An Acad Bras Cienc (2023) 95(2): e20210162 DOI 10.1590/0001-3765202320210162
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

CROP SCIENCE

Genomic relationships of the polyploid rhizoma peanut (*Arachis glabrata* Benth.) inferred by genomic *in situ* hybridization (GISH)

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Abstract: The rhizoma peanut (*Arachis glabrata* Benth., section *Rhizomatosae*) is a tetraploid perennial legume. Although several *A. glabrata* cultivars have been developed as forage and ornamental turf, the origin and genomic constitution of this species are still unknown. In this study, we evaluated the affinity between the genomes of *A. glabrata* and the probable diploid donors of the sections *Rhizomatosae*, *Arachis*, *Erectoides* and *Procumbentes* by genomic *in situ* hybridization (GISH). Single GISH analyses detected that species of the sections *Erectoides* (E_2 subgenome) and *Procumbentes* (E_3 subgenome) were the diploid species with the highest degree of genomic affinity with *A. glabrata*. Based on single GISH experiments and DNA sequence similarity, three species -*A. duranensis*, *A. paraguariensis* subsp. *capibarensis*, and *A. rigonii*-, which showed the most uniform and brightest hybridization patterns and lowest genetic distance, were selected as probes for double GISH experiments. Double GISH experiments showed that *A. glabrata* is constituted by four identical or very similar chromosome complements. In these assays, *A. paraguariensis* subsp. *capibarensis* showed the highest brightness onto *A. glabrata* chromosomes. Thus, our results support the autopolyploid origin of *A. glabrata* and show that the species with E_2 subgenome are the most probable ancestors of this polyploid legume forage.

Key words: *Arachis*, chloroplast *trnT-S* and *trnT-Y* sequences, genomic constitution, genomic *in situ* hybridization (GISH), nuclear ribosomal internal transcribed spacer (ITS), polyploidy.

INTRODUCTION

Arachis L. (Leguminosae) is a South American genus that comprises 83 species (Krapovickas & Gregory 1994, Valls & Simpson 2005, Valls et al. 2013, Santana & Valls 2015, Valls & Simpson 2017, Seijo et al. 2021). According to their morphological characteristics, geographic distribution, and cross-compatibility, these species have been distributed into nine taxonomic sections: *Arachis*, *Caulorrhizae*, *Erectoides*, *Extranervosae*, *Heteranthae*, *Procumbentes*, *Rhizomatosae*, *Trierectoides* and *Triseminatae* (Krapovickas & Gregory 1994). These species are mostly autogamous with geocarpic fruits (Krapovickas & Gregory 1994), and have two basic chromosome numbers: x = 9 and x = 10 (Fernández & Krapovickas 1994, Peñaloza & Valls 2005, Lavia et al. 2008). In these species, seven different genomes (A, B, D, F, G, K, and R) have been formally recognized (Smartt et al. 1978, Stalker 1991, Robledo et al. 2009, Robledo & Seijo 2010, Silvestri et al. 2015, Ortiz et al. 2017), while other five (Am, C, E, Ex and T) have been assigned based on the infrageneric division (Smartt & Stalker 1982). In addition, in the species with E genome, three subgenomes (E_1, E_2 and E_3) have been tentatively suggested

for the series *Trierectoides*, *Erectoides* and *Procumbentes*, respectively (Smartt & Stalker 1982), which are currently homonymous sections (Krapovickas & Gregory 1994).

The genus *Arachis* includes three economically important species: the peanut (*A. hypogaea*), the forage peanut (*A. pintoi*) and the rhizoma peanut (*A. glabrata*). *Arachis hypogaea* L. (section *Arachis*) is an oilseed crop and a direct source of human food, whereas *A. pintoi* Krapov. & W.C. Greg. (section *Caulorrhizae*) and *A. glabrata* Benth. (section *Rhizomatosae*) are forage species used in tropical and subtropical regions. The genome composition and genetic origin of *A. hypogaea* (Seijo et al. 2004, 2007, 2018, Grabiele et al. 2012, Zhang et al. 2016, Bertioli et al. 2019, 2020) and *A. pintoi* (Lavia et al. 2011, Pucciariello et al. 2013) have been deeply investigated at both cytogenetic and molecular levels, whereas those of *A. glabrata* remain largely unknown.

Arachis glabrata is a tetraploid species with 2n=4x=40 (Gregory et al. 1973). It is a perennial warm-season legume, native to the northeast of Argentina, east of Paraguay and Bolivia, and south of Brazil (Krapovickas & Gregory 1994). On the basis of leaf morphology and geographic distribution, two varieties have been recognized: *A. glabrata* var. *glabrata* and var. *hagenbeckii* (Harms ex Kuntze) F. J. Herm. (Krapovickas & Gregory 1994). In North America and Australia, this species is used as a forage crop because of its high tolerance to grazing and cold temperatures, high nutritive value and digestibility (similar to those of alfalfa), good dry matter production, fast coverage rate, and high potential for use in mixtures with vigorous C4 grasses (French et al. 2006, Mullenix et al. 2016). Several cultivars have been developed and are commercialized as forage and ornamental turf (Prine et al. 1981, 1986, 1990, 2010, Muir et al. 2010, Quesenberry et al. 2010). However, since all commercial cultivars derive from a few plant introductions, their genetic variability is reduced (French et al. 1994). Moreover, it is estimated that over 90% of the planted *A. glabrata* corresponds to the cultivar 'Florigraze' (Quesenberry et al. 2010), increasing the concern for the genetic vulnerability of this crop.

The potential productivity and adaptability of *A. glabrata* could be increased by incorporating accessions of this species from diverse origins to the breeding programs as well as by using the available genetic diversity in related *Arachis* species. For the latter purpose, to select the most compatible species from a panel of potential wild species with desirable traits to be introgressed, basic information about the genome composition of *A. glabrata* is still needed. The available information about the genetic origin of *A. glabrata* and its polyploid nature is still controversial. Different genomic constitutions and different degrees of diploidization have been reported (Gregory & Gregory 1979, Raman 1981, Jahnavi & Murty 1985, Singh & Simpson 1994, Ortiz et al. 2011, 2017), and also different wild species have been postulated to be involved in the genetic origin of *A. glabrata*. Therefore, investigating the genomic constitution of *A. glabrata* and its genomic relationships with genetically related *Arachis* species will help in the selection of diploid parents for the genetic improvement of the existing *A. glabrata* commercial cultivars.

Together with other two tetraploid [*A. pseudovillosa* (Chodat and Hassl.) Krapov. & W.C. Greg. and *A. nitida* Valls, Krapov. & C.E.Simpson, 2n=4*x*=40] and one diploid (*A. burkartii* Handro, 2n=2*x*=20) species, *A. glabrata* is included in the section *Rhizomatosae* for having rhizomes (Gregory et al. 1973, Fernández & Krapovickas 1994, Peñaloza & Valls 2005, Valls & Simpson 2005). The relationships of *A. glabrata* with other species of the section *Rhizomatosae* and

with other species of the genus as a whole have been inferred from the analysis of cross-compatibility assays (Gregory & Gregory 1979, Krapovickas & Gregory 1994), field observations (Valls 1996), molecular markers (Gimenes et al. 2002, Nobile et al. 2004, Angelici et al. 2008), genomic sequences (internal transcribed spacers (ITS) and 5.8S ribosomal DNA (rDNA)) (Bechara et al. 2010, Friend et al. 2010), and comparative karyotype analyses (Ortiz et al. 2017). These approaches have demonstrated that *A. glabrata* is not closely related to *A. burkartii*, but revealed some genomic affinity with diploid species of the sections *Arachis*, *Erectoides* and *Procumbentes*. All these data suggest that the section *Rhizomatosae* may not be monophyletic, and have opened questions about the polyploid nature of *A. glabrata*, its genomic constitution, and the species that participated in its origin.

The genomic constitution and genetic origin of many polyploid plants have been investigated by complementary approaches such as genomic *in situ* hybridization (GISH) and DNA sequence polymorphism analysis (Liu et al. 2006, Chester et al. 2010, Mavrodiev et al. 2015, Marques et al. 2018, Wang et al. 2019). GISH has shown to be very useful in clarifying the genomic constitution and potential diploid ancestor of the allopolyploid peanut *A. hypogaea* (Seijo et al. 2007), whereas the variation in the ITS and 5.8 rDNA of nuclear rDNA (Bechara et al. 2010, Friend et al. 2010) and non-coding chloroplast DNA (cpDNA) sequences (Grabiele et al. 2012) has been useful to determine the wild species that most probably participated in the genetic origin of peanut. Therefore, we expect that these techniques could provide useful information to determine the genomic relationships of the polyploid *A. glabrata*.

In the present study, we conducted single and double GISHs onto both varieties of *A. glabrata*, by using ten diploid *Arachis* species representing the A, Am, K, E (E_2 and E_3 subgenomes) and R genomes, with the aim to: 1) gain insights into the genomic affinities between *A. glabrata* and its probable diploid genome donors, and 2) test the genomic composition and polyploid nature of *A. glabrata*. The polymorphisms of the nuclear ribosomal ITS and two non-coding cpDNA regions were used as aid to identify the most probable genome donors of *A. glabrata* from a set of cross-compatible species.

MATERIALS AND METHODS

Plant material

Samples of both *A. glabrata* varieties and ten diploid *Arachis* species were obtained from the Instituto de Botánica del Nordeste (Corrientes, Argentina). The diploid species analyzed were selected among those reported to produce hybrids with *A. glabrata* (Krapovickas & Gregory 1994) and to share karyotype landmarks similar to those of the tetraploid (Ortiz et al. 2017), except *A. pusilla* Benth., which was included as negative control for cytogenetic analyses. The original provenances and collection numbers of the species analyzed are cited in Table I.

Selection of diploid species by genetic distance of nuclear and cpDNA sequences

DNA extraction

Total genomic DNA (gDNA) was extracted from young actively growing leaves by using the cetyl trimethylammonium bromide (CTAB) procedure (Doyle & Doyle 1987). DNA concentrations and qualities were determined by spectrophotometry and gel electrophoresis, respectively.

Table I. List of the *Arachis* species and samples studied, their provenance, chromosome number, genome formula, type of DAPI pattern and number of ribosomal loci (rDNA). Species are ordered first by ploidy level and then by genomic constitution.

Taxon	Provenance [a] and collection number [b]	2n	Genomic constitution [c]	DAPI Pattern [d]	rDNA loci [e]	
					18-26S rDNA	5S rDNA
Sect. *Rhizomatosae* Krapov. & W.C. Greg.						
A. glabrata Benth var. *glabrata*	Argentina, Prov. Misiones, Dept. Candelaria. SeLaSo 2842	40	$E_2E_2E_2E_2$*	Type 2	2 (10,10)	2 (3, 3)
A. glabrata Benth. var. *hagenbeckii* (Harms ex Kuntze) F. J. Herm.	Paraguay, Dept. Paraguari, 2 km N from Caapucú. KGPSc 30107	40	$E_2E_2E_2E_2$*	Type 2	2 (10,10)	2 (3, 3)
A. burkartii Handro	Argentina, Prov. Corrientes, Dept. Monte Caseros. SeSo 2872	20	RR	Type 5	4 (3+, 4, 10^)	1 (3)
Sect. *Erectoides* Krapov. & W.C. Greg.						
A. hermannii Krapov. & W.C. Greg.	Brazil, St. MS, Mun. Coxim. VRGeSv 7560	20	E_2E_2	Type 2	1 (10)	1 (3)
A. major Krapov. & W. C. Gregory	Brazil, St. MS, Mun. Rio Negro. VPoBi 9468	20	E_2E_2	Type 2	1 (10)	1 (3)
A. paraguariensis subsp. *capibarensis* Krapov. & W.C. Greg.	Brazil, St. MS, Mun. Porto Murtinho. HLKHe 565/566	20	E_2E_2	Type 2	1 (10)	1 (3)
Sect. *Procumbentes* Krapov. & W.C. Greg.						
A. appressipila Krapov. & W.C. Greg.	Brazil, St. MS, Corumbá. GKP 9993	20	E_3E_3	Type 2	2 (9, 10)	1 (3)
A. lignosa (Chodat & Hassl.) Krapov. & W.C. Greg.	Brazil, St. MS, Porto Murtinho. VRcSgSv 13570	20	E_3E_3	Type 2	1 (10)	1 (3)
A. rigonii Krapov. & W.C. Greg.	Bolivia, Dept. Santa Cruz, Santa Cruz de la Sierra. GKP 10034	20	E_3E_3	Type 2	1 (10)	1 (3)
Sect. *Arachis*						
A. duranensis Krapov. & W.C. Greg.	Argentina, Prov. Salta, Dept. San Martín, Campo Durán. K 7988	20	AA	Type 1	2 (2, 10)	1 (3)
A. batizocoi Krapov. & W.C. Greg.	Bolivia, Dept. Santa Cruz, Prov. Cordillera, Paja Colorada. KGBPScS 30079	20	KK	Type 1	2 (4, 10)	3 (3, 8, 10)
Sect. *Heteranthae* Krapov. & W.C. Greg.						
A. pusilla Benth.	Brazil, St. MG, Mun. Januária. VFaPzSv 13107	20	AmAm	Type 5	3 (2, 3, 10+)	1 (10)

[a] *NR* National route, *PR* Provincial route, *BR* Brazilian route, *Prov* province, *St* State, *Dept* department, *Mun* Municipality. [b] Collectors: B, D.J. Banks; Bi, L. Bianchetti; Fa, L. Faraco de Freitas; 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; P, J.R. Pietrarelli; Po, A. Pott; Pz, E. Pizarro, R, V.R. Rao; Rc, R.C. Oliveira; S, C.E. Simpson; Sc, A. Schinini; Se, J.G. Seijo; Sg, A.K. Singh; So, V. Solís Neffa; Sv, G.P. Silva; V, J.F.M. Valls. [c] genome designation following Smartt & Stalker (1982), Robledo & Seijo (2010), Ortiz et al. (2017). E_2 and E_3 are subgenomes. * Genome constitution proposed in this work. [d] Patterns of DAPI heterochromatin distribution following Silvestri et al. (2020). [e] Data are extracted from Robledo et al. (2009), Robledo & Seijo (2010), Ortiz et al. (2017) and Silvestri et al. (2020). Data correspond to the haploid complement. Numbers in parentheses are designations of the chromosome pairs bearing ribosomal sites. ^two 18-26S rDNA sites, +co-localization of 5S rDNA and 18-26S rDNA sites, underlined number indicates chromosome pair without DAPI bands.

DNA amplification, sequencing and analysis

ITS1, ITS2, 5.8S of nuclear rDNA and the two chloroplast *trnT-S* and *trnT-Y* sequences from the *Arachis* species analyzed were amplified via polymerase chain reaction (PCR) by using the primers and PCR profiles listed in Table II. The ITS sequences from eight species and the cpDNA sequences from two species used here were downloaded from GenBank (http://www.ncbi.nlm.nih.gov). The remaining ITS and cpDNA sequences were amplified in a 25 µl reaction mixture containing 100 ng template DNA, 1× reaction buffer, 0.5 µM of each primer, 0.2 mM of each dNTP, and 1U GoTaq DNA polymerase (Promega). The PCR products were detected on 1.4% agarose gels and submitted for sequencing to Macrogen Inc. (Seoul, Korea). The basic information about these sequences, including the GenBank identification numbers, is listed in Table SI (Supplementary Material).

The sequences from chloroplast and nuclear markers were analyzed and edited using the Chromas 2.6.6 software (http://technelysium.com.au/wp/wp/chromas/), aligned with ClustalW, and refined manually using the software package MEGA v. 7 (Kumar et al. 2016). Species relationships were analyzed using the unweighted pair group method with arithmetic mean (UPGMA) (Sokal & Michener 1958) to estimate the genetic distances among *Arachis* species with the MEGA v.7 software. These analyses were performed using evolutionary distances calculated by the composite maximum likelihood method (Sneath & Sokal 1973, Tamura et al. 2004). Our objective was not to conduct a phylogenetic analysis but to identify the diploid species with the lowest genetic distance with *A. glabrata*, and thus to reduce the number of possible different combinations of double GISH experiments. The two cpDNA regions were analyzed together in the same sequence matrix because we had no reason to suspect incongruences among different regions of a uniparentally inherited, non-recombining genome. However, the plastid and nuclear sequences were analyzed separately.

GISH experiments

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 & Krapovickas 1994),

Table II. The primers and PCR condition for the three regions analyzed.

Region	Name of primers	Sequence of primers (5”-3”)	Profiles	Source
ITS1-5.8S-ITS2	ITS5m ITS4	GGAAGGAGAAGTCGTAACAAGG TCCTCCGCTTATTGATATGC	1 cycle: 2 min 94°C; 35 cycles: 60s 94°C, 60s 55 °C, 60s 72°C; 1 cycle: 10min 72 °C.	White et al. (1990)
trnT–S	$trnT^{UGU}R$ $trnS^{GGA}$	AGGTTAGAGCATCGCATTTG TACCGAGGGTTCGAATCCCTC T	1 cycle: 5 min 94°C; 35 cycles: 55s 94°C, 25s 56 °C, 25s 72°C; 1 cycle: 10min 72 °C.	Shaw et al. (2005)
trnT–Y	$trnT^{GGU}$ $trnY^{GUA}$	CTACCACTGAGTTAAAAGGG CCGAGCTGGATTTGAACCA	1 cycle: 5 min 94°C; 35 cycles: 55s 94°C, 25s 56°C, 25s 72°C; 1 cycle: 10min 72 °C.	Shaw et al. (2005)

and fixed and stored in absolute ethanol:glacial acetic acid (3:1) at -20 °C. Root apices were digested in 1 % (w/v) cellulase (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.

Design of the GISH experiments

To analyze the degree of genomic homology between *A. glabrata* and diploid species with different genomes/subgenomes (A, E_2, E_3, K and R), single and double GISH experiments were performed. Firstly, single GISH experiments were designed to determine which of the diploid species shared the greatest genomic homology with *A. glabrata* and thus reduce the number of possible combinations of double GISH experiments. For this purpose, gDNA of each *A. glabrata* variety was used as probe and hybridized to chromosomal preparations of the ten diploid species here assayed (Table I). Chromosomal preparations of the genetically distant *A. pusilla* (sect. *Heteranthae*, Am genome) were also included as a negative control of hybridization. Subsequently, the hybridization patterns of at least six metaphases from different individuals of each taxon were compared according to the distribution (from few localized dots to uniformly dispersed), location (proximal, interstitial or distal) and intensity (weak to strong) of the hybridization signals on the chromosomes, and different degrees of genomic hybridization were determined.

Based on the results of single GISH experiments and DNA sequence similarity, the species that showed the most uniform and brightest hybridization patterns and lowest genetic distance were selected to develop genomic probes for double GISH experiments. Also, the karyotype landmarks of the diploid species compared to those in the tetraploid (Ortiz et al. 2017) were considered. *Arachis duranensis* Krapov. & W.C. Greg. was included as a representative of the A genome despite its moderate genomic affinity revealed in the single GISH experiments, because it showed the lowest genetic distance in the cpDNA sequence analysis.

Double GISH experiments were performed onto the chromosomes of both varieties of *A. glabrata* and included two differentially labeled gDNA probes corresponding to diploid species with different genome/subgenomes (A and E_2, A and E_3, E_2 and E_3) and Salmon sperm DNA to block highly repetitive sequences.

Probe labeling and GISH

The gDNAs of the species selected were labeled with digoxigenin-11-dUTP (Roche) or biotin-16-dUTP (Roche) by nick translation. To reduce experimental artifacts due to poor labeling, firstly, each gDNA probe was checked by dot blot. Probes were fixed onto a H+ nitrocellulose membrane and then checked to produce similar colorimetric signal after detection with antibodies conjugated with alkaline phosphate and NBT/BCIP (Moscone et al. 1996) at different concentrations. Only those that produced similar colorimetric signal in the dilution series were used for the GISH experiments. Probes that passed the dot blot experiment were hybridized onto the chromosomes of the same species as a second control. The pretreatment of slides, chromosome and probe denaturation, conditions for the *in situ* hybridization (hybridization mixes containing gDNA probes at a concentration of 2.5-3.5 ng/µl and unlabeled

sonicated DNA of Salmon sperm as blocking agent), post-hybridization washing, blocking, and indirect detection with fluorochrome-conjugated antibodies were performed according to Moscone et al. (1996) and Seijo et al. (2007). Briefly, 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-rodamine isothiocyanate (TRITC) produced in rabbit and anti-mouse conjugated to FITC produced in sheep (all from Sigma-Aldrich). The hybridization mixture and the first post-hybridization wash contained 60% formamide in 2×SSC (saline sodium citrate buffer) at 37 °C, which resulted in a stringency that allowed sequences with 80-85% identity to remain hybridized. Finally, the preparations were counterstained and mounted with Vectashield medium (Vector Laboratories) containing 2 mg/ml of 4,6-diamino-2-phenyl-indole (DAPI, Sigma-Aldrich). Counterstaining with DAPI revealed a C-banding-like pattern, with major heterochromatic bands fluorescing more intensely (cf. Seijo et al. 2004).

Fluorescence microscopy and image acquisition

Chromosomes were viewed and photographed with a Leica (Heerbrugg, Switzerland) DMLB fluorescence microscope equipped with a computer-assisted Leica DC 250 digital camera system. Red, green, and blue images were captured in black and white using appropriate filters for TRITC, FITC, and DAPI excitation, respectively. Digital images were combined, and then the color balance, brightness, and contrast were processed uniformly across the image.

RESULTS

Single GISH experiments using *A. glabrata* gDNA as probe on the chromosomes of different diploid *Arachis* species

As a first approach to gain insights into the genomic relationships between the gDNA of *A. glabrata* and diploid *Arachis* species, single GISH analyses were performed. The experiments consisted in hybridizing labeled gDNA from both varieties of *A. glabrata* onto the chromosomes of the diploid species selected for this study. A total of 20 GISH experiments were performed (two probes × 10 species). The experiments showed various types of localized and well-defined signals (including interstitial and proximal regions and rDNA loci) and dispersed hybridization along whole chromosomes. Similar GISH patterns were obtained using the gDNA probes of both varieties of *A. glabrata* onto the metaphases of the diploid species. Representative somatic metaphases of the 10 diploid *Arachis* species probed with *A. glabrata* var. *glabrata* gDNA (red fluorescence) are shown in Figures 1 and 2. A summary of the hybridization patterns is shown in Table III.

The single GISH experiments on the *A. pusilla* chromosomes (Am genome) using *A. glabrata* gDNA probes revealed only six clear hybridization signals (Figures 1a and 1b) located at the 18-26S rDNA loci described for the species (Silvestri et al. 2020). Similarly, the hybridization onto the metaphases of *A. burkartii* (R genome) revealed two strong signals corresponding to the larger 18-26S rDNA loci of the satellite chromosomes. Additionally, very weak and dispersed hybridization was observed mostly in the proximal and interstitial regions of this rhizomatous diploid species (Figures 1c and 1d).

Within the species of the section *Arachis*, weak hybridization was observed onto the chromosomes of *A. batizocoi* Krapov. & W.C. Greg.

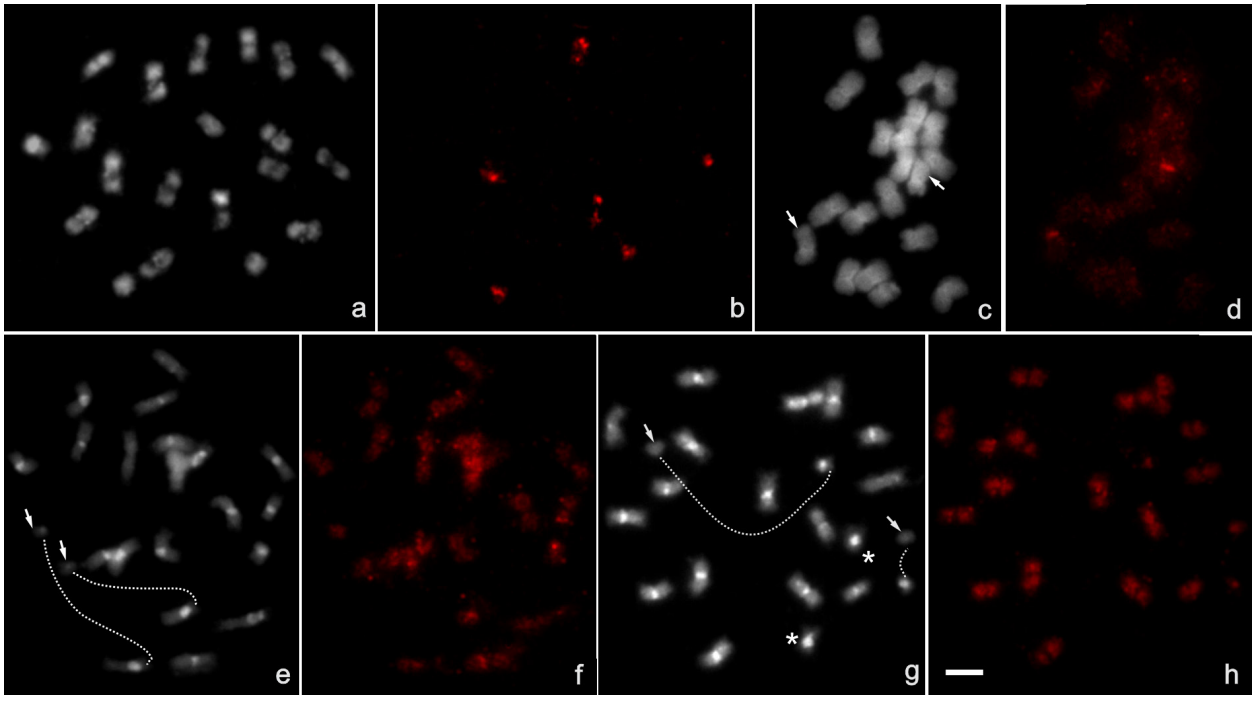


Figure 1. Representative somatic metaphases of diploid *Arachis* species of the sections *Heteranthae*, *Rhizomatosae* and *Arachis* after single GISH experiments using gDNA probe from *A. glabrata* (red) and DAPI staining (gray): (a, b) *A. pusilla*, (c, d) *A. burkartii*, (e, f) *A. batizocoi*, (g, h) *A. duranensis*. The white arrows point to the satellites, which are attached with dotted lines to the proximal regions of their respective chromosomal arms. Pair A9 of *A. duranensis* is indicated by asterisks in Figure 1g. Scale bar = 3 µm.

(K genome), except for the heterochromatic pericentromeric regions, some distal regions and the short arms of the *sm* K9 pair (Figures 1e and 1f). Moderate and uniform signals along the proximal and interstitial regions of 18 chromosomes of *A. duranensis* (A genome) were detected. The A9 pair showed very weak hybridization (Figures 1g and 1h).

All the species of the sections *Erectoides* (E_2 subgenome) and *Procumbentes* (E_3 subgenome) showed a uniform hybridization pattern along all the chromosomes (Figure 2). Among them, *A. lignosa* (Chodat & Hassl.) Krapov. & W.C. Greg. showed hybridization with moderate intensity (Figures 2a and 2b). The most uniform and intense hybridization pattern on the entire length of the chromosomes (except on the heterochromatin of centromeric regions) was observed in *A. rigonii* Krapov. & W.C. Greg. and *A. appressipila* Krapov. & W.C. Greg. of the E_3 subgenome and in *A. hermannii* Krapov. & W.C. Greg., *A. major* Krapov. & W.C. Greg. and *A. paraguariensis* subsp. *capibarensis* Krapov. & W.C. Greg. of the E_2 subgenome (Figures 2c-l).

Identification of diploid species for double GISH experiments by nuclear and plastid DNA sequence data

The amplification of the ITS sequence data produced a fragment of 587 bp in length. Sixty-nine variable sites (11.75%) were detected after alignment of the sequences isolated from 12 taxa. The UPGMA dendrogram (Figure 3a) showed that *A. lignosa* (E_3 subgenome), *A. rigonii* (E_3 subgenome) and *A. paraguariensis* subsp. *capibarensis* (E_2 subgenome) were more closely related to the two *A. glabrata* varieties and that almost all the other species, which

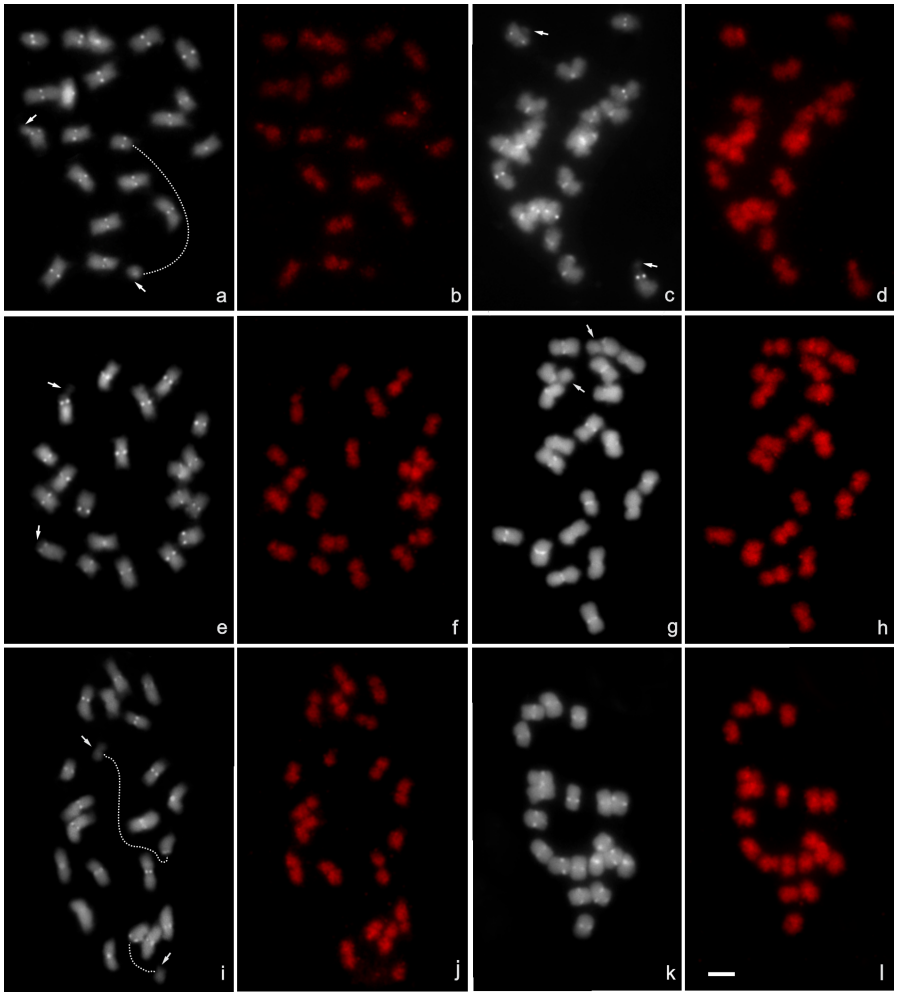


Figure 2. Representative somatic metaphases of diploid *Arachis* species of the sections *Procumbentes* and *Erectoides* after single GISH experiments using gDNA probe from *A. glabrata* (red) and DAPI staining (gray): (a, b) *A. lignosa*, (c, d) *A. rigonii*, (e, f) *A. appressipila*, (g, h) *A. hermannii*, (i, j) *A. major*, (k, l) *A. paraguariensis* subsp. *capibarensis*. The white arrows point to the satellites, which are attached with dotted lines to the proximal regions of their respective chromosomal arms. Scale bar = 3 µm.

were depicted as single branches in the tree, were more distantly related. Both chloroplast regions, trnT–S (1023 bp) and trnT–Y (948 bp), were concatenated into a single alignment for the 12 taxa studied. The aligned sequences contained 1971 characters, with 47 variable sites (2.38%). The varieties of *A. glabrata* differed in one single nucleotide substitution. The UPGMA dendrogram (Figure 3b) showed *A. pusilla* in a distant individual branch. The other species grouped into two major clusters. The first one included the two varieties of *A. glabrata* closely associated with *A. duranensis* (A genome), and *A. batizocoi* (K genome), and with *A. burkartii* (R genome), *A. major* (E_2 subgenome), and *A. hermannii* (E_2 subgenome) associated more distantly. The second cluster included *A. paraguariensis* subsp. *capibarensis* (E_2 subgenome) and the three remaining species of the section *Procumbentes* (E_3 subgenome).

Double GISH experiments using gDNA from diploid species as probes on *A. glabrata* chromosomes

The results of the single GISH experiments suggested that three species of the section *Erectoides* (*A. hermannii*, *A. major* and *A. paraguariensis* subsp. *capibarensis*, all with E_2 subgenome), two species of the section *Procumbentes* (*A. rigonii* and *A. appressipila*, with E_3 subgenome), and the more distantly *A. duranensis* (section *Arachis*, with A genome)

Table III. Summary of the semiquantitative hybridization patterns after single GISH experiments using *A. glabrata* gDNA as probes onto the chromosomes of different diploid *Arachis* species.

Chromosome preparations	Genome	*A. glabrata* gDNA probe			Figures
		Only 18-26S rDNA *loci*	Mostly proximal and interstitial regions*	Dispersed onto all chromosomes*	
Sect. ***Heteranthae***					
A. pusilla	Am	6 sites	-	-	1a-b
Sect. ***Rhizomatosae***					
A. burkartii	R	2 sites	Very weak	-	1c-d
Sect***. Arachis***					
A. batizocoi	K	-	Weak (except *p*K09)	-	1e-f
A. duranensis	A	-	Moderate (except A10)	-	1g-h
Sect. ***Procumbentes***					
A. lignosa	E_3	-	-	Moderate	2a-b
A. rigonii	E_3	-	-	Strong	2c-d
A. appressipila	E_3	-	-	Strong	2e-f
Sect. ***Erectoides***					
A. hermannii	E_2	-	-	Strong	2g-h
A. major	E_2	-	-	Strong	2i-j
A. paraguariensis subsp. *capibarensis*	E_2	-	-	Strong	2k-l

***Different degrees of hybridization are indicated as weak, moderate and strong according to the brightness intensity observed. Lack of or very low hybridization signals are indicated by a dash (-). In all cases the pericentromeric DAPI + bands did not show hybridization under the experimental conditions used here. *p*: short arm.**

were the diploid species with the highest degree of genomic affinity with *A. glabrata*. These diploids constituted the initial set of candidate species for further double GISH experiments to investigate the genomic constitution of the two taxonomic varieties of *A. glabrata*.

The selection of diploid species for double GISH was refined considering the species having the most intense and uniform hybridization patterns in the single GISH experiments (Table III) and the highest genetic similarity with *A. glabrata* in the cluster analyses of the plastid (*A. duranensis*) and nuclear (*A. paraguariensis*, *A. rigonii*) DNA sequences (Figure 3). Additionally, the diploid *A. paraguariensis* subsp. *capibarensis* and *A. rigonii* were selected because they share a larger number of chromosomal markers with *A. glabrata* than the other species of the sections *Erectoides* and *Procumbentes* (Ortiz et al. 2017, Table I and Figure 4).

Differently labeled probes of gDNA were used in three combinations - *A. duranensis* with *A. rigonii*, *A. duranensis* with *A. paraguariensis* subsp. *capibarensis*, and *A. paraguariensis* subsp. *capibarensis* with *A. rigonii* - for double GISH experiments. Hybridizations of all the probes assayed were similarly dispersed in all the 40 chromosomes, and no difference of intensity was observed among the four chromosome sets (Figure 5 and Supplementary Material – Figure S1) of any of the two varieties of *A. glabrata*. Representative somatic metaphases of *A. glabrata* var. *glabrata* probed with gDNA of

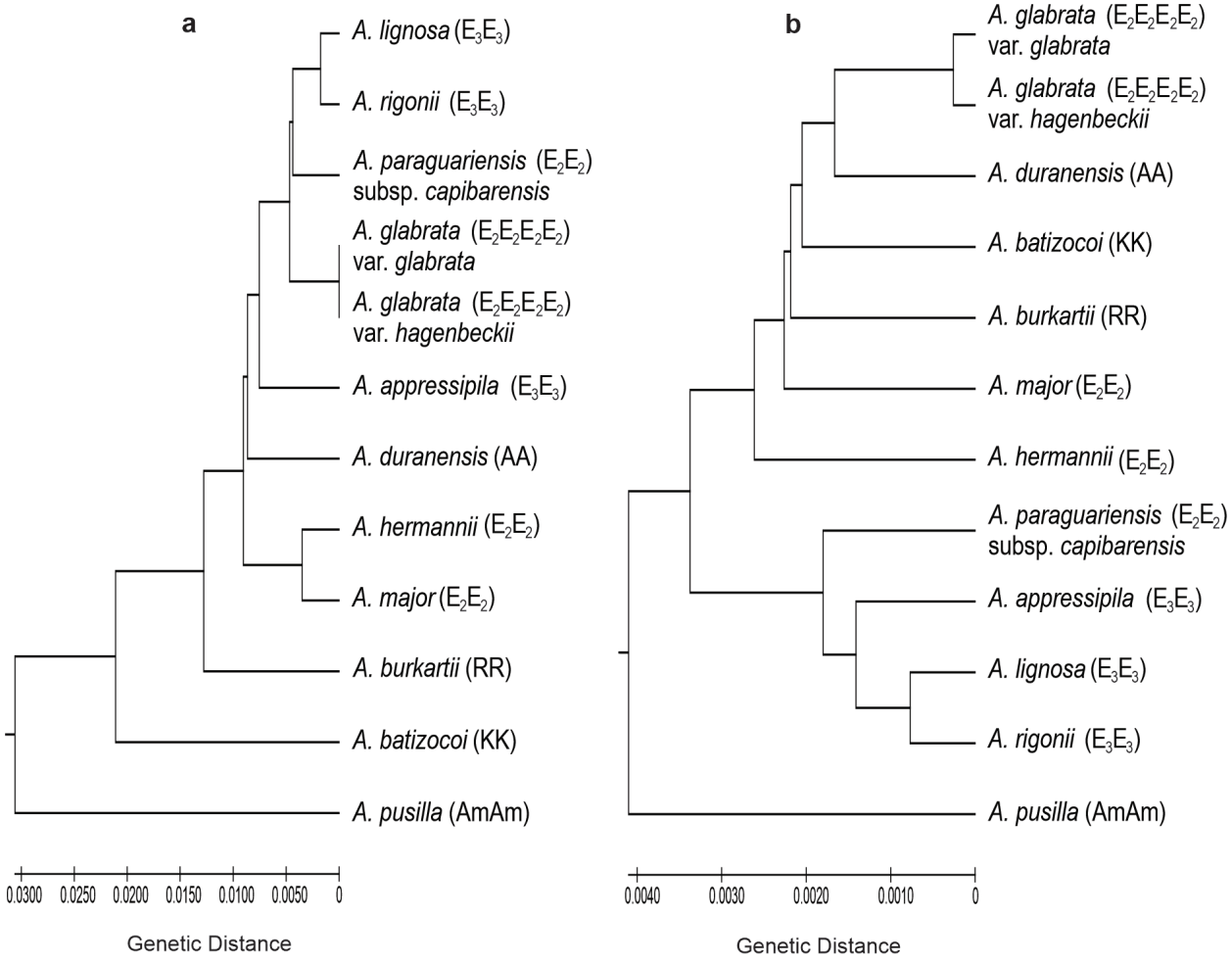
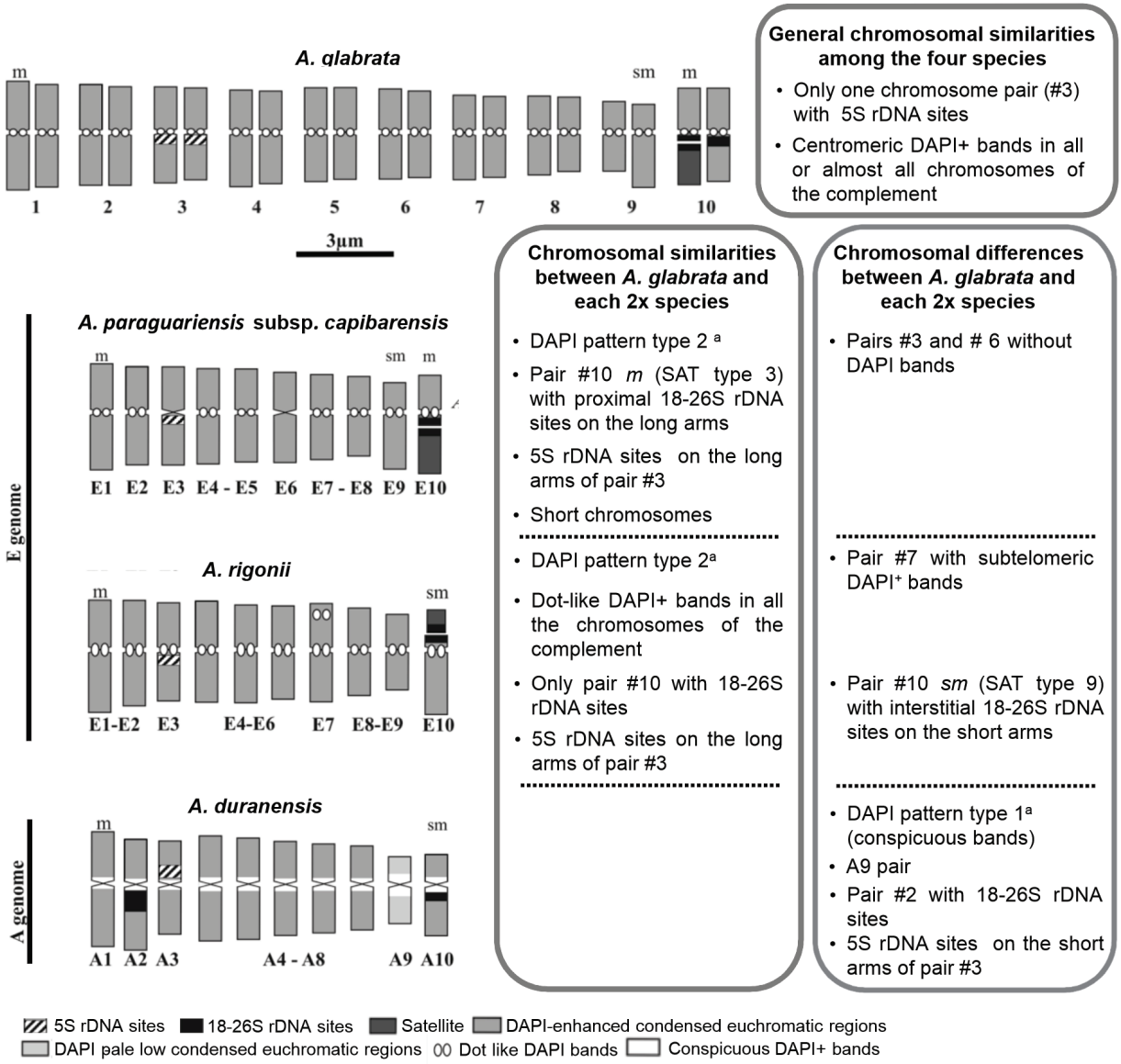


Figure 3. UPGMA dendrograms of *Arachis* species based on the sequences of ITS (a) and two non-coding regions (*trnT–S*, *trnT–Y*) of the cpDNA (b) datasets. The genomic constitution of each taxa is indicated in parentheses.

the three combinations of diploid species are shown in Figure 5.

Although the probes of the different species assayed showed similar distribution of the hybridization dots along the chromosomes (except for their centromeric DAPI heterochromatic bands), differences in the intensity of brightness were observed among the probes. In the two cases, when gDNA of *A. duranensis* (A genome) was simultaneously hybridized with gDNA of *A. rigonii* (E_3 subgenome) and *A. paraguariensis* subsp. *capibarensis* (E_2 subgenome), the gDNA of the former (in green, Supplementary Material – Figures S1d and S1e) hybridized less intensely and more sparsely than that of the E genome species (in red, Supplementary Material – Figures S1g and S1h). As a result, in the merged picture, the chromosomes of *A. glabrata* were observed as intense orange (Figures 5d and 5e). The only exceptions to this pattern were the interstitial regions, where all the probes showed intense hybridization. These regions appeared as more yellowish regions in the merged metaphases of *A. glabrata* (Figures 5d and 5e).

The hybridizations performed with probes of *A. paraguariensis* subsp. *capibarensis* (E_2 subgenome) and *A. rigonii* (E_3 subgenome) resulted in intense labeling of both probes onto the 40 chromosomes, except for their centromeric



Idiograms were redrawn from Robledo and Seijo (2010) and Ortiz et al. 2017.

[a] Patterns of DAPI heterochromatin distribution following Silvestri et al. 2020. 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 are observed as centromeric blocks of similar size in all or almost all the chromosomes, except pairs #9 and #10, which have longer bands. Pattern 2 corresponds to a generalized and homogeneous distribution of tiny bands, which are observed as centromeric blocks in the form of "dots".

Figure 4. Scheme showing the idiograms and the main chromosomal similarities and differences between *A. glabrata* and the three diploid species tested in the double GISH experiments.

DAPI heterochromatic bands (Supplementary Material – Figure S1). However, the former probe (green fluorescence) hybridized more uniformly and with more intensity than the latter (in red, Supplementary Material – Figure S1), which was concentrated mainly in the interstitial regions (Supplementary Material – Figure S1). As a result of the merging of the hybridization with both probes, the metaphase chromosomes of *A. glabrata* were seen as yellow-green chromosomes (Figure 5f).

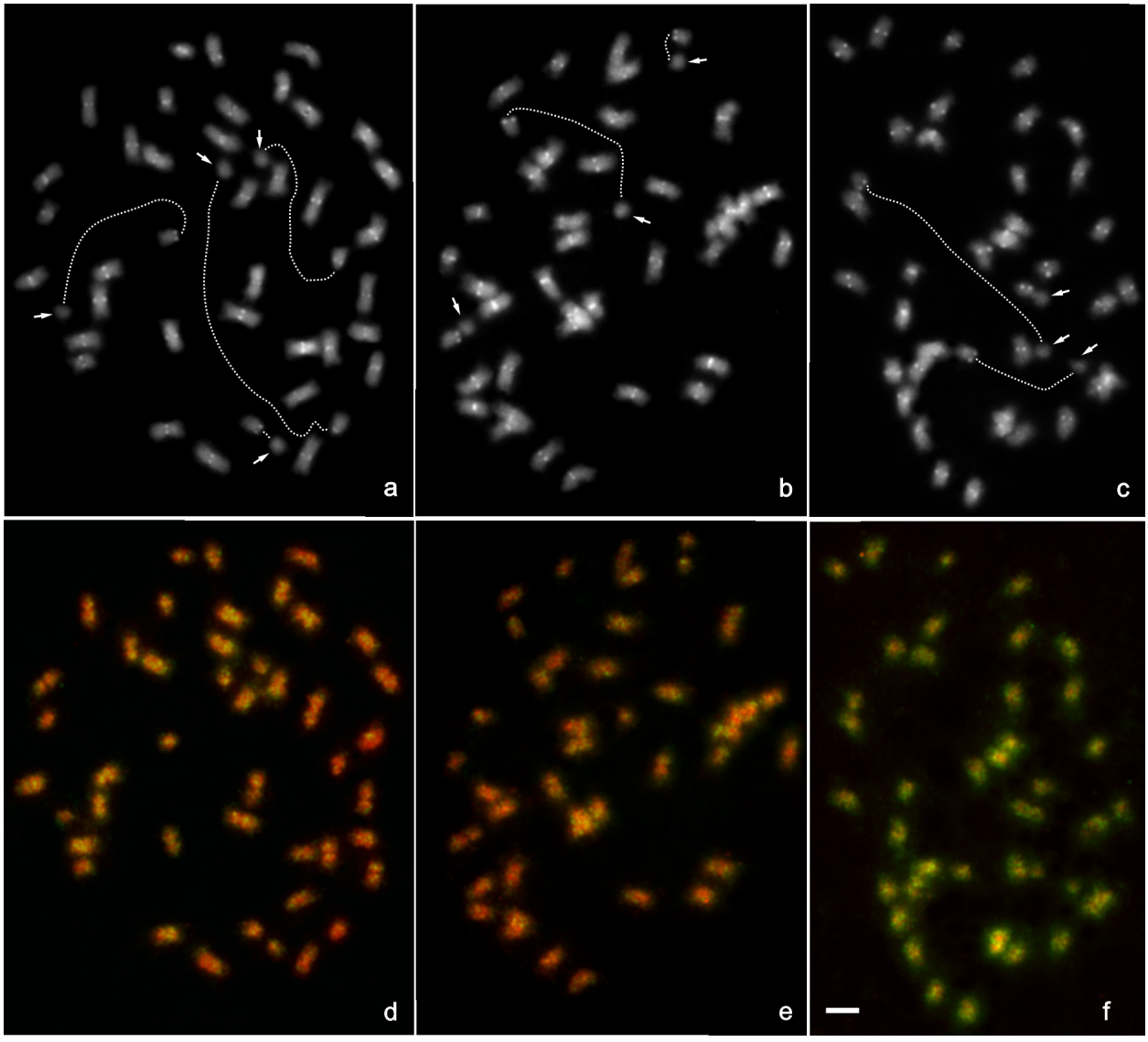


Figure 5. Representative somatic metaphases of tetraploid *A. glabrata* after double GISH. DAPI staining (gray) was used to highlight the heterochromatic bands and to stain euchromatin in Figs. a, b, and c after double GISH using gDNA probes of d: *A. duranensis* (green) and *A. rigonii* (red), e: *A. duranensis* (green) and *A. paraguariensis* subsp. *capibarensis* (red), f: *A. paraguariensis* subsp. *capibarensis* (green) and *A. rigonii* (red). The white arrows point to the satellites, which are attached with dotted lines to the proximal regions of their respective chromosomal arms. Scale bar = 3 µm.

DISCUSSION

Morphological and taxonomic studies have placed all the rhizomatous *Arachis* species (three tetraploids and one diploid) within the taxonomic section *Rhizomatosae* (Krapovickas & Gregory 1994). However, the relationships between the diploid and tetraploid species have been called into question (Angelici et al. 2008, Bechara et al. 2010, Ortiz et al. 2017). Here, we present evidence that the diploid *A. burkartii* is not a parental of the tetraploid *A. glabrata* and that the most probable ancestors of this polyploid legume forage are species with the E_2 subgenome.

Arachis burkartii is not a genome donor of *A. glabrata*

The single GISH experiments using *A. glabrata* gDNA (without specific blocking DNA of any *Arachis* species) onto the chromosomes of *A. burkartii* clearly evidenced that the genomic sequences of the complements of these two species are largely different. The patterns of hybridization observed between these species of the section *Rhizomatosae* showed lower similarities than those detected among different genomes of the section *Arachis* (Seijo et al. 2007), suggesting that the genome of *A. burkartii* (R genome, Ortiz et al. 2017) is different from that of *A. glabrata*. Thus, the results also suggest that the taxonomic section as defined by Krapovickas & Gregory (1994) is not a natural group.

The fact that *A. burkartii* was distantly positioned from *A. glabrata* in the cpDNA and ITS rDNA dendrograms constructed here, as also observed in previous molecular analyses (Gimenes et al. 2002, Nobile et al. 2004, Angelici et al. 2008, Bechara et al. 2010, Friend et al. 2010), show that *A. burkartii* is very distant from both *A. glabrata* varieties. The single GISH experiments clearly demonstrated a very low global sequence similarity between *A. burkartii* and *A. glabrata*. The karyotypes of these two species also differ, mainly in the number and position of the rDNA and in the pattern of heterochromatic bands (Ortiz et al. 2017). All these results clearly demonstrate that *A. burkartii* is not a genome donor of *A. glabrata*.

Differences similar to those found between *A. burkartii* and *A. glabrata* were considered for the arrangement of species of the section *Arachis* in different genomes (Robledo & Seijo 2010). The fact that the hybridization pattern of *A. glabrata* gDNA onto the metaphase of *A. burkartii* was similar to that observed onto the chromosomes of the distant *A. pusilla* (Am genome) supports the existence of a large genomic differentiation between *A. glabrata* and *A. burkartii*. This genome and karyotype differentiation may explain the genetic isolation between these two species, as noted by the lack of success of numerous crossing attempts under experimental (Gregory & Gregory 1979, Krapovickas & Gregory 1994) conditions. Therefore, our results support the assignment of the R genome (Ortiz et al. 2017) only for *A. burkartii*, and not to the tetraploid *A. glabrata*.-

Identification of alternative candidates as diploid genome donors of *A. glabrata*

After discarding *A. burkartii* as a genome donor of *A. glabrata*, the second aim of this study was to identify diploid species with sequence affinity to the chromosomes of this tetraploid species. Among them, *A. batizocoi* (K genome) was evaluated because artificial hybrids have been obtained between this species and *A. glabrata* (Krapovickas & Gregory 1994). However, single GISH assays revealed that *A. glabrata* gDNA only produced dispersed and very tiny signals along the metaphase chromosomes of *A. batizocoi*, evidencing a low genomic affinity between these species. This low hybridization was somewhat expected because this species presents large karyotypic differences (Robledo & Seijo 2010, Ortiz et al. 2017) with *A. glabrata*, and the hybrids obtained have less than 1% of stained pollen (Gregory & Gregory 1979). The cytogenetic results are also in complete agreement with the distance dendrograms based on cpDNA and ITS rDNA here presented and with previous phylogenies based on DNA sequences (Bechara et al. 2010, Friend et al. 2010). Thus, although *A. batizocoi* presents certain degree of cross-compatibility with *A. glabrata*, our results do not support this diploid species as a genome donor of *A. glabrata*.

All the other diploid species here evaluated as potential genome donors having the A, E_2 and

E_3 genomes/subgenomes were better candidates as progenitors of *A. glabrata* than the two species formerly analyzed. In all these diploids, the gDNA probe of *A. glabrata* hybridized along the euchromatic portion of all (or most) of their chromosomes. The absence of hybridization in the heterochromatic DAPI$^+$ regions is an expected result in GISH experiments of *Arachis* species. This phenomenon could be attributed to the high condensation of heterochromatin that inhibits the accessibility of the probe to the target sequences under the hybridization conditions usually used for GISH (Seijo et al. 2007) and/or to the lack of homology of the satellite elements that compose the heterochromatic bands in different species (Samoluk et al. 2019).

Among these potential genome donors, the single GISH experiments using gDNA of *A. glabrata* as probe showed that *A. duranensis* (A genome) was the one that presented the lowest intensity of the hybridization signals. This showed that the genomes differ largely in the nuclear sequences, mainly in the repetitive ones. In addition, although *A. duranensis* was the most genetically similar species at the level of cpDNA sequences analyzed here, the ITS rDNA dendrogram showed a great genetic distance between them. These results eliminate *A. duranensis* as a potential genome donor of *A. glabrata*.

The species of the sections *Procumbentes* and *Erectoides*, despite being distantly related in the cpDNA cluster analysis, showed the highest genome affinities with *A. glabrata* in the ITS rDNA dendrogram constructed. This is in agreement with previous studies in which they also appeared very closely related in analyses based on RAPD markers (Dos Santos et. al. 2003, Nóbile et al. 2004). Consistently with the ITS rDNA dendrogram, the *Procumbentes* and *Erectoides* species showed the highest intensity of hybridization in GISH experiments with *A. glabrata*, although with different degrees of sequence homology. Among them, *A. lignosa* (E_3 subgenome) showed the weakest hybridization, which evidenced a large genomic differentiation with *A. glabrata*. The most uniform and intense hybridization observed in all the other diploid species tested in single GISH experiments showed a higher degree of homology between the genome of any of these diploids and that of *A. glabrata*.

The analysis of mitotic metaphases showed that the chromosomes of the species belonging to the section *Procumbentes* 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 *A. glabrata* (Ortiz et al. 2017). By contrast, the species of the section *Erectoides* have the most similar pattern of chromosome markers expected for the genome donor of *A. glabrata* (Ortiz et al. 2017, Table I). Among them, *A. paraguariensis* subsp. *capibarensis* is the only taxa that has SAT chromosomes type 3 and one pair of 18-26S rDNA sites like those observed in *A. glabrata*. The other subspecies, *A. paraguariensis* subsp. *paraguariensis* was previously discarded as a genome donor of *A. glabrata* mainly because of having five pairs of 18-26S rDNA sites and SAT chromosomes type 4 (Ortiz et al. 2017). Thus, the results of the single and double GISH analyses, together with the chromosome and ITS rDNA sequence data, support the *Erectoides* species and, particularly *A. paraguariensis* subsp. *capibarensis*, as the best candidate as genome donor of *A. glabrata*.

Genomic constitution of *A. glabrata* evaluated by double GISH

To gain further insights into the genome constitution of *A. glabrata* and its genome donor/s, double GISH experiments were performed hybridizing gDNA of the most

probable candidate diploid species onto the chromosomes of *A. glabrata*. This technique has been formerly very useful for genome discrimination of the AABB allopolyploids *A. hypogaea* and *A. monticola* (Seijo et al. 2007, 2018), as well as in polyploids of other plant genera (Yang et al. 2017, Marques et al. 2018, Wang et al. 2019). Here, to perform the double GISH experiments, we selected two of the diploid species (*A. rigonii* and *A. paraguariensis*) that had shown the most brightful and uniform hybridization signals with *A. glabrata* gDNA in single GISH experiments and the lowest genetic distance in ITS rDNA and cpDNA analyses. *Arachis duranensis* was used as a negative control of low but uniform hybridization onto the chromosomes.

The double GISH experiments revealed a similar pattern of hybridization in all the chromosomes of both *A. glabrata* varieties despite the gDNA of diploid species used as probes. The probe derived from *A. duranensis* showed a similar low affinity in the four complements of the tetraploid regardless of whether it was co-hybridized with gDNA of species with the E_2 or E_3 subgenomes. Similarly, the gDNA of the E_2 and E_3 subgenomes species hybridized homogeneously and with high intensity in the four complements of *A. glabrata* despite the gDNA used for the co-hybridizations. These results are compatible with the patterns expected for autopolyploids like *Avena* (Leggett & Markhand 1995) or allopolyploids derived from species with very similar genomes like *Miscanthus* (Hodkinson et al. 2002).

Different reports have proposed *A. glabrata* as an autopolyploid species (Singh 1985, Singh & Simpson 1994). Meiotic analysis in different accessions of *A. glabrata* has shown 0-8 quadrivalents and, on this base, we have previously postulated that different populations may have varied degrees of diploidization (Ortiz et al. 2011). Other authors, however, have postulated this species as an EERR allopolyploid (Bechara et al. 2010) originated by crossing between one species from *Erectoides* group (including the species of the sections *Trierectoides*, *Erectoides* and *Procumbentes*) and one species of the section *Rhizomatosae* with a genome constitution different from that of *A. burkartii* (Gregory & Gregory 1979, Bechara et al. 2010). However, our GISH experiments revealed a high degree of similarity in the sequence composition of the four chromosome sets of *A. glabrata*, despite the probe used in the experiments. These results support the autopolyploid origin of *A. glabrata*. Alternatively, if different diploid species had been involved in its origin, they must have had a very similar genome sequence, especially at the repetitive level and also a similar karyotype structure as that observed among the *Erectoides* species studied here (Ortiz et al. 2017). Accordingly, we suggest an $E_2E_2E_2E_2$ genome constitution for *A. glabrata*.

CONCLUSIONS

The results obtained from the GISH experiments evidence that the tetraploid *A. glabrata* is constituted by four identical or very similar chromosome complements. The similar pattern of genomic hybridization observed in both varieties of this species suggests that the same diploid species participated in their origin. These results also confirm that 1) the R genome of the diploid *A. burkartii* is not a genome donor of *A. glabrata*, 2) the species with E_2 subgenome have the highest degree of genomic homology with *A. glabrata* and the most similar karyotypes, and 3) among the species tested, *A. paraguariensis* subsp. *capibarensis* showed the highest brightness in all the double GISH experiments using DNA of diploid species as probes onto *A.*

glabrata chromosomes and also in single GISH experiments using *A. glabrata* as probe onto the chromosomes of diploid species.

Thus, our results increase the knowledge about the genomic relationships of *A. glabrata* with diploid species that showed different degrees of crossability and other species selected for this study. We envision that the data here provided enlarge the germplasm sources useful for breeders to increase the genetic variability of *A. glabrata* and for the selection of diploid parents for the genetic improvement of the existing *A. glabrata* commercial cultivars.

Acknowledgments

This work was supported by grants the Secretaría General de Ciencia y Técnica de la Universidad Nacional del Nordeste (PI No. 18P005 and PI No.16P003) and the Agencia Nacional de Promoción Científica y Tecnológica (PICT No. 2015-2802).

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SUPPLEMENTARY MATERIAL

Figure S1.
Table SI.

How to cite
ORTIZ AM, CHALUP L, SILVESTRI MC, SEIJO G & LAVIA GI. 2023. Genomic relationships of the polyploid rhizoma peanut (*Arachis glabrata* Benth.) inferred by genomic *in situ* hybridization (GISH). An Acad Bras Cienc 95: e20210162. DOI 10.1590/0001-3765202320210162.

Manuscript received on February 2, 2021;
accepted for publication on May 11, 2021

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