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# PHYSICAL MAPPING OF THE 5S AND 18S–25S rRNA GENES BY FISH AS EVIDENCE THAT *ARACHIS DURANENSIS* AND *A. IPAENSIS* ARE THE WILD DIPLOID PROGENITORS OF *A. HYPOGAEA* (*LEGUMINOSAE*)[1]

J. GUILLERMO SEIJO,[2,6] GRACIELA I. LAVIA,[3] AVELIANO FERNÁNDEZ,[3,4] ANTONIO KRAPOVICKAS,[3] DANIEL DUCASSE,[5] AND EDUARDO A. MOSCONE[2]

[2]Instituto Multidisciplinario de Biología Vegetal, Casilla de Correo 495, 5000 Córdoba, Argentina; [3]Instituto de Botánica del Nordeste, Casilla de Correo 209, 3400 Corrientes, Argentina; [4]Facultad de Ciencias Exactas y Naturales y Agrimensura, Universidad Nacional del Nordeste, Av. Libertad 5450, 3400 Corrientes, Argentina; and [5]Instituto de Fitopatología y Fisiología Vegetal, INTA, Camino a 60 Cuadras Km 5 ½, 5119 Córdoba, Argentina

The 5S and the 18S–25S rRNA genes were physically mapped by fluorescent in situ hybridization (FISH) in all botanical varieties of cultivated peanut *Arachis hypogaea* ($2n = 4x = 40$), in the wild tetraploid *A. monticola*, and in seven wild diploid species considered as putative ancestors of the tetraploids. A detailed karyotype analysis including the FISH signals and the heterochromatic bands was carried out. Molecular cytogenetic landmarks are provided for the construction of a FISH-based karyotype in *Arachis* species. The size, number, and chromosome position of FISH signals and heterochromatic bands are similar in all *A. hypogaea* varieties and *A. monticola*, but vary among the diploid species. Genome constitution of the species is discussed and several chromosome homeologies are established. The bulk of the chromosome markers mapped, together with data on geographical distribution of the taxa, suggest that peanut originated upon domestication of *A. monticola* and evidence that the diploids *A. duranensis* and *A. ipaensis* are the most probable ancestors of both tetraploid species. Allopolyploidy could have arisen by a single event or, if by multiple events, always from the same diploid species.

**Key words:** *Arachis*; FISH mapping; heterochromatin; karyotypes; *Leguminosae*; peanut origin; rRNA genes.

*Arachis hypogaea* (v.n. ''peanut,'' ''groundnut'') is a cultigen that has become the third most important grain legume crop of the world (Duke, 1981) because of its multiple use as human food, vegetable oil, feedstock, and ground cover (Wynne and Halward, 1989). This species is adapted to a wide ecological range of tropical and subtropical regions and is cultivated under diverse agricultural production systems in Asia, Africa, and the Americas (Holbrook and Isleib, 2001).

On the bases of morphological features, crossing experiments, and seed protein electrophoretic profiles, Krapovickas and Gregory (1994) recognized two subspecies within the cultigen, *hypogaea* and *fastigiata*. Additionally, six botanical varieties were described, two of them within subsp. *hypogaea* (*hypogaea* and *hirsutea*) and four within subsp. *fastigiata* (*fastigiata*, *aequatoriana*, *peruviana*, and *vulgaris*). Moreover, the numerous land races within varieties are diverse in their vegetative and reproductive traits (cf. Krapovickas and Rigoni, 1960; Grosso et al., 1994; Krapovickas and Gregory, 1994; Krapovickas et al., 2001).

Even though *A. hypogaea* has considerable morphological variation, its genetic variability is low (Kochert et al., 1991; Paik-Ro et al., 1992; Stalker and Mozingo, 2001). Great effort has been made to build germplasm collections (Krapovickas and Rigoni, 1960; Valls et al., 1985; Holbrook, 2001; Upadhyaya et al., 2001); however, the gene pool available within the species has not been adequate for plant breeders to solve major agricultural problems, such as susceptibility to various pests and diseases. Therefore, interest has been directed toward a group of closely related wild species that possess agronomically useful characters for cultigen breeding (Johnson et al., 1977; Foster et al., 1981; Singh, 1986b; Burow et al., 2001; Simpson, 2001). In this sense, many studies have been centered upon the understanding of the origin, organization, and evolution of the *A. hypogaea* genome, with particular attention to determining its possible ancestors (cf. Singh and Smartt, 1998, and references therein).

All *Arachis* species grow in South America and were taxonomically arranged into nine sections (Krapovickas and Gregory, 1994). Peanut in particular is considered an allotetraploid ($2n = 4x = 40$) with an AABB genome constitution (Smartt et al., 1978) and included in the section *Arachis* together with a wild allotetraploid entity, *A. monticola*, and 25 wild diploid species (Krapovickas and Gregory, 1994). Two diploid species belonging to the section *Arachis* are speculated to have originated *A. monticola*, which upon domestication gave rise to the cultigen *A. hypogaea* (Krapovickas and Rigoni, 1957; Krapovickas and Gregory, 1994). The close relationship between the two taxa is supported by crossing experiments (Krapovickas and Rigoni, 1954, 1957; Hammons, 1970), as well as by conventional cytogenetic (Fernández and Krapovickas, 1994) and molecular studies (Halward et al., 1991; Kochert et al., 1991). On the other hand, the issue of the actual diploid progenitors of *A. monticola* and *A. hypogaea* is still unresolved. Since the early fifties, when the first hybrid

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[6] E-mail: seijo@agr.unne.edu.ar.

between *A. hypogaea* and the diploid *A. correntina* was obtained (Krapovickas and Rigoni, 1954), several other diploid species, with either the A or the B genome, have produced hybrids with *A. hypogaea* and, thus, been proposed as putative progenitors of the tetraploids (Krapovickas and Rigoni, 1957; Raman, 1960; Smartt and Gregory, 1967; Krapovickas, 1973; Stalker and Wynne, 1979; Singh and Moss, 1984).

According to morphological similarities and interspecific cross-compatibilities, Gregory and Gregory (1976) were the first to suggest *A. duranensis* and *A. cardenasii* as possible progenitors of the cultigen, but both are currently considered to have the A genome. On the other hand, Smartt et al. (1978) advanced the possibility that *A. cardenasii* and *A. batizocoi*, with the proposed A and B genome, respectively, have many of the characters expected for the donors to *A. hypogaea*. The hypothesis that *A. duranensis* and *A. batizocoi* are the most probable ancestors of peanut was further proposed by Singh (1986b, 1988). Nevertheless, restriction fragment length polymorphism (RFLP) data suggested that *A. duranensis* and *A. ipaensis* are the closest diploid relatives of *A. hypogaea* and that *A. batizocoi* did not appear to be closely related to the cultigen (Kochert et al., 1991). This contention was supported by Fernández and Krapovickas (1994) who found that the karyotype of *A. ipaensis* lacks the ''A chromosome'' pair, which is characteristic of the A genome. Thus, *A. ipaensis* became an alternative to *A. batizocoi*, both without the ''A chromosome'' pair, as the B genome donor to *A. monticola*/*A. hypogaea*. A newer revision of the origin of the cultigen has revalidated the candidacy of *A. batizocoi* as a putative parent of *A. hypogaea* based on the ability to obtain synthetic allotetraploids (Singh and Smartt, 1998). More recently, Raina and Mukai (1999a, b) even proposed *A. villosa* as the most probable A genome donors instead of *A. duranensis* according to genome painting data and the number of rDNA loci revealed by fluorescent in situ hybridization (FISH). On the other hand, *A. trinitensis* and *A. williamsii*, with A and B genome, respectively, were also included in the list of probable ancestors of the cultigen considering their geographical distribution (Lavia, 1996).

In this context, we see first that the identity of the real progenitors of *A. hypogaea* is uncertain and second that the cultigen could have multiple origins because of the existence of two *A. hypogaea* subspecies and several diploid candidates as genome donors (cf. Singh, 1986a; Lavia, 1999). A comprehensive intraspecific study of *A. hypogaea*/*A. monticola* and the putative diploid parents could bring light to this long controversy. Physical mapping of ribosomal rRNA genes by in situ hybridization has already been a valuable tool in studies of plant genome organization, cytotaxonomy, introgression, and evolution (Zhang and Sang, 1999; Adams et al., 2000; Benabdelmouna et al., 2001) and may be useful here. In particular, chromosome homeologies could be established with detailed rDNA sequence mapping in some groups of plants (Jiang and Gill, 1994; Moscone et al., 1999). But, although species of *Arachis* are diverse in their number of 5S and 18S–25S ribosomal DNA loci (Raina and Mukai, 1999a), no precise cytogenetic mapping analysis of these sequences has been attempted for the genus.

Therefore, we used FISH to physically map the 5S and the 18S–25S rDNA sites in all botanical varieties of the cultivated peanut, in the wild tetraploid *A. monticola*, and in seven of the eight diploid species so far considered as putative ancestors of the tetraploids with the objectives of (1) proving whether *A. hypogaea* originated from a unique or multiple events of poliploidization by analyzing the *A. hypogaea* varieties, (2) determining chromosome homeologies between species, particularly between *A. hypogaea* and *A. monticola*, to gain insight into whether *A. monticola* is the tetraploid ancestor of *A. hypogaea*, (3) casting light on the actual diploid genome donors of *A. hypogaea* by comparing the rDNA loci patterns of the putative wild progenitors and the cultigen, and (4) finding molecular cytogenetic landmarks to facilitate the construction of a FISH-based map of the *Arachis* genome.

## MATERIALS AND METHODS

***Plant material***—*Arachis hypogaea* varieties and wild species of *Arachis* were obtained from the peanut germplasm collection at the INTA Manfredi in Córdoba, Argentina, and at the Instituto de Botánica del Nordeste in Corrientes, Argentina. The original procedences, voucher specimens, and life cycle of the samples studied are cited in Table 1. In the tables, species are ordered first according to their ploidy level and secondarily after their genomic constitution. The geographical distribution of the wild species as well as the center of major diversity of *A. hypogaea* subspecies *hypogaea* are represented in Fig. 1.

***Chromosome preparations***—Plants obtained from seeds were grown in pots. Collected root tips (5–10 mm long) were pretreated with 2 mmol/L 8-hydroxyquinoline for 3 h at room temperature (Fernández and Krapovickas, 1994) and then fixed in 3 : 1 absolute ethanol : glacial acetic acid for a minimum of 12 h at 4°C. Somatic chromosome spreads were prepared according to Schwarzacher et al. (1980). Root apices were macerated in 1% (m/v) cellulase Onozuka R-10 (from *Trichoderma viridae*; Serva, Heidelberg, Germany) plus 10% (v/v) pectinase dissolved in 40% glycerol (from *Aspergillus niger*, Sigma, St. Louis, Missouri, USA) in 0.01 mol/L citric acid/sodium citrate buffer, pH 4.8, at 37°C for 2 h, and then squashed in 45% acetic acid. After removal of the coverslip with $CO_2$, slides were air dried, aged for 1–2 d at room temperature, and then kept at −20°C until use.

***Probe labeling and fluorescent in situ hybridization***—The 5S rDNA and 18S–25S rDNA repeated sequences were localized using the following DNA probes: pXV1, a 349-base pair (bp) fragment of the 5S rRNA gene repeated unit from *Beta vulgaris*, including the adjacent intergenic spacer (Schmidt et al., 1994), and R2, a 6.5-kilobase (kb) fragment of the 18S–5.8S–25S rDNA repeat unit from *Arabidopsis thaliana*, including internal transcribed spacers ITS1 and ITS2 and a short 5′ segment of the intergenic region (IGR) (Wanzenböck et al., 1997). The first probe was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) and the second one with biotin-11-dUTP (Sigma), both by nick translation.

Pretreatment of preparations, chromosome and probe denaturation, conditions for the in situ hybridization (hybridization mixes contained DNA probes at a concentration of 2.5–3.5 ng/μL), post-hybridization washing, blocking, and indirect detection by fluorochrome conjugated antibodies were performed according to Moscone et al. (1996). The first set of antibodies consisted of mouse anti-biotin (Dakopatts, Dako, Carpinteria, California, USA) and sheep anti-digoxigenin conjugated to fluorescein isothiocyanate (FITC) (Boehringer Mannheim) in PBS (0.13 mol/L NaCl, 0.007 mol/L $Na_2HPO_4$, 0.003 mol/L $NaH_2PO_4$), 3% (m/v) bovine serum albumin (BSA). The second set of antibodies consisted of rabbit anti-mouse conjugated to tetramethyl-rodamine isothiocyanate (TRITC) (Dakopatts) and FITC-conjugated rabbit anti-sheep (Dakopatts) in PBS, 3% (m/v) BSA. Preparations were counterstained and mounted with Vectashield medium (Vector Laboratories, Burlingame, California, USA) containing 2 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI).

The DAPI counterstaining subsequent to FISH resulted in a C banding-like pattern with major heterochromatin bands fluorescing more intensely, thus aiding chromosome identification (cf. Moscone et al., 1996, 1999).

***Fluorescence microscopy and image acquisition***—Chromosomes were viewed and photographed with a Leica DMLB fluorescence microscope (Lei-

TABLE 1. List of the *Arachis* species studied, their provenance, and life cycle.

| Taxon | Provenance and collection number[a] | Life cycle[b] |
|---|---|---|
| *A. hypogaea* L. subsp. *hypogaea* var. *hypogaea* (race Guaycurú) | Argentina, Corrientes. FCA 27 | a |
| *A. hypogaea* subsp. *hypogaea* var. *hirsuta* Köhler (race Mitad del Mundo) | Ecuador, San Antonio. B, P, Va 732 (MDI 86-2769) | a |
| *A. hypogaea* subsp. *fastigiata* Waldron var. *fastigiata* (race Colorado) | Argentina, Corrientes. FCA 31 | a |
| *A. hypogaea* subsp. *fastigiata* var. *aequatoriana* Krapov. and W. C. Gregory (race Zaruma) | Ecuador, Quito. B, P, Z 683 (MDI 86-2535) | a |
| *A. hypogaea* subsp. *fastigiata* var. *peruviana* Krapov. and W. C. Gregory (race Tingo María) | Perú, Casma. MDI 87-2753 (PI 393641) | a |
| *A. hypogaea* subsp. *fastigiata* var. *vulgaris* C. Harz (race Blanco Manfredi) | Argentina. MDI 68-2551 | a |
| *A. monticola* Krapov. and Rigoni | Argentina, Prov. Jujuy, Dept. Capital, Yala. Se, Sn 2774 | a |
| | Argentina, Prov. Jujuy, Dept. Capital, Lozano. Se, Sn 2775 | a |
| | Argentina, Prov. Jujuy, Dept. Capital, Yala. K 30062 (PI 468196) | a |
| *A. cardenasii* Krapov. and W. C. Gregory | Bolivia, Dept. Santa Cruz, Prov. Chiquitos, 17 km S San José. K, S, Sc 36033 (PI 476012) | p |
| | Bolivia, Dept. Santa Cruz, Prov. Chiquitos, Roboré. G, K, P 10017 (PI 262141) | p |
| *A. correntina* (Burkart) Krapov. and W. C. Gregory | Argentina, Prov. Corrientes, Dept. Ituzaingó. K 7897 (PI 331192) | p |
| | Argentina, Prov. Corrientes, Dept. San Miguel, La Palmira. Se, Sn 2844 | p |
| *A. duranensis* Krapov. and W. C. Gregory | Argentina, Prov. Salta, Dept. San Martin, Campo Durán. K 7988 (PI 219823) | a |
| *A. villosa* Benth. | Argentina, Prov. Corrientes, Dept. Paso de los Libres, Laguna Mansa. Se, Sn 2866 | p |
| *A. ipaensis* Krapov. and W. C. Gregory | Bolivia, Dept. Tarija, Prov. Gran Chaco, Ipa. K, G, B, P, Sc, S 30076 (PI 468322) | a |
| *A. williamsii* Krapov. and W. C. Gregory | Bolivia, Dept. Beni, Prov. Cercado, Trinidad. W 1118 | a |
| *A. batizocoi* Krapov. and W. C. Gregory | Bolivia, Dept. Santa Cruz, Prov. Cordillera, Parapeti. K 9484 (PI 298639) | a/b |

[a] B = D. Banks, G = W. C. Gregory, K = A. Krapovickas, P = J. Pietrarelli, S = C. E. Simpson, Sc = A. Schinini, Se = J. G. Seijo, Sn = V. G. Solís Neffa, Va = F. Valenzuela, W = D. E. Williams, Z = H. Zurita; FCA = plant introduction number of Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste, Argentina; MDI = plant introduction number of Instituto Nacional de Tecnología Agropecuaria (INTA) Manfredi, Argentina; PI = plant introduction number of United States Department of Agriculture (USDA), USA. Prov. = province, dept. = department.

[b] a = annual, b = biannual, p = perennial.

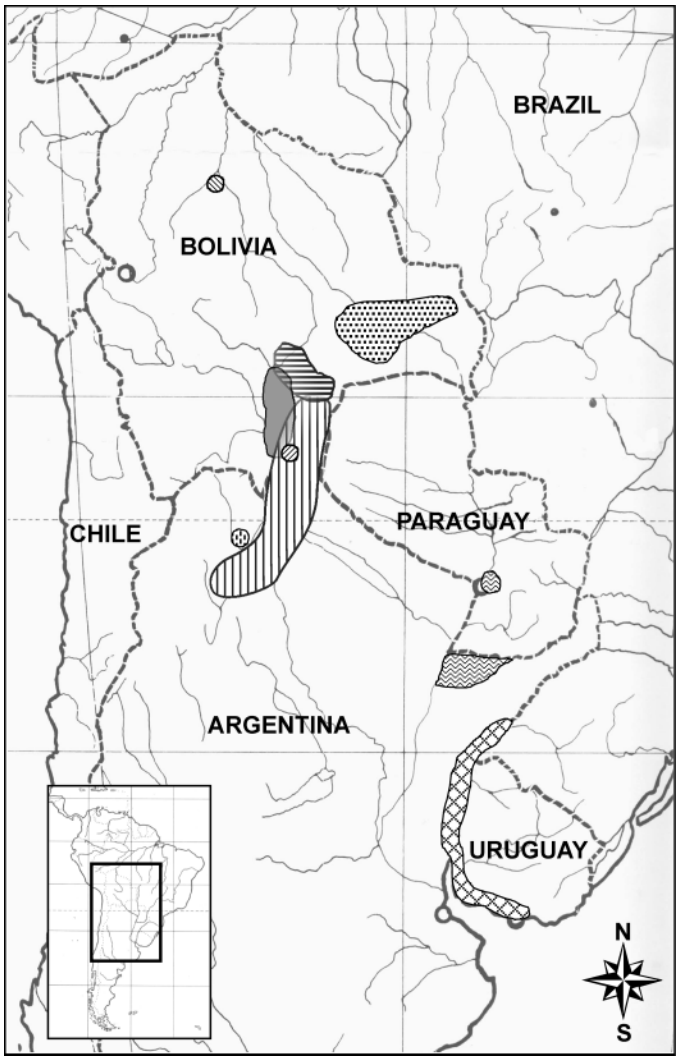



Fig. 1. Geographical distribution of the wild *Arachis* species studied and the major center of variability of *Arachis hypogaea* var. *hypogaea*. = *A. batizocoi*, = *A. cardenasii*, = *A. correntina*, = *A. duranensis*, = *A. hypogaea* var. *hypogaea*, = *A. ipaensis*, = *A. monticola*, = *A. villosa*, = *A. williamsii*.

ca, Heerbrugg, Switzerland) 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 pseudo-colored and combined using IM 1000 Leica software, then imported into Photoshop, version 7.0 (Adobe, San Jose, California, USA) for final processing.

***Karyotypic analysis***—At least five metaphase plates per species were used for chromosome measurements. The centromeric index (i = short arm length × 100/chromosome length) was used to classify the chromosomes according to Levan et al. (1964) in metacentric (m, i = 50–37.51) and submetacentric (sm, i = 37.50–25.10). Different lengths of the same arm (and band/locus, where applicable) from homologous chromosomes were combined to mean values and represented in the haploid complements of the idiograms. Intercalary markers were mapped using the index di = $d \times 100/a$ ($d$ = distance of band center from the centromere, $a$ = length of the corresponding chromosome arm) after Greilhuber and Speta (1976).

## RESULTS

General karyotype features, genome formula, number of chromosomes with heterochromatic DAPI+ bands, presence of ''A chromosome,'' and number of 5S and 18S–25S rDNA loci are listed for each *Arachis* species in Table 2. Illustrations of representative somatic metaphases are in Figs. 2–10, and the respective idiograms are in Fig. 11.

***General karyotype features***—The metaphase chromosomes in *Arachis* are comparatively small and range from 2 to 5 μm. In *A. hypogaea*, all varieties have similar karyotypes, with a tetraploid chromosome number $2n = 4x = 40$, an haploid karyotype formula of 19 m + 1 sm, and a mean haploid karyotype length of 67.28 ± 4.02 μm (Table 2). One sm pair bears conspicuous paracentromeric secondary constrictions in long arms and large satellites (''SAT chromosomes'' after Fernández and Krapovickas [1994]). Usually, the secondary constrictions of this pair are extended and the satellites remain far from the corresponding proximal arm segments. In all peanut accessions, the smallest m pair (''A chromosomes'' after Husted [1936]) displays a lower level of euchromatin condensation in comparison to the remaining chromosomes of the complement. *Arachis monticola*, $2n = 4x = 40$, has similar karyotype to the one observed in *A. hypogaea*.

All diploid species have $2n = 2x = 20$ with a karyotype mostly composed by m chromosomes of similar size (Table 2), one ''SAT chromosome'' pair (m or sm), and a total haploid karyotype length varying between 28.37 μm in *A. cardenasii* and 41.97 μm in *A. duranensis*. *Arachis cardenasii*, *A. correntina*, *A. duranensis*, and *A. villosa* possess an ''A chromosome'' pair, which is missing in *A. batizocoi*, *A. ipaensis*, and *A. williamsii*.

***Heterochromatin distribution***—In the complements of the tetraploid species, *A. hypogaea* (Fig. 2) and *A. monticola* (Fig. 3), half of the chromosomes—those belonging to the A genome—have centromeric DAPI+ bands, while the remainder (with the B genome) lack banding (Table 2, Fig. 11). The bands vary in size slightly, with the most conspicuous being those borne by the small A9 pair. All *A. hypogaea* varieties and *A. monticola* have a similar distribution and amount of heterochromatin, which accounts for about 7% of the karyotype length.

Among the diploid species with the A genome analyzed here, *A. correntina* (Fig. 8), *A. duranensis* (Fig. 7), and *A. villosa* (Fig. 9) display centromeric bands in all chromosomes, while *A. cardenasii* (Fig. 10) lacks banding in pair A4. Those entities considered as having the B genome, *A. ipaensis* (Fig. 4) and *A. williamsii* (Fig. 5), are devoid of heterochromatic bands, while in *A. batizocoi* (Fig. 6) all chromosomes are banded except pair number 4 (Table 2, Fig. 11).

In the diploid taxa with banded chromosomes, the percentage of heterochromatin ranges from 14.10% of the karyotype length in *A. duranensis* to 16.56% in *A. cardenasii*. In the species with the A genome, the A9 pair has the largest bands, which comprise an average of 45% of the chromosome length. Few remarkable heteromorphisms in banding pattern between homologous chromosomes were found, i.e., difference in band size of pair A4 in *A. villosa* and *A. correntina*.

***Cytological mapping of the 5S and 18S–25S rRNA genes by FISH***—All accessions of *A. hypogaea* (six) and *A. mon-*

TABLE 2. Karyotype features of *Arachis* species.[a]

| Species | 2n | Karyotype formula[b] | Karyotype length (μm), mean (SD) | Genome formula | Hc amount[c] (%) | No. chromosomes with hcb[c] (%) | A chromosome[d] | No. rDNA sites[e] | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 5S | 18S-25S |
| *A. hypogaea* | 40 | 19 m + 1 sm | 67.28 (4.02) | AB | 6.89 | 50 | + | 2 (A3, B3*) | 5 (A2, A10, B3, B7, B10) |
| *A. monticola* | 40 | 19 m + 1 sm | 66.93 (3.98) | AB | 6.96 | 50 | + | 2 (A3, B3*) | 5 (A2, A10, B3, B7, B10) |
| *A. cardenasii* | 20 | 10 m | 28.37 (1.38) | A | 16.56 | 90 | + | 1 (A3) | 3 (A2, A7, A10) |
| *A. correntina* | 20 | 9 m + 1 sm | 36.28 (1.53) | A | 14.11 | 100 | + | 1 (A3) | 2 (A2, A10) |
| *A. duranensis* | 20 | 9 m + 1 sm | 41.97 (2.27) | A | 14.10 | 100 | + | 1 (A3) | 2 (A2, A10) |
| *A. villosa* | 20 | 9 m + 1 sm | 33.88 (1.75) | A | 14.34 | 100 | + | 1 (A3) | 2 (A2, A10) |
| *A. ipaensis* | 20 | 10 m | 29.29 (1.23) | B | 0.00 | 0 | – | 1 (B3*) | 3 (B3, B7, B10) |
| *A. williamsii* | 20 | 9 m + 1 sm | 34.85 (2.32) | B | 0.00 | 0 | – | 1 (B3) | 1 (B10) |
| *A. batizocoi* | 20 | 9 m + 1 sm | 34.52 (1.56) | ? | 14.13 | 90 | – | 1 (3) | 3 (2, 7, 10) |

[a] Data correspond to the haploid complement except chromosome number.
[b] m = metacentric, sm = submetacentric.
[c] Heterochromatin (Hc) refers to DAPI+ bands (hcb).
[d] Presence/absence of A chromosome is indicated by +/−, respectively.
[e] Figures in parentheses are the designations of the chromosomes involved; see Fig. 11. Synteny of 5S site to 18S-25S site is indicated by an asterisk.

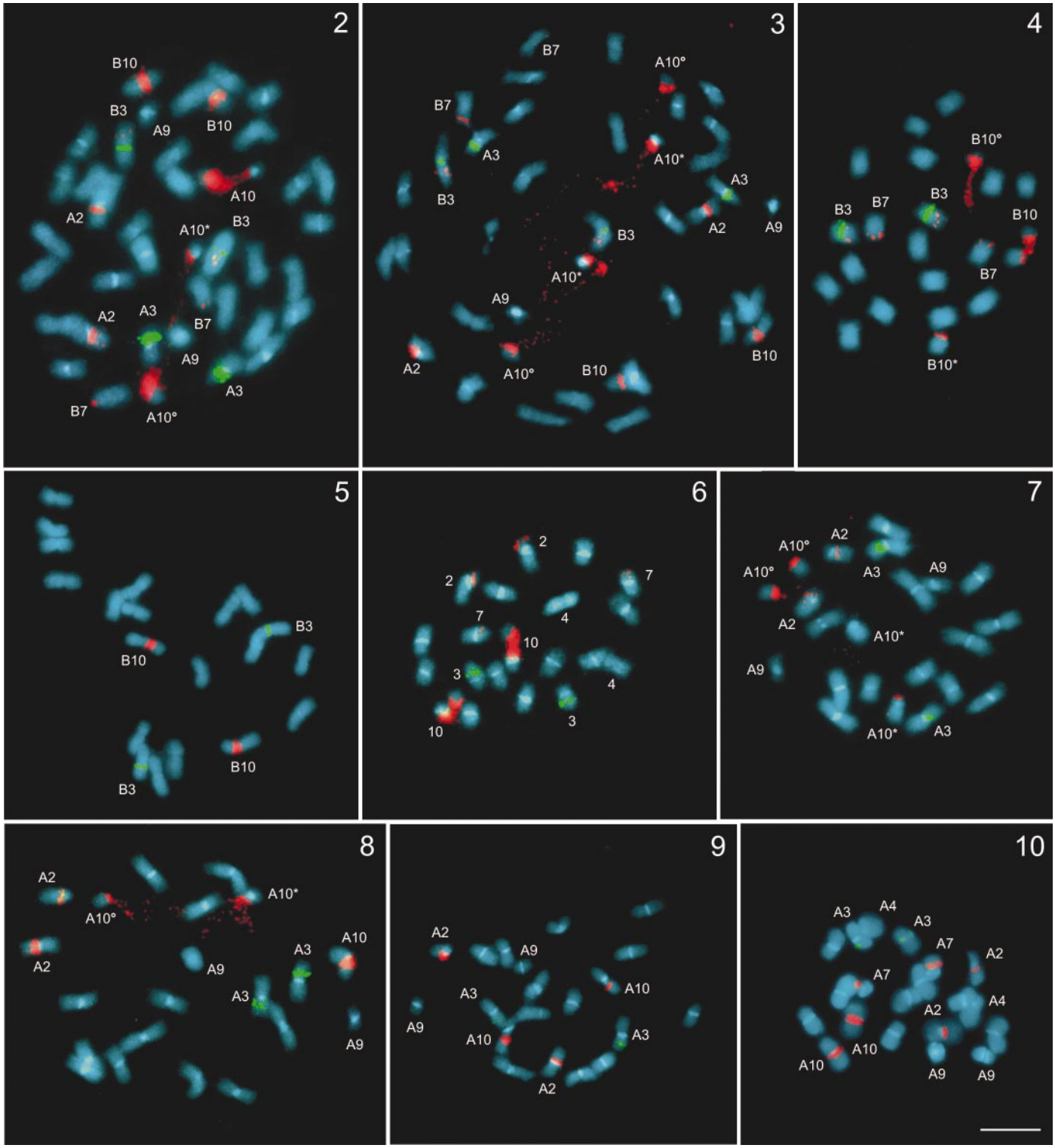
*ticola* (three) have two pairs of 5S and five pairs of 18S–25S rDNA sites (Table 2, Figs. 2, 3). In both species, the 5S loci are intercalarily located in short arms near the centromere of chromosome pairs A3 and B3 (Fig. 11). All 18S–25S rDNA clusters are interstitially placed, four pairs in long arms—three next to the centromere (A2, A10, and B10) and another in the middle of the arm (B3)—and the remaining pair in subtelomeric position of short arms (B7). One 5S site is syntenic to an 18S–25S site (B3).

In the diploid species, only one pair of 5S rDNA loci was found, while the number of 18S–25S sites varies from one in *A. williamsii* to three in *A. cardenasii*, *A. ipaensis*, and *A. batizocoi* (Table 2, Figs. 4–11). The 5S gene clusters are placed close to the centromere in the short arms of pairs A3 (*A. correntina*, *A. villosa*, and *A. duranensis*) and B3 (*A. ipaensis*), and in the long arms of pairs A3 (*A. cardenasii*), B3 (*A. williamsii*), and 3 (*A. batizocoi*). Concerning the 18S–25S rRNA genes, in the three species with the A genome, *A. correntina*, *A. duranensis*, and *A. villosa*, two paracentromeric pairs of loci in long chromosome arms (A2 and A10) were observed. *Arachis cardenasii* has an additional pair of 18S–25S clusters in the long arms next to the centromere (A7). Among the remaining species, *A. williamsii* has only one pair of 18S–25S sites in the long arms, intercalarily placed near the centromere (B10). *Arachis ipaensis* and *A. batizocoi* possess two additional pairs of loci, one of them subterminal in short arms (B7 and 7, respectively). The remaining pair of 18S–25S sites is interstitially placed in the long chromosome arms in *A. ipaensis* (B3) and in the short arms in *A. batizocoi* (2). In the former entity, the B3 chromosome pair has one cluster of each rRNA gene family.

Homomorphy in the FISH pattern of homologous chromosomes is high. In general, the size of the 5S loci is similar between species, except for those in pair A3 of *A. hypogaea* and *A. monticola*, which are larger. The 18S–25S sites vary in length among and within complements. In all species, the largest 18S–25S loci are located in chromosome pair 10 and correspond to the unique active nucleolar-organizing regions. In the tetraploid entities, the A10 pair bears active 18S–25S sites and the B10 inactive ones.

## DISCUSSION

***Chromosomal patterns of 5S and 18S–25S rDNA loci and heterochromatin***—Physical mapping by FISH of the 5S and 18S–25S rDNA sequences in the two subspecies and six botanical varieties of *A. hypogaea* demonstrates that both ribosomal RNA gene families have a similar number, size, and chromosomal distribution of signals between taxa. Furthermore, a high intraspecific homogeneity in heterochromatin amount and distribution was found, with a common pattern of centromeric bands in only half of the chromosomes, while the other half are unbanded. In a commercial sample of peanut for which no taxonomic data was provided, Raina and Mukai (1999a) also found two pairs of 5S rDNA sites but, in disagreement with our findings, just four pairs of 18S–25S loci. The additional locus of the latter gene family we found is placed in synteny with a 5S site in the B3 chromosome. The constancy in the FISH signal patterns observed here in all *A. hypogaea* accessions agrees with the low variability of molecular markers reported for the species (Kochert et al., 1991; Paik-Ro et al., 1992), although it contrasts with the wide mor-




Figs. 2–10. Somatic metaphases of *Arachis* species after double fluorescent in situ hybridization (FISH), showing yellow-green fluorescein isothiocyanate (FITC) signals from the 5S rDNA probe (pXV1) and red tetramethyl-rodamine isothiocyanate (TRITC) signals from the 18S–25S rDNA probe (R2). The 4′, 6-diamidino-2-phenylindole (DAPI) counterstaining (light blue) subsequent to the FISH procedure was used to highlight the heterochromatin bands and to stain euchromatin. In each species, homologous chromosomes identified in the A and B genomes are marked with the same number. The same number in different species indicate possible homeologies. **2.** *A. hypogaea* ($2n = 40$). **3.** *A. monticola* ($2n = 40$). **4.** *A. ipaensis* ($2n = 20$). **5.** *A. williamsii* ($2n = 20$). **6.** *A. batizocoi* ($2n = 20$). **7.** *A. duranensis* ($2n = 20$). **8.** *A. correntina* ($2n = 20$). **9.** *A. villosa* ($2n = 20$). **10.** *A. cardenasii* ($2n = 20$). In the cases in which the secondary constriction of chromosome number 10 is extended, the short arm and the proximal segment of the long arm are indicated by an asterisk and the separated satellite is marked by a degree sign. Scale bar = 5 μm.

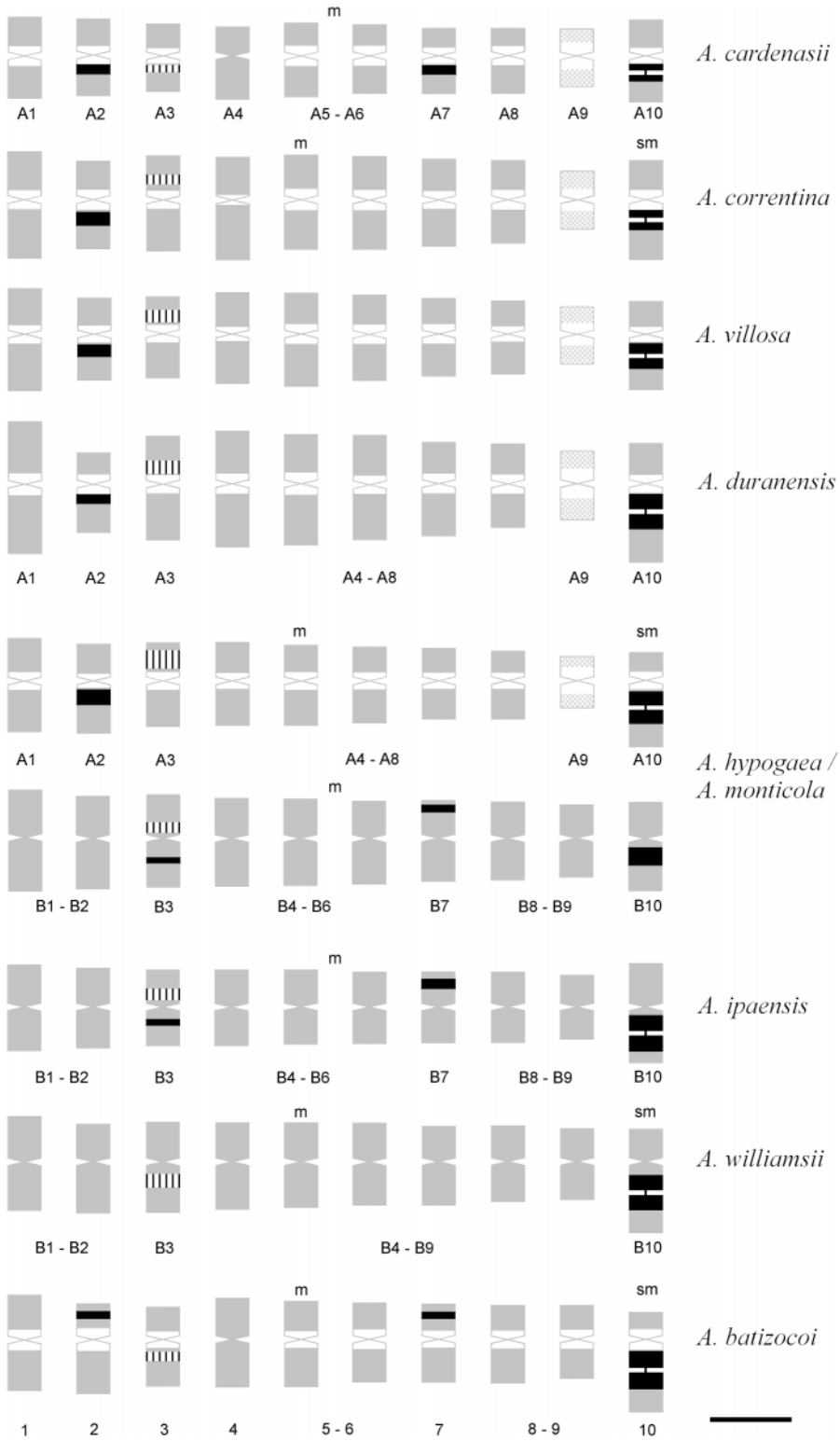



Fig. 11. Idiograms of *Arachis* species showing the distribution of 5S (▥) and 18S–25S rDNA loci (■), 4′, 6-diamidino-2-phenylindole (DAPI)-enhanced heterochromatic bands (□), DAPI-pale low condensed euchromatic regions (▨), and indifferent DAPI-stained normally condensed euchromatin (▩). Genes were mapped by fluorescent in situ hybridization (FISH), heterochromatic and differentially condensed euchromatic regions were distinguished by DAPI counterstaining of the FISH preparations. In *A. hypogaea*/*A. monticola*, the chromosomes were organized according to their genome (A genome with heterochromatic bands and B genome without them). The chromosomes of the A genome were ordered primarily by morphology and secondarily by decreasing size. The chromosomes of the B genome were arranged by decreasing size, except those with rDNA loci that were placed after their possible homeologies with chromosomes of the A genome. In the diploid species, the chromosomes were ordered primarily by morphology and secondarily according to decreasing size, except those with tentative homeologies with chromosomes of the tetraploid species. In the different species, chromosomes that have the same number on the idiograms are not necessarily homeologous. Chromosomes with similar morphology that lack any characteristic landmark were arranged in groups. Scale bar = 3 μm.

phological variation described between varieties (Krapovickas, 1968; Krapovickas and Gregory, 1994).

*Arachis monticola* has a similar pattern to the one observed in *A. hypogaea* with respect to number, size, and distribution of rDNA clusters and heterochromatic bands. The report of only two pairs of 18S–25S sites in *A. monticola* by Raina and Mukai (1999a) instead of the five pairs we found could not be explained by the presence of intraspecific variability, because our analysis demonstrates the same pattern between old and new collections from the two populations so far known for this taxon. The high degree of homeology between *A. monticola* and *A. hypogaea* inferred from the chromosome markers here examined (cf. infra) suggests that both tetraploid taxa are very closely related.

Concerning the putative diploid ancestors of *A. hypogaea* studied by us (all except *A. trinitensis*), this is the first report on rRNA gene localization by FISH in *A. correntina* and *A. williamsii*. In the remaining species, previous data on loci number without precise mapping descriptions were given by Raina and Mukai (1999a). We found the same number of 5S sites they cited, except for *A. batizocoi* (one pair instead of two). With respect to the 18S–25S loci, the numbers we observed agree with those of Raina and Mukai (1999a) only for *A. duranensis* and *A. batizocoi*, but differ for *A. cardenasii* (four pairs after Raina and Mukai, 1999a), *A. villosa* (1), and *A. ipaensis* (1). Such discrepancies can hardly be attributed to intraspecific variation because our preliminary assays are highly consistent in rDNA FISH pattern between populations. Sample misidentifications could indeed cause these differences because Raina and Mukai (1999a, b) consider section *Arachis* as being composed of only 10 instead of the 25 diploid species so far taxonomically recognized within the section (Krapovickas and Gregory, 1994).

The diploid species could be grouped according to their karyotype features. In this sense, the entities with A9 pair—the ''A chromosome'' pair that defines the A genome (Smartt et al., 1978)—also have heterochromatic bands in (almost) all chromosomes; thus, they are homogeneous in their gross karyotypic structure. On the other hand, the species without A9 pair, traditionally considered as having the B genome, are karyotypically more diverse because some of them lack heterochromatic banding. Concerning the FISH patterns of rRNA genes in the diploid species analyzed, the 5S loci has low variation, with just one pair per complement, while the 18S–25S sites are diverse in size, number, and location. The variability in the 18S–25S arrays is mainly noticed in the group of taxa without the A9 chromosome where it allows species discrimination. On the other hand, within the group of species with the A genome, only *A. cardenasii* is clearly differentiated as the other taxa (*A. duranensis*, *A. correntina*, and *A. villosa*) display similar FISH signal patterns.

According to the chromosome markers analyzed, the following homeologies between A genome species including the tetraploid taxa are proposed: A2 and A10 (with 18S–25S sites), A3 (with 5S loci), and A9 pairs (with differential euchromatin condensation) of each taxon. On the other hand, among B genome species, B7 and B10 (with 18S–25S clusters) and B3 pairs (with both rRNA gene arrays) of *A. ipaensis* and the tetraploids should be considered homeologous. Also homeology between pairs B3 (with 5S loci) and B10 (with 18S–25S sites) of *A. williamsii* and B3 and B10 of the species mentioned earlier is likely, although the former pair in particular lacks 18S–25S arrays. The establishment of homeologies between *A. batizocoi* and the other entities is less clear due to its uncertain genome composition. However, pairs 2 and 10 (with 18S–25S loci) and 3 (with 5S sites) could be homeologous to pairs A2, A10, and A3 of A genome species, respectively. In addition, pairs 4 (without heterochromatic banding) and 7 (with 18S–25S loci) could match with pairs A4 and A7 of *A. cardenasii*, respectively. Finally, rDNA-bearing chromosome pairs from species with different genome constitution, which are indicated by the same figure in the idiograms (cf. Fig. 11), are suggested to be homeologous, although more distantly related than those from species with the same genome.

***Genome analysis in section Arachis***—Based on conventional karyotypic studies in the members of section *Arachis* and on crossing experiments and chromosome pairing analyses in interspecific hybrids, three different genomes have been proposed for the section, i.e., the A and B genome, which are each in nearly half of the diploid species, and the D genome, which is only present in *A. glandulifera* (cf. Smartt et al., 1978; Singh and Moss, 1984; Stalker, 1991; Fernández and Krapovickas, 1994; Lavia, 1999). Additionally, an AABB genomic constitution has been suggested for the tetraploids, *A. hypogaea* and *A. monticola* (Smartt et al., 1978). In this sense, the bulk of chromosome markers analyzed here supports the proposed genomic formula and demonstrates a striking genomic similarity between both taxa, which also show high cross-compatibility producing fertile $F_1$ (Krapovickas and Rigoni, 1954, 1957) and display similar genomic painting patterns after probing the total DNA of the same diploid species (Raina and Mukai, 1999b).

The affinities in rDNA loci and heterochomatic banding patterns we found between diploids are in accordance with data on crossability, because species with the A genome more readily produce interspecific hybrids than species with the B genome (Krapovickas and Gregory, 1994; Singh and Smartt, 1998). Within the group of diploid species with the A genome, *A. correntina*, *A. villosa*, and *A. duranensis* constitute a homogeneous subgroup with similar karyotype formulae and heterochromatin and rRNA gene array FISH patterns. These data agree with conclusions on the taxonomic affinities between the two former species in particular, because *A. correntina* was formerly considered a variety of *A. villosa* (cf. Burkart, 1939; Krapovickas and Gregory, 1994). On the other hand, *A. cardenasii*, although with similar karyotypic formula and heterochromatic banding pattern than the former species, may be included in a different subgroup because it has one chromosome without heterochromatic bands and an additional 18S–25S rDNA site in the haploid complement.

The species without the ''A'' chromosome, supposedly bearing the B genome, are more diverse and, in particular, *A. batizocoi* can be clearly separated by having heterochromatic bands in all chromosomes except one. On the other hand, *A. ipaensis* and *A. williamsii* form a subgroup by being deprived of banding, although both species differ in the number of 18S–25S rDNA loci. It should be noted that different RFLP patterns between *A. batizocoi* and *A. ipaensis* were found (Kochert et al., 1991). Additionally, *A. batizocoi* is the species from this group that show greater cross-compatibility with the A genome species (Smartt and Gregory, 1967; Krapovickas and Gregory, 1994; Singh and Smartt, 1998). In this sense, the similarities in heterochromatic banding patterns and in the number of rDNA clusters between *A. batizocoi* and *A. car-*

*denasii* are remarkable. Therefore, the genome constitution of *A. batizocoi* should be clarified.

On the other hand, the relationship between *A. ipaensis* and *A. williamsii* is still not completely determined. In this context, further studies on chromosome structure using FISH and additional species are needed to obtain more information about the genomic relationships in the section and to help in the genomic characterization of the *Arachis* germplasm in general.

***Origin of the cultigen***—Our data confirm that *A. hypogaea* is an allotetraploid (cf. Husted, 1936) with half of its chromosomes having heterochromatic bands and with only one small ''A'' chromosome pair (A9). The fact that all varieties of both *A. hypogaea* subspecies have equal number, size, and distribution of rDNA loci, together with morphological and geographical data (cf. infra), suggests that the cultivated peanut originated from a single event of allopolyploidization or, if from multiple events, always involving the same parental diploid species. Furthermore, genomic rearrangements comprising the analyzed markers during infraspecific differentiation and domestication did not seem to occur. These phenomena could explain the limited variability detected at DNA level by RFLP and randomly amplified polymorphic DNA (RAPD) markers (Halward et al., 1991; Kochert et al., 1991) among varieties. Furthermore, the high homeology we found between the *A. hypogaea* and *A. monticola* karyotypes suggests that the cultigen arose upon domestication of the wild allotetraploid species.

Data on geographical distribution show that *A. monticola* grows in a very restricted area of less than 10 $km^2$ in northwestern Argentina, San Salvador de Jujuy (Fig. 1). On the other hand, the subspecies *hypogaea* of the cultigen, with the most primitive features in the species (i.e., creeping habit, small fruit with a well-marked constriction, reduced reticule, and two latent seeds), has its main center of variation in southeastern Bolivia, in the first spurs of Los Andes mountains, Chuquisaca and Tarija departments (Krapovickas and Gregory, 1994; Simpson et al., 2001). On this basis, both allotetraploid species have been proposed to have originated somewhere near the cited regions (Krapovickas, 1968; Krapovickas and Gregory, 1994).

According to the current distribution of the tetraploid taxa, we should search for possible diploid ancestors among the extant diploid species of the section *Arachis* that grow within the same geographic area, i.e., *A. duranensis*, *A. batizocoi*, and *A. ipaensis* (Fig. 1). From these candidates, *A. duranensis* and *A. ipaensis* are the most probable progenitors of *A. hypogaea*/*A. monticola*, because the former is the only species with the A genome and the latter the only one without centromeric bands. The exclusion of *A. batizocoi* as putative parent is supported by molecular marker data, which show *A. hypogaea* more distantly related to this species than to any other proposed diploid ancestor (Kochert et al., 1991; Paik-Ro et al., 1992). Our results on rDNA loci localization clearly point out the closest genetic affinity of *A. duranensis* and *A. ipaensis* with *A. hypogaea*, because the sum of the rDNA sites and their positions in the diploids are equal to those found in the tetraploid.

Concerning the remaining diploid species studied, *A. cardenasii* and *A. williamsii* should be discarded for having a different number of 18S–25S rDNA sites in comparison to those expected for the parentals of the tetraploids. On the other hand, *A. villosa* and the closely related *A. correntina* possess a karyotype similar to *A. duranensis* but are perennials with taproots, characters that are absent in *A. hypogaea*. Additionally, both diploid species are geographically separated from the proposed center of origin of the tetraploids by the Chaco region—more than 1000 km apart—which lacks *Arachis* species in most of its extension (Fig. 1). This distance itself is very significant for *Arachis* species because the calculated seed dispersion for individual plants is about 1 m per year (i.e., 1000 km in one million years), due to geocarpy. Moreover, because all *Arachis* species studied so far have an autogamous reproductive system with a small amount of cross-pollination, the estimated gene flow is very restricted, mainly between allopatric populations (cf. Krapovickas and Gregory, 1994; Simpson et al., 2001). Fortuitous dispersion of *A. villosa* and *A. correntina* by fluviatile hydrochory toward the region of probable origin of the cultigen can be also discarded because their habitats in the La Plata river basin are downstream (Fig. 1). Therefore, even though the FISH pattern of rDNA loci and the general karyotype features of *A. villosa* and *A. correntina* are similar to those of *A. duranensis*, the two former species can be excluded as putative ancestors of *A. hypogaea* on the basis of geographical and morphological data.

In summary, we have physically localized the rRNA gene loci and provided the heterochromatin pattern of all the botanical varieties of *A. hypogaea* and its wild relatives, achieving chromosomal landmarks and refined banded karyotypes for ongoing studies on comparative genomics and for the germplasm characterization of *Arachis* species. On the other hand, our results, together with those from classical cytogenetic (Fernández and Krapovickas, 1994), geographical, morphological (Krapovickas and Gregory, 1994), and molecular approaches (Kochert et al., 1991), support the hypothesis that *A. duranensis* and *A. ipaensis* are the most probable species to have participated in the origin of the cultigen and of *A. monticola*.

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