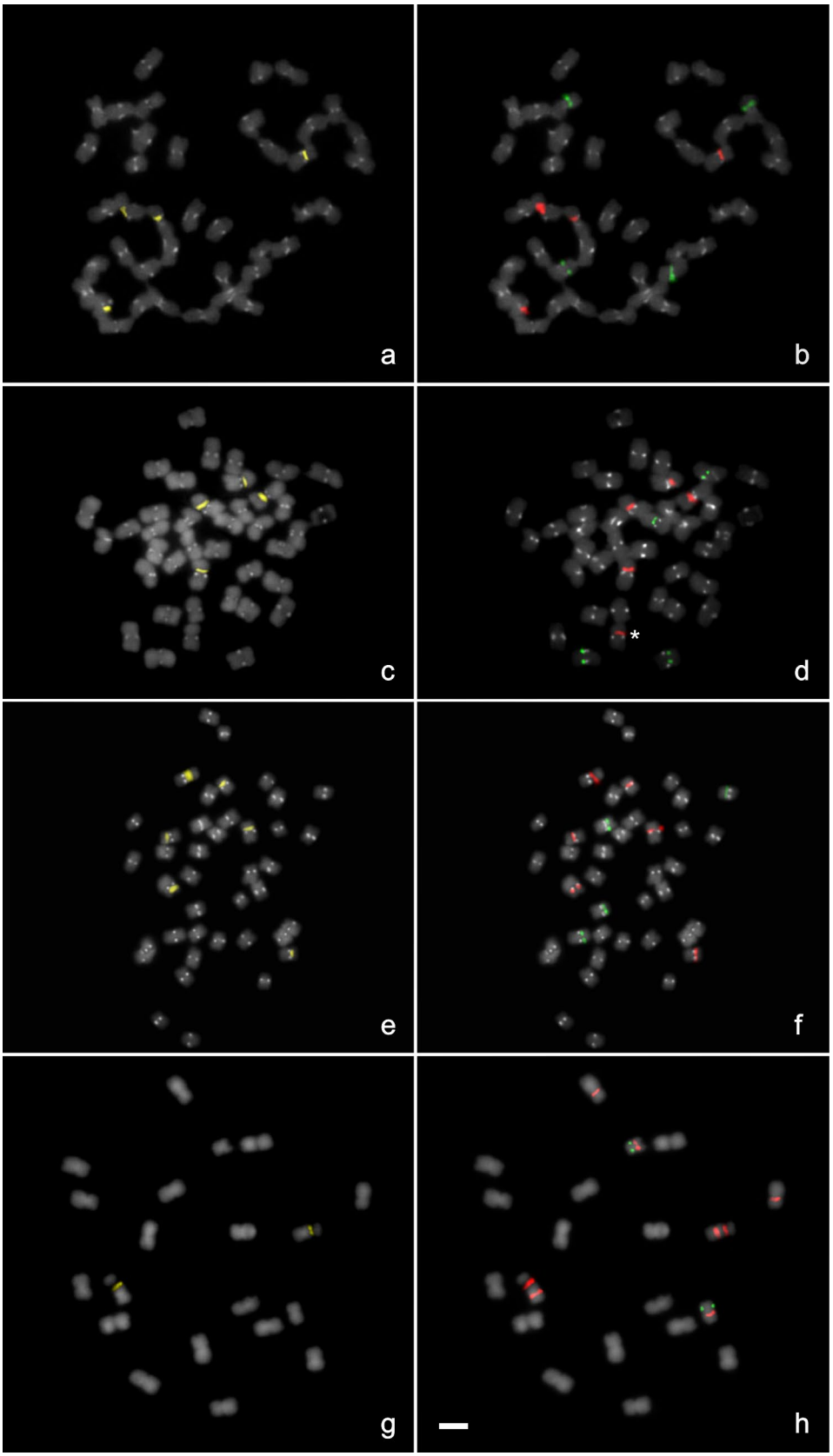


Fig. 2 Somatic metaphases of *Arachis* species belonging to section *Rhizomatosae* after DAPI/CMA double staining (*left*) and double FISH (*right*). *Yellow signals* correspond to the $CMA^{+}DAPI^{-}$/C-$DAPI^{-}$ heterochromatin bands, *gray signals* to the $DAPI^{+}CMA^{-}$/C-$DAPI^{+}$ heterochromatin bands, *green signals* to the 5S rDNA sites, and *red signals* to the 18–26S rDNA sites. The *asterisk* indicates the extra 18S–26S rDNA locus detected on one chromosome # 2 of *A. pseudovillosa*. **a, b** *A. glabrata*, **c, d** *A. pseudovillosa*, **e, f** *A. nitida*, **g, h** *A. burkartii*. *Scale bar* 3 μm

K) and *A. burkartii* remained as independent OTUs while the other ones formed a large cluster.

Within the large cluster, the two A genome species (*A. duranensis* and *A. stenosperma*) of the section *Arachis* grouped together because they have one A9 pair and centromeric conspicuous DAPI$^+$ heterochromatin in all the chromosomes of the complement. All the other species had centromeric dot-like DAPI$^+$ heterochromatin. Among them, three subgroups were recognized: the first included the three species of the section *Procumbentes*, the second the two subspecies of *A. paraguariensis* (sect. *Erectoides*), and the third all the tetraploid species of the section *Rhizomatosae* together with *A. hermannii* and *A. major* (sect. *Erectoides*).

Discussion

The establishment of species relationships through chromosomal analysis is based on the premise that closely related species may have more similar karyotypes than distantly related ones (Acosta et al. 2016; Chalup et al. 2015; Robledo et al. 2009; Robledo and Seijo 2010; Seijo et al. 2004; Silvestri et al. 2015; Weiss-Schneeweiss et al. 2008). Here, we combined DAPI/CMA banding and FISH techniques to generate useful chromosomal markers for the identification of chromosome homeologies among the three rhizomatous tetraploid species and between them and the diploid species from different sections of the genus *Arachis*. The first detailed karyotypes of the species of the sections *Rhizomatosae, Erectoides* and *Procumbentes* here provided allowed the genome characterization of all the *Rhizomatosae* species and to make inferences on the origin of the tetraploid ones.

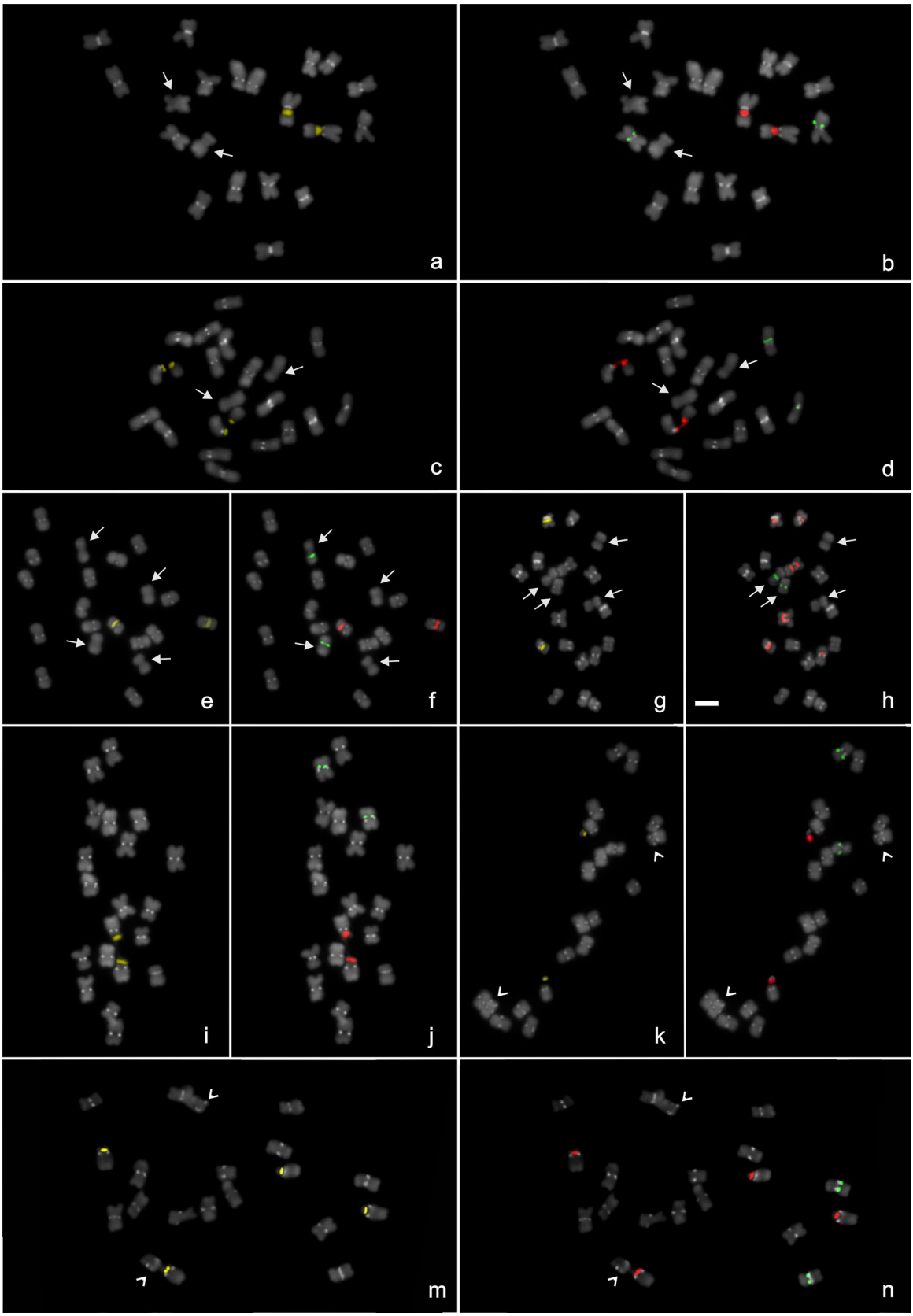
Heterochromatin distribution, rDNA loci localization and karyotype patterns

The physical mapping of DAPI/CMA heterochromatin and 5S and 18S–26S rDNA loci provided strong evidence for the existence of two different karyotype patterns within the *Rhizomatosae* species. The common karyotype pattern for the three tetraploid rhizomatous species can be defined by the presence of SAT chromosomes type 3 (#10), centromeric dot-like DAPI$^+$ bands in all the chromosomes of the complement, four chromosomes (#3) with proximal 5S rDNA sites on their long arms, and four to six chromosomes (#2 and #10) with proximal 18–26S rDNA sites. Although all the species presented one 18–26S rDNA site proximally localized in the four chromosomes #10, the presence of additional sites was a useful landmark for the differentiation of the species. Indeed, *A. pseudovillosa* and *A. nitida* could be recognized by having one or two additional sites, respectively, in chromosomes #2.

Among the tetraploid species, the facts that *A. glabrata* and *A. nitida* had similar chromosome length, similar amount of DAPI$^+$ heterochromatin and the same heteromorphism in chromosomes #9 (two *sm* and two *m*) suggest that they are more closely related to each other than to *A. pseudovillosa*, as revealed in the cluster analysis. This is concordant with their morphological features (Krapovickas and Gregory 1994; Valls and Simpson 2005) and the genetic similarity analysis done using Random Amplified Polymorphic DNA (RAPD) markers (Valente et al. 2003).

The unique karyotype pattern of the diploid rhizomatous *A. burkartii* was evidenced in the cluster analysis. The karyotype of this species was different from all the other diploid *Arachis* species by the co-localization of one 5S rDNA site with one 18–26S rDNA site on pair #3, the co-localization of two 18–26S rDNA loci on pair #10, and the lack of DAPI$^+$ heterochromatin bands.

Species of the sections *Procumbentes* and *Erectoides* showed similar karyotype patterns, mainly characterized by the presence of centromeric dot-like DAPI$^+$ heterochromatin in all or almost all the chromosomes of the complement. The similarity observed in the cluster analysis is consistent with the genetic similarity detected using microsatellite data (Hoshino et al. 2006), by which they clustered together. The three species of the section *Procumbentes* showed a karyotype pattern characterized by having DAPI$^+$ heterochromatin in all the chromosomes of the complement, submetacentric SAT chromosomes type 9, and interstitial 18–26S rDNA sites on the short arms of pair #10. In the species of the section *Erectoides*, two karyotype subgroups were recognized. One was represented by the two subspecies of *A. paraguariensis*, which have short chromosomes, eight pairs with DAPI$^+$ heterochromatin, proximal 18–26S rDNA sites on the long arms of pair #10 and the chromosomes harboring the 5S rDNA sites (#3) without DAPI$^+$ heterochromatin. The other karyotype subgroup included the species *A. hermannii* and *A. major*, with large chromosomes with the proximal 18–26S rDNA sites on the short arms of pair #10 but with DAPI$^+$ heterochromatin bands in the chromosomes harboring the 5S rDNA sites. The two karyotype subgroups detected in the species of the section *Erectoides* are supported by the morphological differences and the low genetic affinity reported between them (Krapovickas and Gregory 1994). The two subspecies of *A. paraguariensis*, together with *A. stenophylla*, are differentiated from the other species of the section *Erectoides* by the absence of thickened branches on the roots, and by the disposition of the flowers. Although they are more frequent at the base of the plant in *A. paraguariensis* and *A. stenophylla*, they do not form a dense cluster as in the other *Erectoides* species.


a
b
c
d
e
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n

◂**Fig. 3** Somatic metaphases of *Arachis* species belonging to sections *Erectoides* and *Procumbentes* after DAPI/CMA double staining (*left*) and double FISH (*right*). *Yellow signals* correspond to the CMA^+DAPI^-/C-$DAPI^-$ heterochromatin bands, *gray signals* to the $DAPI^+CMA^-$/C-$DAPI^+$ heterochromatin bands, *green signals* to the 5S rDNA sites, and *red signals* to the 18–26S rDNA sites. *Arrows* indicate chromosomes without $DAPI^+$ bands and *arrowhead* indicates chromosomes with subtelomeric $DAPI^+$ bands. **a**–**h** section *Erectoides*, **i**–**n** section *Procumbentes*. **a, b** *A. hermannii*, **c, d** *A. major*, **e, f** *A. paraguariensis* subsp. *capibariensis*, **g, h** *A. paraguariensis* subsp. *paraguariensis*, **i, j** *A. lignosa*, **k, l** *A. rigonii*, **m, n** *A. appressipila. Scale bar* 3 µm

Polyploidy nature of the rhizomatous tetraploids

The heterochromatin banding and physical mapping of the 5S and 18S–26S rRNA genes by FISH have been widely used to make inferences about the nature of polyploids in several plant groups (Lavia et al. 2011; Li et al. 2001; Nielen et al. 2010; Seijo et al. 2004; Souza et al. 2012; Tomas et al. 1997). Molecular cytogenetic approaches have shown that polyploid plant genomes usually undergo rapid and dynamic genomic changes upon or immediately following polyploidization (Krishnan et al. 2001; Lim et al. 2007; Piellicer et al. 2013; Weiss-Schneeweiss et al. 2007, 2008), while others appear to be the sum of their parental diploid species (Garcia et al. 2009; Pires et al. 2004; Seijo et al. 2004). Cytogenetic analyses in *Arachis* polyploids—*A. hypogaea, A. monticola* and *A. pintoi*—have shown that polyploidy by itself does not induce significant changes in the organization of ribosomal loci or heterochromatic bands, since their karyotypes are equivalent to the sum of those of the diploid progenitor species, regardless of whether they are autopolyploid as *A. pintoi* 3*x* (Lavia et al. 2011) or allopolyploids as *A. monticola* and *A. hypogaea* (Samoluk et al. 2015; Seijo et al. 2004).

Concerning the rhizomatous tetraploids analyzed here, controversial hypotheses have been put forward for the nature of *A. glabrata*. Some authors have proposed that this species is an autopolyploid but have not provided a genome formula (Singh and Simpson 1994), while others have suggested that it may be an allopolyploid with the EERR genome constitution (Bechara et al. 2010; Gregory and Gregory 1979). Although *A. glabrata* has an heteromorphism in chromosomes #9 (two *sm* and two *m*), the similar patterns of CMA-DAPI heterochromatin and rDNA sites observed here in the four chromosome sets are compatible with the hypothesis that considers this species as an autopolyploid. The detection of one to eight quadrivalents in the meiotic analyses of several accessions (Ortiz et al. 2011) also supports the autopolyploid nature of *A. glabrata*. Moreover, the similar patterns of the chromosome landmarks detected here in the two varieties of *A. glabrata* suggest that they may have originated from a single event of polyploidization, or if multiple, from the same diploid species.

The meiotic chromosome pairing in 20II in most (65%) of the cells and the detection of configurations including up to four multivalents (trivalents and quadrivalents) in low frequencies (0.14 III and 0.45 IV) suggest that *A. nitida* may have two different chromosomes sets but with partial homology (Ortiz et al. 2011). The presence of one 18–26S rDNA site in only two of the four chromosomes #2 and the different morphology among chromosomes #9 (two *m* and two *sm*) support that this species has two partially different chromosome complements. Therefore, *A. nitida* may be considered as a segmental allopolyploid that may have resulted from the hybridization of two species with the same pattern of $DAPI^+$ heterochromatin and the 5S rDNA sites, but different number and position of the 18–26S rDNA sites and morphology of chromosomes #9.

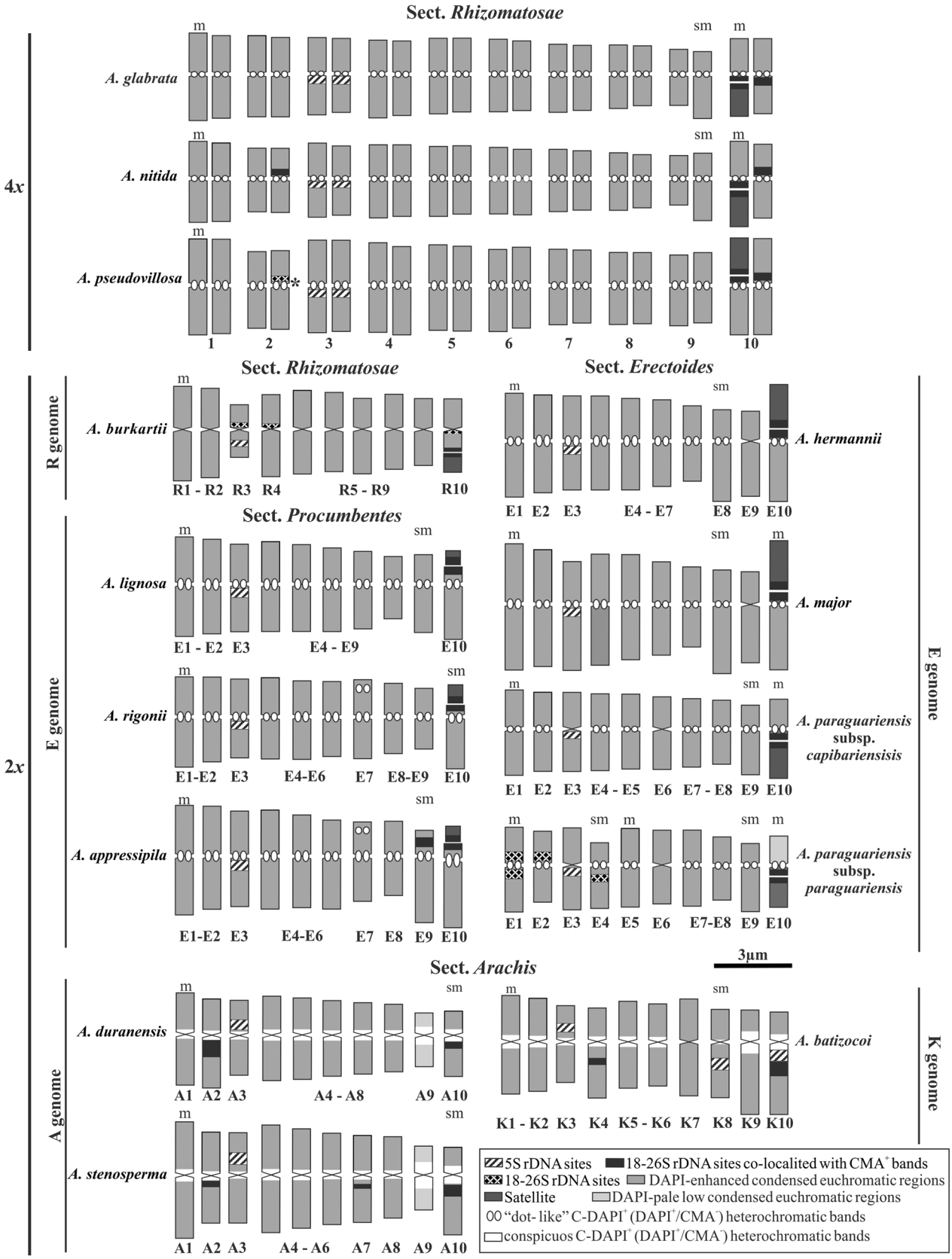
The overall chromosome morphology, the similar distribution pattern of CMA-DAPI bands in the four chromosome sets, and the presence of one 5S and one 18–26S rDNA site in the four chromosomes #3 and #10, respectively, suggest that *A. pseudovillosa* may be an autopolyploid. Under this assumption, the extra 18S–26S rDNA locus detected in only one chromosome #2 (out of four) may have arisen *de novo* by transposition (or other genomic mechanism) after polyploidization. However, analysis of the meiotic behavior of *A. pseudovillosa* is needed to fully understand the polyploid nature of this species.

Evolutionary relationships between diploid and tetraploid *Rhizomatosae* species

The comparative cytogenetic analysis evidenced that the rhizomatous tetraploid species are closely related because they have a common karyotype pattern. This close relationship has also been evidenced by RAPD (Nóbile et al. 2004) and simple sequence repeat (SSR) analyses (Angelici et al. 2008). Thus, the karyotype similarity and the genetic affinity revealed by molecular markers suggest that the three tetraploids belong to a natural group and that they may have at least one common diploid ancestor.

As the section *Rhizomatosae* is taxonomically defined (Krapovickas and Gregory 1994), the most parsimonious hypothesis would consider *A. burkartii* as one of the diploid donors of the rhizomatous tetraploids. However, our cytogenetic analysis evidenced that the karyotype of *A. burkartii* is very different from those of the tetraploids and does not support that hypothesis. The complete reproductive isolation reported between *A. burkartii* and the tetraploids (Krapovickas and Gregory 1994) is in accordance with the cytogenetic results.

Morphologically, *A. burkartii* has the standard petal of the papilionaceous flower with reddish lines on both


Sect. Rhizomatosae
4x
A. glabrata
A. nitida
A. pseudovillosa
1 2 3 4 5 6 7 8 9 10
Sect. Rhizomatosae
Sect. Erectoides
2x
R genome
A. burkartii
R1 - R2 R3 R4 R5 - R9 R10
A. hermannii
E1 E2 E3 E4 - E7 E8 E9 E10
Sect. Procumbentes
E genome
A. lignosa
E1 - E2 E3 E4 - E9 E10
A. major
A. rigonii
E1-E2 E3 E4-E6 E7 E8-E9 E10
A. paraguariensis subsp. capibariensisis
E1 E2 E3 E4 - E5 E6 E7 - E8 E9 E10
A. appressipila
E1-E2 E3 E4-E6 E7 E8 E9 E10
A. paraguariensis subsp. paraguariensis
E1 E2 E3 E4 E5 E6 E7-E8 E9 E10
E genome
3µm
Sect. Arachis
A genome
A. duranensis
A1 A2 A3 A4 - A8 A9 A10
A. batizocoi
K1 - K2 K3 K4 K5 - K6 K7 K8 K9 K10
K genome
A. stenosperma
A1 A2 A3 A4 - A6 A7 A8 A9 A10
5S rDNA sites
18-26S rDNA sites co-localited with CMA+ bands
18-26S rDNA sites
DAPI-enhanced condensed euchromatic regions
Satellite
DAPI-pale low condensed euchromatic regions
"dot- like" C-DAPI+ (DAPI+/CMA-) heterochromatic bands
conspicuos C-DAPI+ (DAPI+/CMA-) heterochromatic bands

◂**Fig. 4** Idiograms of *Arachis* species belonging to sections *Rhizomatosae, Erectoides* and *Procumbentes* showing the distribution of chromosome landmarks mapped by CMA-DAPI banding and FISH. Asterisk in one pair #2 of *A. pseudovillosa* indicates the 18–26S rDNA site observed in only one homologous chromosome. The chromosomes were ordered by morphology and then according to decreasing size. Some chromosomes within each idiogram 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 and Seijo 2010). In the tetraploid species, the tentative homeologous chromosome pairs are represented by the same number. The idiograms of A and K genome species were redrawn from Robledo and Seijo (2010); the morphology of the satellites is not available. *Scale bar* 3 μm

surfaces and coriaceous leaflets with the margin marked on both surfaces (Krapovickas and Gregory 1994). These characters are not present in any of the rhizomatous tetraploids. Furthermore, *A. burkartii* grows mainly in the lower part of the Uruguay River basin, covering great part of the Rio Grande do Sul state (Brazil), the north of Uruguay, and northeast of Argentina (Krapovickas and Gregory 1994). *Arachis glabrata* var. *glabrata* grows from the south of Goiás state, west of Minas Gerais state, and northern half of São Paulo state in Brazil to the south of Paraguay and northeast of Argentina. More recently, a few populations have been found in the east of Bolivia (Seijo et al. unpublished, Herbarium CTES, Argentina). *Arachis glabrata* var. *hagenbeckii* grows in the southeast of Paraguay and northeast of Argentina, in a restricted area that overlaps the range of the variety *glabrata*. The other two tetraploid species, *A. pseudovillosa* and *A. nitida*, have more restricted distribution areas and grow in the Amambay and Concepción departments in Paraguay and the south of Mato Grosso do Sul state in Brazil (Krapovickas and Gregory 1994; Valls and Simpson 2005). Only *A. glabrata* overlaps with the diploid *A. burkartii* in a small area in the extreme northeast of Argentina (Fig. 1), but they grow in different environments and have never been found growing together. Thus, the morphological differences and the parapatric and allopatric distribution of *A. burkartii* with *A. glabrata* and *A. nitida*/*A. pseudovillosa*, respectively, support the rejection of the hypothesis that considers *A. burkartii* as one of the diploid progenitors of the rhizomatous tetraploids.

Molecular marker analyses including the four *Rhizomatosae* species, like RAPD (Nóbile et al. 2004) and SSR (Angelici et al. 2008), showed that the tetraploid species clustered together, while *A. burkartii* was distantly segregated in the trees. In the amplified fragment length polymorphism (AFLP) analysis, *A. burkartii* was associated with the two species of the section *Caulorrhizae* (Gimenes et al. 2002) but not with the rhizomatous tetraploids. In addition, molecular phylogenies based on ITS sequences (Bechara et al. 2010; Friend et al. 2010), which only included *A. burkartii* and *A. glabrata*, showed that *A. burkartii* is distantly related to *A. glabrata*. Thus, molecular markers and sequence analyses strongly support the conclusion obtained from the cytogenetic data about the relationships among the rhizomatous species.

Genomic considerations in the section *Rhizomatosae* and genome donors of the tetraploids

Traditionally, the *Rhizomatosae* species were assigned to the R genome based on the infrageneric divisions (Smartt and Stalker 1982). Considering the cross-compatibility reported among *Rhizomatosae* species (Gregory and Gregory 1979), Smartt and Stalker (1982) advanced the tentative assignation of R_1 to the diploid *A. burkartii* and of R_2 and R_3 to the tetraploid species. However, according to the cytogenetic results provided here, the tetraploid species should be excluded from the R genome, which might remain exclusively for *A. burkartii*. The conservation of the R genome for *A. burkartii* is based on its strong reproductive isolation from the other species of the genus (Krapovickas and Gregory 1994), its particular chromosome characteristics (this work), and the isolated position in the phylogenetic analyses (Bechara et al. 2010; Friend et al. 2010).

With the tetraploids excluded from the R genome, their genome affinities were searched among those described for the other *Arachis* species. For this purpose, the intersectional hybrids reported among the rhizomatous tetraploids and diploid species of the genus (Krapovickas and Gregory 1994; Smartt and Stalker 1982) were considered, particularly among representatives of the A, K, and E genomes.

Based on crossing assays (Krapovickas and Gregory 1994) and meiotic chromosome pairing analyses of the F1 hybrids (obtained by embryo rescue) between *A. glabrata* and some A genome species (*A. diogoi, A. duranensis* and *A. stenosperma*), it has been suggested that one of the diploid progenitors of this tetraploid should belong to the A genome (Mallikarjuna 2004). However, the cytogenetic analysis done here evidenced that the complements of the rhizomatous tetraploids do not have the characteristic A9 pair and that the pattern of $DAPI^+$ heterochromatin bands (dot-like instead of conspicuous bands) is different from that published for the A genome species (Robledo et al. 2009; Seijo et al. 2004). Thus, although some A genome species form F_1 hybrids and partially overlap their distribution areas with the tetraploids, the cytogenetic data suggest that they should be discarded as probable progenitors of the rhizomatous tetraploids.

Concerning the K genome species, although F_1 hybrids between *A. batizocoi* and the three rhizomatous tetraploid species have been reported, they never or poorly flowered. The exception is one hybrid with *A. nitida* that has 0.03% of pollen viability (Krapovickas and Gregory 1994; Valls

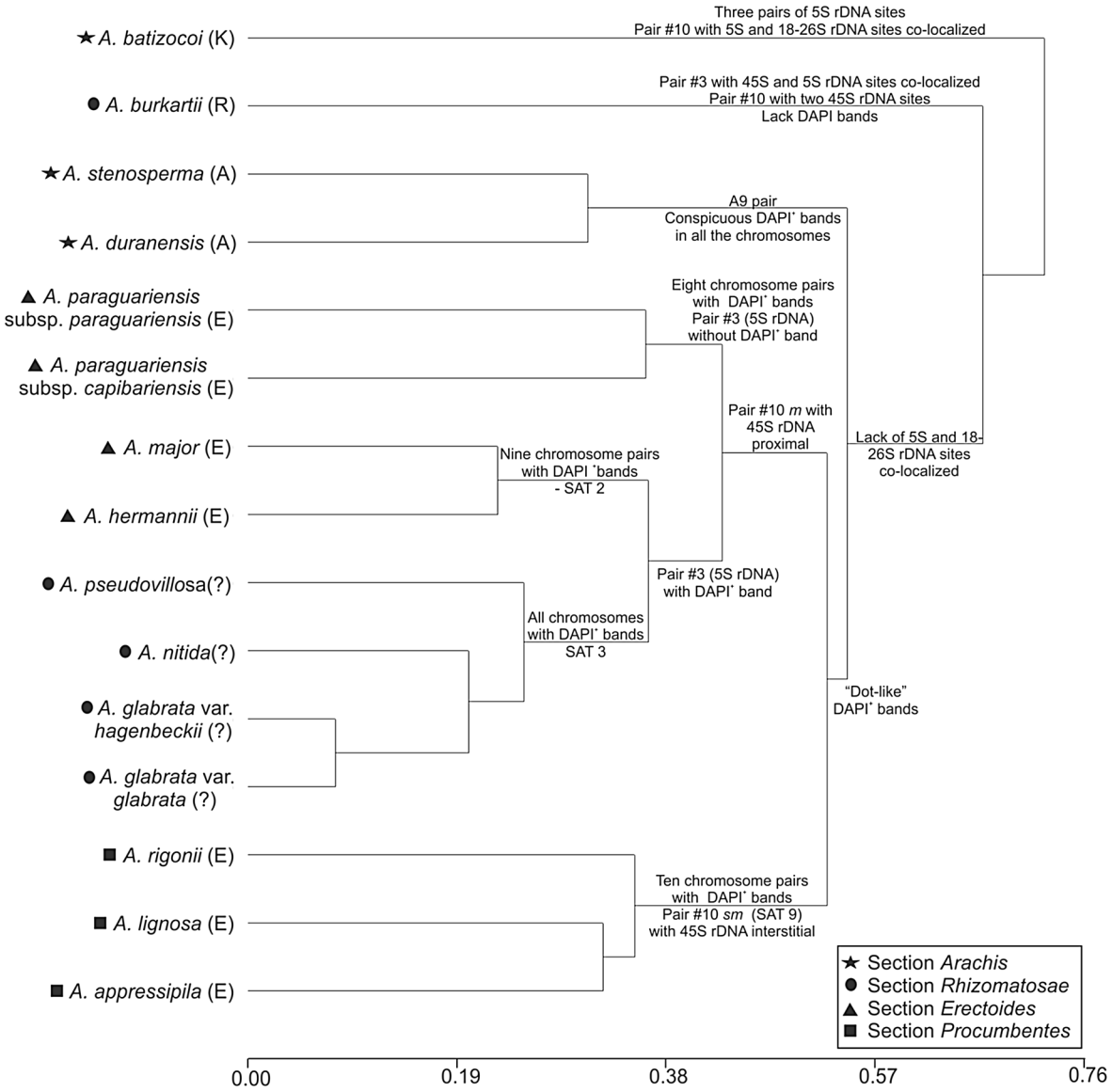


Fig. 5 Dendogram representing the relationship among the karyotypes of several *Arachis* species from sections *Rhizomatosae, Erectoides, Procumbentes* and *Arachis*. *Letters in parentheses* are the designations of genome type. *m* metacentric, *sm* submetacentric chromosomes. Clustering was performed using unweighted pair-group linkage type, with the Gower distance method and standard deviation for scaling. Cophenetic correlation coefficient (*r*) 0.95

and Simpson 2005). Cytogenetically, *A. batizocoi* has a higher number of 5S rDNA and 18–26S rDNA sites, one pair of each type of rDNA sites co-localized in pair #10 and a pattern of DAPI$^+$ heterochromatin (Robledo and Seijo 2010) different from that of the chromosome complements of the tetraploids. The K genome species have conspicuous DAPI$^+$ bands distributed in nine chromosome pairs of the complement (Seijo et al. 2004; Robledo and Seijo 2010). Thus, the hypothesis that *A. batizocoi* (K genome) is one of the genome donors of the tetraploids should also be discarded. The distribution of *A. batizocoi* in a distant geographical area from those of the tetraploid *Rhizomatosae* species supports this proposal.

Gregory and Gregory (1979) suggested that rhizomatous tetraploids consist of many poorly differentiated forms of an ancestral amphidiploid between one diploid species from *Rhizomatosae* and one from the E genome group. The E genome includes all species of the sections *Procumbentes, Erectoides* and *Trierectoides*. Rhizomatous tetraploids produce F_1 hybrids with diploid species of the sections *Erectoides* (four species) and *Procumbentes* (two species) (Krapovickas and Gregory 1994; Stalker 1985; Valls and Simpson 2005). Moreover, some of these

F_1 produce normal flowers with different percentage of pollen viability, such as *A. hermannii* × *A. pseudovillosa* (46.1%), *A. major* × *A. nitida* (29.6%), *A. appressipila* × *A. glabrata* (22.2%) and *A. rigonii* × *A. glabrata* (14.9%). This evidences that the rhizomatous tetraploids are genetically more compatible with the E genome species than with the other species of *Arachis*.

Our results revealed that *Procumbentes* and *Erectoides* species (karyotypes by FISH of *Trierectoides* species are not known yet) have centromeric dot-like $DAPI^+$ bands distributed in almost all the centromeres, one 5S rDNA site localized on the long arm of a medium sized chromosome, and one 18–26S rDNA site on the SAT chromosomes in each chromosome set. This pattern is the most compatible with those observed in the rhizomatous tetraploids, although the chromosome landmarks observed in the tetraploid complements cannot be fully reconstructed from the sum of the characters of any pair of diploid species analyzed in this work.

Concerning *A. glabrata*, the molecular phylogenies evidence that this species is closely associated with species of the section *Procumbentes* (Bechara et al. 2010; Friend et al. 2010). However, the latter have SAT chromosomes type 9 (pair #10) and a chromosome pair with subtelomeric $DAPI^+$ bands (*A. rigonii* and *A. appressipila*), which were not found in this rhizomatous tetraploid. Thus, from a cytogenetic point of view, to consider *Procumbentes* species as genome donors of *A. glabrata* would have required the occurrence of a paracentric inversion in the short arm of pair #10. It is also remarkable that the *Procumbentes* species here analyzed are allopatric both with *A. glabrata* and among themselves (Fig. 1).

On the other hand, the four *Erectoides* species reported to produce F_1 hybrids with *A. glabrata* are sympatric with this tetraploid species (Fig. 1). Among them, *A. paraguariensis* subsp. *capibariensis*, in spite of having fewer chromosomes with $DAPI^+$ heterochromatic bands, has the most similar pattern of chromosome markers expected for the genome donor of *A. glabrata*.

The karyotype of *A. pseudovillosa* has a pattern of heterochromatic bands and rDNA sites similar to that of the *Erectoides* species with the largest chromosomes (*A. major* and *A. hermannii*). The moderate pollen viability of the F_1 hybrids obtained between *A. hermannii* and this tetraploid (Kraprovickas and Gregory 1994) supports the close relationships between these species. Furthermore, the distribution areas of these two diploids are close to that of *A. pseudovillosa*, but the area of *A. major* overlaps with that of this tetraploid in Amambay and Concepción departments in Paraguay and south of Mato Grosso do Sul state in Brazil (Fig. 1). These results suggest that *A. hermannii, A. major* or a species with a similar karyotype may be considered the most probable diploid genome donor of *A. pseudovillosa*.

Arachis nitida has the two types of chromosomes with the 18–26S rDNA sites observed in *A. glabrata* and *A. pseudovillosa*. This suggests a first hypothesis that *A. nitida* may have originated by hybridization between these two tetraploids. Besides the karyotype similarities, the three tetraploid species have overlapping distributions in Amambay and Concepción departments in Paraguay (Fig. 1). Moreover, hybrids between them have been reported, but without data on the fertility of pollen (Krapovickas and Gregory 1994).

An alternative hypothesis for the origin of *A. nitida* may consider hybridization between *A. paraguariensis* subsp. *capibariensis* and a diploid species of the section *Erectoides* with two metacentric chromosome pairs having rDNA sites on the short arms. However, the latter chromosomal features were not found in the diploid *Erectoides* species analyzed in this work. Thus, to test the latter hypothesis, a larger survey including additional *Erectoides* species is required for a more compressive understanding of the origin of *A. nitida*.

In summary, the comparative chromosome analysis presented here, supported by the reproductive and geographic isolation and the genetic differences suggests that (1) the section *Rhizomatosae* is a polyphyletic group constituted by two groups of species, one of them composed of the three rhizomatous tetraploid species and the other by the distant diploid *A. burkartii*, (2) the R genome of *A. burkartii* is not homologous to that of any of the rhizomatous tetraploids, (3) the E genome diploid species are the most closely related to the tetraploid *Rhizomatosae* species, and (4) rhizomes may have arisen at least twice in the genus *Arachis*. We envision that these data will be useful for breeders to select diploid parents in the genetic improvement of the existing *A. glabrata* commercial cultivars and for the development of new ones.

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