

# Karyotype Stability and Genome-Specific Nucleolar Dominance in Peanut, Its Wild 4× Ancestor, and a Synthetic AABB Polyploid

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## ABSTRACT

Allopolyploidy is a significant evolutionary process involved in the origin of many crops, including peanut (*Arachis hypogaea* L.). The process usually results in a series of chromosome, genomic and epigenetic rearrangements in the derived polyploids. Here, we examined the chromosomal consequences undergone by AABB tetraploids of *Arachis* after the genome merger. For that objective, different chromosome markers and DNA contents were compared among peanut, its wild tetraploid ancestor, and the diploid genome donors *A. duranensis* Krapov. & W.C. Gregory (AA, female) and *A. ipaënsis* Krapov. & W.C. Gregory (BB, male). The analysis also included an artificially synthesized allotetraploid using *A. ipaënsis* as a female [(*A. ipaënsis* × *A. duranensis*)<sup>4×</sup>]. The karyotypes in the natural (originated ~10,000 yr ago) and newly synthesized allopolyploids have largely maintained the patterns of heterochromatin and ribosomal RNA loci detected in the diploid progenitors. Intergenomic translocations were not evident using genome in situ hybridization, and the DNA contents of the allotetraploids corresponded to the expected sum of those observed in their parental species. The analysis of ribosomal DNA loci and their association with nucleolar organizing regions revealed a rapid establishment of nucleolar dominance in favor of the A genome. The large macrostructural stability of karyotype observed here after polyploidization has not been frequently cited for polyploid crop plants. This stability is significant for peanut breeding, since it suggests that effective introgression of wild useful alleles into cultivated peanut may potentially occur in most of the extension of the A and B chromosome complements.

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**Abbreviations:**  $C_x$ , monoploid genome size; DAPI, 4,6-diamidino-2-phenylindole; FISH, fluorescent in situ hybridization; FITC, fluorescein isothiocyanate; GISH, genome in situ hybridization; NOR, nucleolar organizing region; rDNA, ribosomal DNA; TRITC, tetramethylrhodamine isothiocyanate.

**P**EANUT (*Arachis hypogaea* L.) is one of the main oilseed crops in the world (Stalker, 2017), cultivated under diverse agricultural production systems in Asia, Africa, Australia, and the Americas (Holbrook and Isleib, 2001). It is largely accepted that *A. hypogaea* ( $2n = 4x = 40$ ) originated recently (10,000–15,000 yr ago) (Dillehay et al., 2007; Bertoli et al., 2016) from a single allopolyploidization event (Seijo et al., 2007; Bertoli et al., 2011; Grabiele et al., 2012). Therefore, the six botanical varieties and the hundreds of peanut land races so far recognized (Krapovickas et al., 2009, 2013) are probably derived from only one or a few plants.

The wild diploid species *A. duranensis* Krapov. & W.C. Gregory (A genome) and *A. ipaënsis* Krapov. & W.C. Gregory (B genome) are the most probable ancestors of *A. hypogaea* (Fernández and Krapovickas 1994; Kochert et al., 1996; Seijo et al., 2004). However, it has been postulated that these diploid ancestors could first have originated a wild allotetraploid plant, which may have resembled the extant AABB wild tetraploid *A. monticola* Krapov. & Rigoni (Krapovickas and Gregory, 1994; Grabiele et al., 2012). The wild characteristics of this species are

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based mainly on its ability to persist in nature spontaneously and its fruit structure. In *A. monticola*, each seed has its own shell (separated by an isthmus), whereas in *A. hypogaea*, the seeds (two or more) are always contained in a single shell (Krapovickas and Rigoni, 1957). Besides the ecological and morphological differences, molecular and cytogenetic data demonstrated that both the tetraploid species are closely related (Seijo et al., 2004, 2007; Grabile et al., 2012; Moretzsohn et al., 2013).

Due to its single origin from one or a few plants, peanut has a limited genetic diversity (Kochert et al., 1996; He et al., 2003), particularly for many traits of agricultural value. In contrast, the 82 wild diploid *Arachis* species are very diverse (Krapovickas and Gregory, 1994; Tallury et al., 2005; Valls and Simpson, 2005, 2017; Moretzsohn et al., 2013; Valls et al., 2013) and have been selected during evolution by a range of environments and biotic stresses, providing a rich source of genetic variation in agronomically important traits. Attempts to introduce useful variability from wild diploid species into peanut have taken several paths and have frequently been met with difficulty (Stalker et al., 2016; Sharma et al., 2017; Stalker, 2017). These attempts included hybridization of diploid and tetraploid species directly, somatic doubling of a diploid followed by crossing with a cultivated tetraploid, diploid by tetraploid crosses using  $2n$  gametes, or formation of triploids followed by doubling to hexaploids and elimination of chromosomes either spontaneously or through repeated backcrossing with the tetraploid (Simpson, 2001). Independently of the method, in all of them, one step of polyploidization is needed to create tetraploid introgression lines and to restore fertility of the crossed materials.

An expected consequence of the merger of two nuclear genomes during artificial polyploidization is that the resulting polyploid will contain the full genomic complement of both parents and all their traits. However, allopolyploidy often leads to unexpected departures from predicted genomic additivity. The mechanisms by which polyploidy contributes to novel variation are not fully understood, in part because chromosome doubling involves a complex and variable spectrum of different chromosomal, genomic, and epigenetic changes (Kashkush et al., 2002, 2003; Osborn et al., 2003; Soltis et al., 2004; Madlung and Wendel, 2013). Thus, the use of diploids with desired traits in breeding programs is puzzling, since often the attributes of diploids cannot be recovered in the synthetic or introgressed materials (Leal-Bertioli et al., 2012, 2015). Therefore, understanding the effects of polyploidy on chromosome structure and gene function is a priority in any breeding program that includes polyploidization.

The additivity of the karyotype and genome size is an expectation that serves as a testable null hypothesis of

predicted genomic contributions to the polyploid nucleus. However, naturally occurring spontaneous polyploids may not be ideal models to test the hypothesis because the initial conditions may have been hidden by the independent evolution of their genomes and those of their progenitors since the polyploidization event. Thus, synthetic experimental allopolyploids are used to gain insights into the earliest stages of polyploid genome evolution (Lim et al., 2007). In this sense, the crossability between *A. ipaënsis* and *A. duranensis* has been demonstrated, and tetraploidization has been accomplished through the use of colchicine, followed by successful crosses of the resulting synthetic amphidiploid with several accessions of *A. hypogaea* (Fávero et al., 2006). This newly synthesized amphidiploid, together with the well-established AABB polyploids, provides an appropriate biological model to make inferences on the effects of the allopolyploidization in *Arachis*.

Comparisons of the chromosome complements of tetraploid species and the most closely related diploid species revealed a variety of events associated with allopolyploid divergence, including the presence of chromosome translocations, divergence in repetitive sequences, differential ribosomal DNA (rDNA) silencing, and wide epigenetic changes (Adams et al., 2003; Soltis et al., 2004; Adams and Wendel, 2005; Doyle et al., 2008; Madlung and Wendel, 2013). Although chromosomes of *Arachis* species are relatively small and morphologically little differentiated, application of fluorescence in situ hybridization (FISH) with repetitive sequences of DNA (Seijo et al., 2004; Nielen et al., 2009; 2011; Bertioli et al., 2013; Samoluk et al., 2015b, 2017; Zhang et al., 2012) and with total genomic DNA (genome in situ hybridization [GISH]) (Seijo et al., 2007) has provided several chromosome landmarks for the analysis of chromosome structure and evolution.

To address the impact of allopolyploidization in the chromosome complements of *Arachis* AABB polyploids, we present a comparative cytogenetic analysis of the cultivated peanut, the wild AABB tetraploid *A. monticola*, an artificial allotetraploid [ $(A. ipaënsis \times A. duranensis)^{4x}$ ] and the diploid progenitors of the tetraploids by localizing repetitive DNA loci (rDNA and heterochromatin) on chromosomes by FISH, identifying genomes using GISH, and estimating DNA  $C$  values by flow cytometry. The results provided reveal long-term stability in the macrostructure of the karyotypes of the AABB tetraploids of *Arachis*, but unidirectional dominance of the nucleolar organizing regions (NORs).

## MATERIALS AND METHODS

### Plant Material

The material analyzed in this study is listed in Table 1. The spontaneous amphidiploids *A. monticola* (wild) and *A. hypogaea* (cultivated) are considered as mesopolyploids originated

**Table 1. *Arachis* species analyzed with their status, provenance, ploidy, and genome size (2C and monoploid genome size [Cx]).**

Species	Status†	Collection no.‡ and origin	Ploidy (x)	2C	Cx
<i>A. hypogaea</i> L. subsp. <i>hypogaea</i> var. <i>hypogaea</i> (race Guaycurú)	C	CTES 27. Argentina, Corrientes	4	5.610 ± 0.050	1.402
<i>A. hypogaea</i> L. subsp. <i>hypogaea</i> var. <i>hirsuta</i> Köhler (race Mitad del Mundo)	C	B, P, Va 732 (MDI 86–2769). Ecuador, San Antonio	4	5.652 ± 0.112	1.413
<i>A. hypogaea</i> L. subsp. <i>fastigiata</i> Waldrom var. <i>fastigiata</i> (race Colorado)	C	CTES 31. Argentina, Corrientes	4	5.757 ± 0.088	1.439
<i>A. hypogaea</i> L. subsp. <i>fastigiata</i> var. <i>peruviana</i> Krapov. and W.C. Greg. (race Tingo María)	C	MDI 87–2753 (PI 393641). Perú, Casma	4	5.792 ± 0.252	1.448
<i>A. hypogaea</i> L. subsp. <i>fastigiata</i> var. <i>aequatoriana</i> Krapov. and W.C. Greg. (race Zaruma)	C	B, P, Z 683 (MDI 86–2535). Ecuador, Quito	4	5.801 ± 0.131	1.450
<i>A. hypogaea</i> L. subsp. <i>fastigiata</i> var. <i>vulgaris</i> C. Harz (race Blanco Manfredi)	C	MDI 68–255. Argentina	4	5.678 ± 0.124	1.419
<i>A. monticola</i> Krapov. and Rigoni	W	SeSn 2774 Argentina, Jujuy, Yala (24°67' S, 65°23' W)	4	5.772 ± 0.089	1.443
Amphidiploid ( <i>A. ipaënsis</i> × <i>A. duranensis</i> ) <sup>4x</sup>	S		4	5.711 ± 0.084	1.427
<i>A. duranensis</i> Krapov. and W.C. Greg.	W	SeSn 2741. Argentina, Salta, Río Seco (23°01' S, 63°51' W)	2	2.585 ± 0.125	1.292
	W	V14167. Argentina, Salta, Capital (24°45' S, 65°26' W)	2	2.553 ± 0.099	1.276
<i>A. ipaënsis</i> Krapov. and W.C. Greg.	W	KGbpsSchS 30076. Bolivia, Tarija, Ipa (21°00' S, 63°24' W)	2	3.205 ± 0.084	1.602

† C, cultivated; S, synthetic; W, wild.

‡ Collectors: B, D. Banks; G, W.C. Gregory; K, A. Krapovickas; P, J. Pietrarelly; Sch, A. Schinini; Se, J.G. Seijo; S, C.E. Simpson; Sn, V.G. Solís Neffa; V, J.F.M. Valls; Va, F. Valenzuela; Z, H. Zurita. Germplasm banks: CTES, plant introduction number of the Instituto de Botánica del Nordeste (IBONE), Argentina; MDI, plant introduction number of the Instituto Nacional de Tecnología Agropecuaria (INTA), Manfredi, Argentina; PI, plant introduction number of the USDA, USA.

~10,000 to 15,000 yr ago. Representative specimens of the six botanical varieties of peanut, *A. monticola*, and plants (F8–F9) of the artificially synthesized amphidiploid [(*A. ipaënsis* × *A. duranensis*)<sup>4</sup>] (Fávero et al., 2006) were analyzed. The F1 diploid hybrid was sterile and died soon after tetraploidization assays were performed. The plants of *A. ipaënsis* used for the synthesis of the amphidiploid belonged to the only population known for the species, which, according to the latest records (Seijo, personal observation), may be extinct from nature. The two populations of *A. duranensis* used here are the one identified by chloroplast DNA and nuclear markers as the most similar to the A genome of peanut (Se 2741) and the one used for the synthesis of the amphidiploid (V 14167).

## Chromosome Preparations

Plants obtained from seeds were grown in pots under laboratory conditions. Healthy root tips (5–10 mm long) obtained from seedlings were pretreated with 2 mM 8-hydroxyquinoline for 3 h at room temperature (Fernández and Krapovickas, 1994) and fixed in 3:1 absolute ethanol/glacial acetic acid for at least 12 h at 4°C. Root apices were digested in 1% (w/v) cellulose (Onozuka) plus 10% (v/v) pectinase (Sigma) solution in 0.01 at 37°C for 2 h. Subsequently, the meristematic cells were removed from the root tip and squashed in 45% acetic acid.

## Probe Labeling and Fluorescent in Situ Hybridization

For FISH experiments, the 5S rDNA and 18S to 26S rDNA loci were localized using probes isolated from genomic DNA of *A. hypogaea* subsp. *fastigiata* Waldrom var. *aequatoriana* Krapov. & W.C. Gregory (race Zaruma; Ecuador, Quito, collection: Banks, Pietrarelly, and Zurita 683) (Robledo and Seijo, 2008). For GISH, total genomic DNA used as a probe was extracted from young actively growing leaves of the diploid species

assayed. Probes were labeled by nick translation with digoxigenin-11-dUTP (Boehringer Mannheim) or biotin-11-dUTP (Sigma Aldrich). Mixes for GISH analysis were prepared with equimolar concentrations of labeled genomic DNA of both diploid species without any species-specific blocking DNA, but Salmon Sperm DNA was used as general blocking DNA (Moscone et al., 1996).

## Hybridization, Detection, and Analysis

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 L<sup>-1</sup>, with a stringency to allow sequences with 80–85% identity to remain hybridized), posthybridization washing, blocking, and indirect detection with fluorochrome-conjugated antibodies were performed according to Moscone et al. (1996) and Seijo et al. (2004, 2007). Briefly, the first set of antibodies consisted of mouse anti-biotin and sheep anti-digoxigenin conjugated to fluorescein isothiocyanate (FITC) (Sigma). The second set of antibodies consisted of rabbit anti-mouse conjugated to tetramethylrhodamine isothiocyanate (TRITC) and FITC-conjugated rabbit anti-sheep (Sigma). Preparations were counterstained and mounted with Vectashield medium (Vector Laboratories) containing 2 mg mL<sup>-1</sup> of 4,6-diamidino-2-phenylindole (DAPI). The counterstaining with DAPI revealed a C-banding-like pattern, with major heterochromatic bands fluorescing more intensely (Seijo et al., 2004). Chromosomes were analyzed and photographed with an epifluorescence microscope equipped with a 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.

Karyotype stability was analyzed by comparing the chromosome markers in the corresponding chromosome complements between diploid and tetraploid specimens. The chromosome secondary constrictions after FISH were analyzed to detect the active NORs. The presence of secondary constrictions in metaphase of *Arachis* species (Fernández and Krapovickas, 1994; Seijo et al., 2004) as in other plants (Lim et al., 2008) correlates strongly with low levels of chromatin condensation at interphase and almost certainly reflects transcriptional activity at the preceding interphase (Pikaard, 2000). Thus, the analysis of extended secondary constrictions provides an effective method to record activation of ribosomal loci in *Arachis* species.

## Determination of DNA Content

The genome size was determined using flow cytometry of isolated nuclei according to Doležel et al. (1989). Newly expanded leaves of *Arachis* species were chopped together with leaves of standard species using 0.5 mL buffer (high-resolution DNA kit, Partec GmbH Münster). Nuclei of *Paspalum dilatatum* Poir. cultivar Chirú Q4081 ( $2C = 3.57$  pg; Vaio et al., 2007) and *Paspalum intermedium* Munro ex Morong accession Sch 28857 ( $2C = 1.42$  pg; Vaio et al., 2007) were used as internal standards (Samoluk et al., 2015a). The suspension was filtered through a 30- $\mu$ m nylon mesh and stained with 50 mg mL<sup>-1</sup> of propidium iodide and 50 mg mL<sup>-1</sup> ribonuclease. Measurements were performed on a Partec PAS flow cytometer using a linear scale. Approximately 5000 nuclei per sample were measured, and at least four repetitions were made for each species. The Flomax software (Partec) was used to calculate the positions of G0/G1 peaks.

Arbitrary values were converted to picograms from the ratio of the mean absorbency of the *Arachis* test species to that of the calibration standard. The expected mean genome sizes for the tetraploids were calculated as averages of all the possible combinations of the measurements done in the diploid progenitors. Statistical significance of the differences among the tetraploid DNA contents was analyzed using the Tukey test in INFOSTAT version 10.0 (Di Rienzo et al., 2017).

## RESULTS

### Heterochromatin Distribution and rDNA Loci Mapping

A summary of the chromosome landmarks of the analyzed species is presented in Table 2. The population of *A. duranensis* analyzed showed centromeric bands of heterochromatin in all the chromosomes (Fig. 1A). Those of the A9 pair (A chromosomes) comprised almost half of the chromosome length, whereas those of the other chromosomes ranged between 7 and 15% of the chromosome length. This species had two interstitial loci of 18S to 26S rDNA on the A2 and A10 chromosome pairs (those of A10 correspond to the secondary constriction of the satellite chromosomes) and one proximal locus of 5S rDNA on the A3 pair (Fig. 1A). By contrast, all the chromosomes of *A. ipaënsis* lacked conspicuous heterochromatic bands (few displayed faint centromeric bands after FISH, Fig. 1B). Characteristically, this species showed three pairs

of 18S to 26S rDNA loci: those of the B10 pair (which correspond to the secondary constriction of the satellite chromosomes) and B3 pair located interstitially, and those of the B7 pair located in the subtelomeric region. The 5S rDNA loci were located proximally in the B3 pair (Fig. 1B), on the same arm as the 18S to 26S rDNA loci.

All six varieties of the two subspecies of *A. hypogaea* (Fig. 1C–1H) and *A. monticola* (Fig. 1I) had half of their chromosomes with centromeric bands of heterochromatin (those of the A genome) and half without them (those of the B genome). The bands varied in size as in *A. duranensis*, being those of the A9 pair the most conspicuous ones. Five pairs of 18S to 26S rDNA loci and two pairs of 5S rDNA loci were detected in *A. hypogaea* (Fig. 1C–1H) and *A. monticola* (Fig. 1I). Four of the 18S to 26S rDNA loci were placed on long arms (three next to the centromere [A2, A10, and B10] and the other in the middle of the arm [B3]), whereas the remaining pair was located in the subtelomeric region of short arms (B7).

In both species, the 5S loci were located in short arms near the centromere of the A3 and B3 pairs (Fig. 2C–2I). The 5S rDNA loci of the B genome (B3 pair) were placed on the same arm as the 18S to 26S loci.

The analysis of the synthetic amphidiploid metaphases (Fig. 1J) showed that the pattern of heterochromatin and rDNA loci (in number, size, and distribution) was the same as those observed in *A. hypogaea* and *A. monticola*. The comparison of tetraploids with their diploid progenitors (AA and BB) revealed that the complements of the former corresponded to the sum of the complements of the latter.

### Genome in Situ Hybridization

The pattern of GISH in the amphidiploids was used to test the occurrence of genome turnover and intergenomic rearrangements. In each of the polyploids analyzed, the specificity of the genomic probes was validated by the presence or absence of conspicuous heterochromatic bands in the A and B genomes, respectively. In the spontaneous tetraploids (*A. hypogaea* [Fig. 2A and 2B] and *A. monticola* [Fig. 2C and 2D] and in the synthetic amphidiploid [Fig. 2E and 2F]), there was clear discrimination of the parental origin of chromosomes. The 20 chromosomes of the A genome (with heterochromatic bands) hybridized with *A. duranensis* genomic DNA (green fluorescence), and the 20 chromosomes of the B genome (without large heterochromatic bands) hybridized with *A. ipaënsis* genomic DNA (red fluorescence). Genome in situ hybridization was equally effective in the spontaneous (Fig. 2B and 2D) and in the artificial allopolyploids (Fig. 2F). Some dotted cross-hybridization of probes between parental genomes was detected, appearing as yellow-green (instead of green) and orange (instead of red) spots onto the chromosomes. The intensity of cross-hybridization spots was similar in all the tetraploids analyzed. Moreover, similar

**Table 2. Karyotype features of diploid and AABB allopolyploids *Arachis* species.†**

Species	2n	Karyotype formula‡	Genome formula	Pairs with H§	A pair¶	No. of pairs with rDNA loci#	
						5S	18S-25S
<i>A. hypogaea</i> (all varieties)	40	19m + 1 sm	AB	50	+	2 (A3, B3††)	5 (A2, A10, B3, B7, B10)
<i>A. monticola</i>	40	19m + 1 sm	AB	50	+	2 (A3, B3††)	5 (A2, A10, B3, B7, B10)
Amphidiploid	40	19m + 1 sm	AB	50	+	2 (A3, B3††)	5 (A2, A10, B3, B7, B10)
<i>A. duranensis</i>	20	9m + 1 sm	A	100	+	1 (A3)	2 (A2, A10)
<i>A. ipaënsis</i>	20	10m	B	0	–	1 (B3††)	3 (B3, B7, B10)

† Cytogenetic data correspond to the haploid complement except chromosome number.

‡ m, metacentric, sm, submetacentric.

§ Pairs with centromeric heterochromatin.

¶ Presence and absence of A chromosome are indicated by + and –, respectively.

# rDNA, ribosomal DNA. Numbers in parentheses are the designations of the pairs of chromosomes involved.

†† 5S and 18S-25S rDNA loci were in the same chromosome.

cross-hybridization appeared when the genomic DNA probes were tested onto the diploid metaphases, that is, the *A. duranensis* DNA probe tested onto the chromosomes of *A. ipaënsis*, and the *A. ipaënsis* DNA probe tested onto the *A. duranensis* chromosomes (data not shown).

Within each complement of the amphidiploids, the hybridization intensity was homogeneous, except for the centromeric DAPI<sup>+</sup> bands of the A genome chromosomes, the whole A9 pair, and the short arm and proximal segment of the long arm of the A10 chromosomes (Fig. 2B–2D), in which the hybridization signal was weaker. This pattern was also observed in the chromosome complement of the diploid *A. duranensis* (data not shown). No intergenomic translocations or differences in the fluorescent intensity of the probes were observed when compared with each set of parental chromosomes.

## C-DNA Content

The genome sizes of the species analyzed are presented in Table 1. No significant differences were evident between the six botanical varieties of peanut (5.610–5.801 pg) or between them and *A. monticola* (5.772 pg) and the artificial amphidiploids (5.711 pg). The genome sizes of *A. hypogaea* and *A. monticola* were not statistically different from the expected sum (mean 5.758 pg) of the genome sizes of their putative diploid progenitors *A. duranensis* (SeSn 2741) and *A. ipaënsis* ( $p < 0.005$ ). Similarly, the genome size of the AABB artificial amphidiploid was almost equal ( $p < 0.005$ ) to the expected sum of the genome sizes (mean 5.79 pg) of its diploid progenitors *A. duranensis* (V14167 f) and *A. ipaënsis*.

## Analysis of Secondary Constrictions

Despite the conserved pattern of the chromosome markers analyzed, a detailed analysis of the metaphases showed that diploid and tetraploid species generally have only one (or, rarely, two or three) pair of chromosomes with extended secondary constriction hybridized with the 18S to 26S rDNA probe (Fig. 1, Table 3). In the metaphases of the

diploid species, only the A10 pair of *A. duranensis* (Fig. 1A) and the B10 pair of *A. ipaënsis* (Fig. 1B) carried extended secondary constriction (Table 3). Occasionally (7% of the cells), one of the homologs of the A2 pair in *A. duranensis* also showed extended secondary constriction. By contrast, the B3 and B7 pairs of *A. ipaënsis* were never observed with secondary constriction.

All the allotetraploids showed the A10 pair with extended secondary constriction in 100% of their cells (Table 3). In addition, one or two A2 chromosomes were observed with extended secondary constriction in *A. monticola* (20% of the cells), *A. hypogaea* var. *hypogaea* (100% of the cells), *A. hypogaea* var. *aequatoriana* (45% of the cells), and the amphidiploid (100% of the cells). In *A. hypogaea* var. *aequatoriana*, the B10 pair was also observed with extended secondary constriction (27% of the cells, Fig. 1F), whereas in the synthetic amphidiploid, the B10 pair showed shortened or condensed secondary constriction with conspicuous hybridization signals in most cases (Fig. 1J). More rarely (27%), the secondary constriction in one of the B10 homologs was slightly extended (Table 3).

## DISCUSSION

### Stability of Chromosome Markers

Collectively, the chromosome morphology and the patterns of the three tandem repetitive DNA loci analyzed (heterochromatic bands, 18S–26S and 5S rDNA) allowed the differentiation of the chromosomes of the A and B complements and the identification of several chromosome pairs of the diploid parents (10 pairs out of 20) in the AABB polyploids. Thus, these markers were valuable to track individual chromosomes of the diploid progenitors in the polyploid derivatives.

The number and location of the heterochromatic bands in the *Arachis* AABB natural allopolyploids analyzed here appeared to be directly inherited from their corresponding diploid ancestors without any detectable organizational or distributional changes. Similarly, the additive pattern of

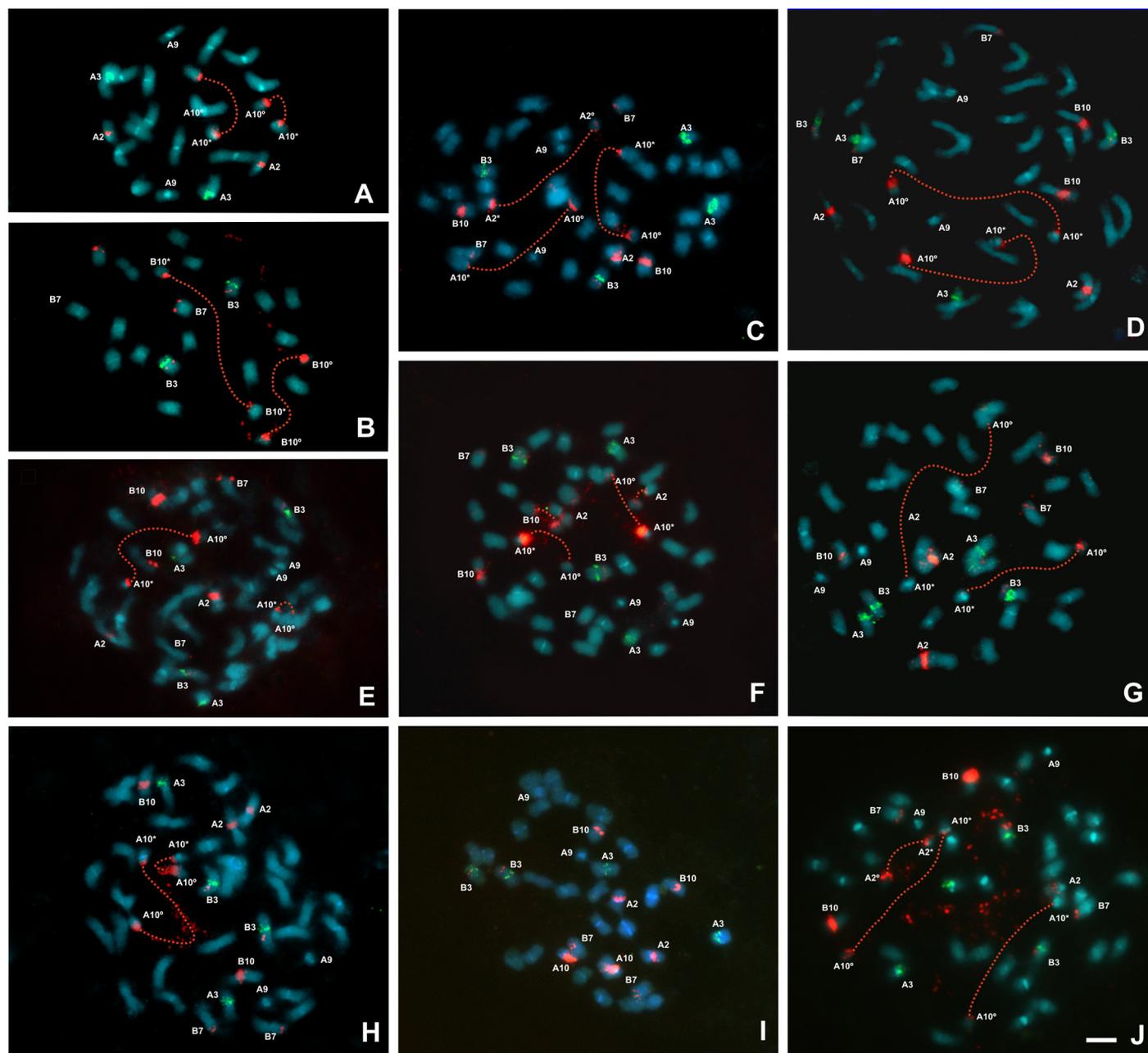


Fig. 1. Somatic metaphases of *Arachis* species after double fluorescent in situ hybridization, showing yellow-green fluorescein isothiocyanate signals from the 5S ribosomal DNA (rDNA) probe, and red tetramethylrhodamine isothiocyanate signals from the 18S to 26S rDNA probe. 4,6-Diamidino-2-phenylindole counterstaining (light blue) was used to highlight the heterochromatic bands and to stain euchromatin. The secondary constrictions of the satellite chromosomes are indicated with a dot line in red, the short arm and the proximal segment of the long arm are indicated by \*, and the separated satellite is marked by °. (A) *A. duranensis* metaphase ( $2n = 20$ , AA) showing centromeric bands of heterochromatin in all the chromosomes, two pairs of 18S to 26S rDNA (A2 and A10), and one pair of 5S rDNA (A3). (B) *A. ipaënsis* metaphase ( $2n = 20$ , BB) showing chromosomes deprived of conspicuous heterochromatic bands, three pairs of 18S to 26S rDNA (B3, B7, and B10), and one pair of 5S rDNA (B3). (C) H-Somatic metaphases of *Arachis hypogaea* varieties (all  $2n = 40$ , AABB). All of the varieties showed half of the chromosomes (those belonging to the A genome) with conspicuous centromeric bands and half (those of the B genome) without them, five pairs of 18S to 26S rDNA (A2, A10, B3, B7, and B10), and two pairs of 5S rDNA (A3 and B3). (D) *A. hypogaea* subsp. *hypogaea* var. *hypogaea*, (E) *A. hypogaea* subsp. *hypogaea* var. *hirsuta*, (F) *A. hypogaea* subsp. *hypogaea* var. *fastigiata*, (G) *A. hypogaea* subsp. *hypogaea* var. *aequatoriana*, (H) *A. hypogaea* subsp. *hypogaea* var. *peruviana*, (I) *A. hypogaea* subsp. *hypogaea* var. *vulgaris*, (J) *A. monticola* ( $2n = 40$ , AABB), and (K) the artificial amphidiploid [*A. ipaënsis* × *A. duranensis*]<sup>4x</sup> showed similar patterns of rDNA loci and heterochromatin than *A. hypogaea*. In all the tetraploids, the 18S to 26S loci with extended secondary constrictions (dot line) were those of the A10 pair, more rarely those of the A2 pair, and only in *A. hypogaea* subsp. *hypogaea* var. *aequatoriana*, those of the B10 pair showed shortly extended secondary constrictions. Scale bar = 5  $\mu$ m.

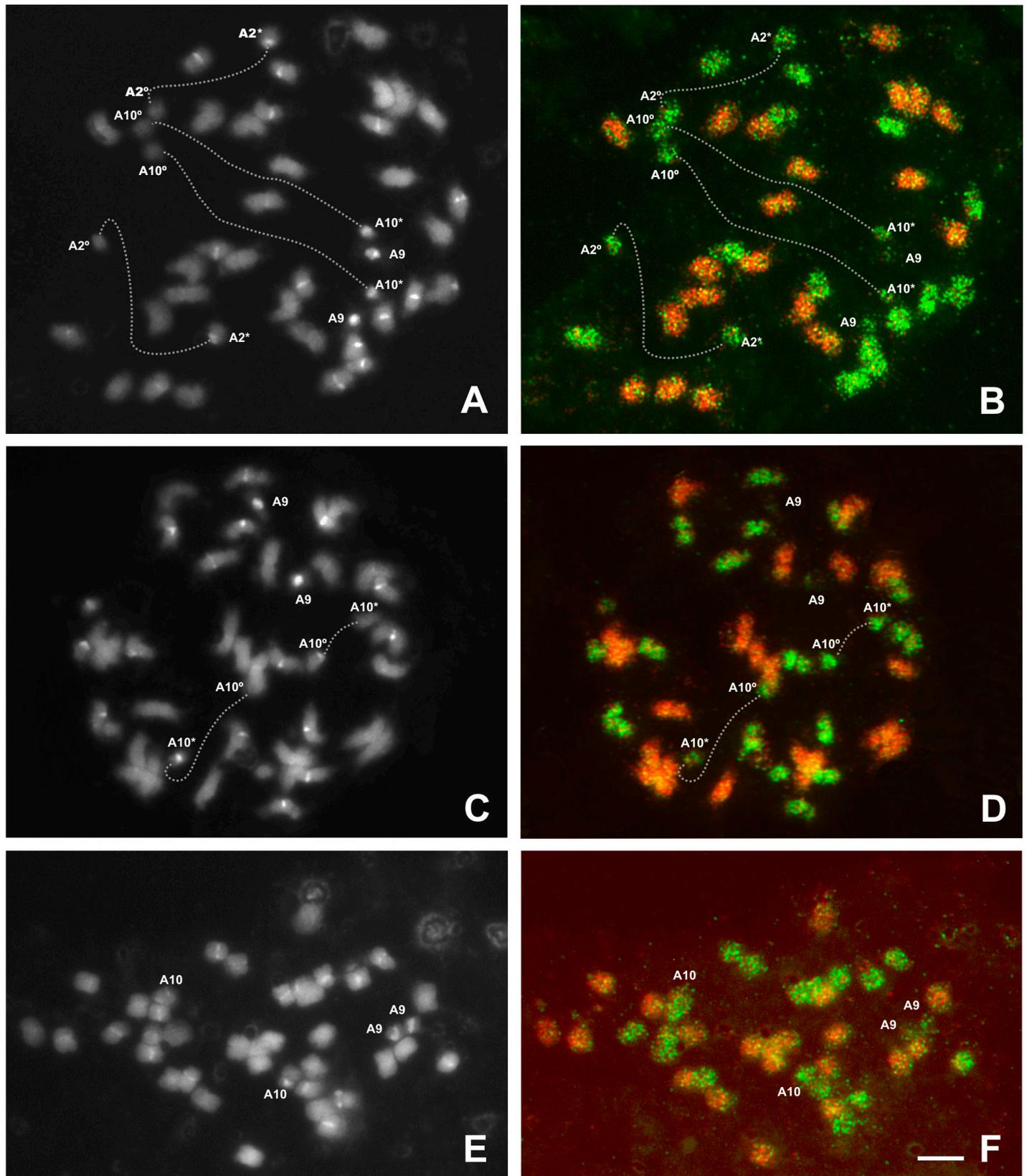


Fig. 2. Representative somatic metaphases AABB polyploid of *Arachis* after double genomic in situ hybridization (B, D, F): (A, B) *Arachis hypogaea* subsp. *hypogaea* var. *hypogaea*, (C, D) *A. monticola*, (E, F) amphidiploid [*A. ipaënsis* × *A. duranensis*]<sup>4x</sup>. 4-6-Diamidino-2-phenylindole after the genomic in situ hybridization (GISH) was used to highlight the heterochromatic bands and to stain euchromatin in Fig. 1A, 1C, and 1E. Fig. 1B, 1D, and 1F show the somatic chromosomes of the allopolyploids (same metaphase as in Fig. 1A, 1C, and 1E, respectively) after GISH with DNA probes from *A. duranensis* labeled with fluorescein isothiocyanate (green) and from *A. ipaënsis* labeled with tetramethylrhodamine isothiocyanate (red). In metaphases where the secondary constriction of chromosome A10 is extended, the short arms and the proximal segment of the long arm are indicated by an asterisk and the separated satellite is marked by °. Scale bar = 5 μm.

**Table 3. Extended secondary constrictions (NORs) recorded in diploid and AABB allopolyploids of *Arachis*. The analyses of secondary constrictions were done in *n* metaphases (or rarely in prometaphases) after being probed for 18S to 25S ribosomal DNA by fluorescent in situ hybridization.**

Species	<i>n</i>	Pairs with extended secondary constrictions observed at metaphase†					Max. no. of NORs
		A2	A10	B3	B7	B10	
		%					
<i>A. duranensis</i>	14	7 (1)	100 (2)				3
<i>A. ipaënsis</i>	15					100 (2)	2
<i>A. monticola</i>	10	20 (2)	100 (2)				4
<i>A. hypogaea</i> var. <i>hypogaea</i>	10	60 (2), 40 (1)	100 (2)				4
<i>A. hypogaea</i> var. <i>hirsuta</i>	6	–	100 (2)				2
<i>A. hypogaea</i> var. <i>fastigiata</i>	12	–	100 (2)				2
<i>A. hypogaea</i> var. <i>aequatoriana</i>	22	27 (1), 18 (2)	100 (2)		0	18 (2), 9 (1)	6
<i>A. hypogaea</i> var. <i>peruviana</i>	16	–	100 (2)				2
<i>A. hypogaea</i> var. <i>vulgaris</i>	18	–	100 (2)				2
Amphidiploid ( <i>A. ipaënsis</i> × <i>A. duranensis</i> ) <sup>4x</sup>	22	100 (2)	100 (2)	0	0	27 (1)‡	5

† Values in parentheses indicate the number of homologs of each chromosome pair with extended secondary constrictions. Example: 20 (1) = 20% of the analyzed cells with one homologous chromosome of this pair with extended secondary constriction.

‡ Bright and large signals but slightly extended.

heterochromatic bands observed in the synthetic AABB allopolyploid compared with its putative diploid parents indicates structural stability of this fraction during allopolyploidization. This stability is in disagreement with many model polyploid plants in which the appearance and/or disappearance of novel heterochromatic bands have often occurred during hybridization or chromosome doubling. Examples of this instability have been reported for the DAPI<sup>+</sup> heterochromatic bands in *Triticale* polyploids (Bento et al., 2011) and for the number of AT- and GC-rich bands in *Artemisia* (García et al., 2009).

The patterns of rDNA loci observed in the diploid *Arachis* species analyzed are in complete accordance with previous cytogenetic analyses (Seijo et al., 2004; Robledo et al., 2009; Robledo and Seijo, 2010). This conserved pattern is not frequent among the most cytogenetic studied model species like the synthetic *Arabidopsis suecica* (Fries) Norrlin, Meddel. (Pontes et al., 2004), *Nicotiana tabacum* L. (tobacco; Skalická et al., 2003), *Tragopogon* (Lim et al., 2008), and *Avena sativa* L. (Ueno and Morikawa, 2007). Therefore, the stability observed in rDNA loci after polyploidization in AABB *Arachis* polyploids arises as one of the few examples in crops in which the inheritance of the rDNA pattern (number and distribution) is not perturbed by hybridization and chromosome doubling events.

### Absence of Genomic Turnover and Intergenic Translocations Analyzed by GISH

The stability of the chromosome markers observed in the AABB *Arachis* polyploids analyzed may have at least two possible explanations. An obvious possibility is that no rearrangements occurred, whereas another possibility is that allopolyploidization-induced changes did occur, but in the genome fractions that had not been covered

by the chromosome markers analyzed in this study. To afford a more complete analysis, we performed double GISH keeping in mind that, in AABB *Arachis* allopolyploids, this technique clearly discriminates between the chromosomes of each diploid parental species (Seijo et al., 2007). The GISH in the synthetic AABB *Arachis* amphidiploid revealed that no detectable genome turnover of the parental chromosome complements occurred. Moreover, GISH was equally effective in the natural allopolyploids, suggesting that no major genome turnover in the dispersed repetitive fraction occurred since the spontaneous origin of *A. monticola* and *A. hypogaea* (dated 10,000–15,000 yr ago; Dillehay et al., 2007; Bertoli et al., 2016). This result is opposed to the case reported for other synthetic and spontaneous amphidiploids. For instance, GISH was less effective in natural tobacco than in synthetic tobacco, particularly with the chromosomes derived from the paternal progenitor *Nicotiana tomentosiformis* L. (Lim et al., 2007). That genomic turnover was accompanied by instability of paternal genomic DNA as revealed by sequence-specific amplification polymorphism analysis of retrotransposon populations (Petit et al., 2007). The results observed in *Arachis* evidenced that no intergenomic colonization by tandem repeats or mobile elements between the parental genomes of the AABB amphidiploids may have occurred massively, at a scale detectable by the GISH technique, in newly synthesized amphidiploids or in the spontaneous and well-stabilized species, *A. monticola* and *A. hypogaea*. The comparison of the retrotransposon fraction between *A. hypogaea* and its wild diploid progenitors supports the low activity of mobile elements during or after allopolyploidization, since no signs of massive reactivation of Ty3-gypsy (Nielen et al., 2009) or Ty1-copia retroelements were detected (Nielen et al., 2011). Similarly, the analysis of the L1 family of LINEs in diploid and

polyploid AABB *Arachis* species revealed no significant changes in the copy number or in the pattern of chromosome distribution (Samoluk et al., 2015b).

The low proportion of cross-hybridization observed in different chromosomes of the *Arachis* tetraploids as dispersed spots revealed that the A and B genomes of these polyploids share, to some extent, small and dispersed regions of homology. The homology between these two genomes may be explained by two alternative hypotheses: that the regions of homology may have arisen by intergenomic crosstalk after polyploidization (Lim et al., 2007), or that they may correspond to remains of ancestral conserved sequences in the two genomes. The fact that the cross-hybridization detected between the A and B genomes in natural polyploids was similar to that observed in the artificial amphidiploid and to the hybridization observed when genomic probes were tested onto diploid metaphases (i.e., DNA of *A. duranensis* onto metaphases of *A. ipaënsis* and vice versa, data not shown) supports the second hypothesis.

In this study, GISH was also used to detect intergenomic translocations. The results obtained evidenced no detectable chromosome rearrangements between the A and B complements in any of the AABB tetraploids analyzed. These results are in agreement with the conserved positions of the 18 to 26S rDNA and 5S rDNA loci in the A and B complements of the tetraploids compared with those observed in the diploid progenitors. Although distinct signals of tetrasomic recombination between the A and the B genomes have been recently inferred from sequence analysis (Leal-Bertioli et al., 2015), the interchanges were not large enough to be detected by GISH. The lack of large intergenomic translocations in *Arachis* AABB polyploids is in contrast with most cases in which a clear discrimination of the parental genome was possible by GISH. In most cases, intergenomic translocations were observed to occur after polyploidization. For example, GISH analysis identified nine intergenomic translocations in spontaneous tobacco (Kenton et al., 1993; Moscone et al., 1996), at least five intergenomic translocations in the allotetraploid *Avena maroccana* Gand., and as many as 18 in the allotetraploid *A. sativa* (Hayasaki et al., 2000). Thus, available evidence indicates that varying degrees of polyploidy-induced translocations occur as a rule in well-established allopolyploids and that, in many model plant species, they occur in the first generations. Therefore, the karyotype additivity detected here in newly synthesized and well-established AABB tetraploids of *Arachis* arises as one of the few cases among well-studied polyploids.

### Genome Size in Natural and Artificial AABB Polyploids of *Arachis*

The analysis of monoploid genome size (Cx) values in a broad range of Angiosperms has demonstrated that the amount of nuclear DNA in well-established polyploid

taxa is usually significantly less than the sum of DNA amounts of the parental species, suggesting that genome downsizing may be a widespread phenomenon of considerable biological significance (Leitch and Bennett, 2004; Eilam et al., 2008). In this study, the measurements in spontaneous *Arachis* AABB tetraploids showed that their 2C-DNA contents, even lower, did not depart significantly from the sum of the genome sizes of their diploid progenitors (*A. duranensis* and *A. ipaënsis*), as reported previously (Samoluk et al., 2015a). The artificially synthesized new AABB polyploid also reflected approximately the sum of genome sizes of its parents, suggesting that no significant changes in genome size have occurred in the early generations of this amphidiploid. Therefore, *Arachis* AABB spontaneous allotetraploids and the resynthesized amphidiploid should be included in the group of polyploid species with complete or nearly complete additivity (1–5% different) of DNA content with respect to their progenitors, like AD *Gossypium* allopolyploids (Hendrix and Stewart, 2005) and tobacco (Leitch and Bennett, 2004).

### Nucleolar Dominance of the A Genome

Nucleolar dominance refers to the phenomenon in hybrids or allopolyploids whereby nucleoli formation occurs in chromosomes inherited from only one of the two parents (Pikaard, 2000). Cytogenetically, this phenomenon has been described as differential amphiplasty that involves the disappearance of the satellite and secondary constriction of a chromosome (Navashin, 1934). Here, the presence of an extended secondary constriction in metaphase of *Arachis* species was used to estimate activation of ribosomal loci in the preceding interphase (Fernández and Krapovickas 1994; Pikaard, 2000; see Material and Methods).

To better understand the intergenomic interactions of the 18S to 26S rDNA loci in *Arachis* polyploids, an analysis was conducted among the loci within the diploid progenitors (intra-genomic analysis). The presence of extended secondary constriction in only the A10 pair (rarely in A2) in *A. duranensis* and in only the B10 pair (never in the B3 or B7) in *A. ipaënsis* suggests a strong intra-genomic dominance of those loci. The analysis of spontaneous AABB polyploids demonstrated a similar pattern of intra-genomic dominance of the 18S to 26S rDNA than in the parental diploids. That is, the A10 pair was active in all the cells, whereas those of the A2 pair were rarely expressed only in two of the six varieties of *A. hypogaea*. Similarly, whenever the NORs of the B complement were active in the tetraploids (only in *A. hypogaea* var. *aequatoriana*), they corresponded to those of the B10 pair.

The analysis of the spontaneous AABB allopolyploids also showed the occurrence of intergenomic dominance. That is, the A10 (and less frequently the A2) pair showed extended secondary constriction in all the cells, whereas those of the B10 pair were very rarely extended. Moreover,

the quantitative analysis of the metaphases suggests that the 18S to 26S rDNA loci follow a hierarchical pattern of activation: those of the A10 pair were the first to be transcriptionally activated, then those of the A2, and finally those of the B10 chromosomes. This phenomenon was more completely evident in *A. hypogaea* var. *aequatoriana*, since in the other varieties of peanut and in *A. monticola*, only the A10 and the A2 chromosomes or solely the A10 pair presented secondary constriction. The variability of the rDNA activation observed among different varieties of *A. hypogaea* and in *A. monticola* may be determined by small differences in the mechanism required for cytosine methylation and repressive histone modifications to establish a heterochromatic state that inactivates ribosomal RNA gene loci on a multimegabase scale (Preuss et al., 2008).

The analysis of the artificial AABB amphidiploid evidenced that the A genome nucleolar dominance was established in the first generations. Moreover, the presence of extended secondary constriction in the A2 pair in all the cells analyzed suggests that the demand of rDNA in the newly polyploid nuclei was mainly covered by these loci rather than by those of the B10, as expected. The establishment of nucleolar dominance in the first generations is a general phenomenon reported for other allopolyploids such as *Brassica*, *Triticum aestivum* L., *Hordeum*, and *Triticale* (reviewed in Chen and Pikaard, 1997; Pikaard 2000). However, the facts that the hybridization signals in the B10 chromosomes were large and more frequently extended and that the A2 loci were more frequently active in the artificial amphidiploid than those of the spontaneous AABB allopolyploids suggest that the process of stabilizing rDNA expression in the former required further medium-term genetic and epigenetic rearrangements (Chen and Pikaard, 1997; Lim et al., 2008; Książczyk et al., 2011) to reach the pattern observed in the spontaneous polyploids.

Pioneering research with synthetic polyploids suggested that the dominance of the 18S to 26S rDNA loci evolved toward the maternal version (Song et al., 1995), whereas in other polyploids, it was demonstrated that the dominance is independent of the maternal effect and ploidy level (Chen and Pikaard, 1997; Liu et al., 1998; Joly et al., 2004). The whole analysis done in the present study showed that the 18S to 26S rDNA loci of *A. duranensis* were preferentially expressed in the AABB polyploids, irrespective of whether this species acted as the female (*A. hypogaea* and *A. monticola*; Grabile et al., 2012) or the male (artificial AABB amphidiploid; Fávero et al., 2006) progenitor. These results suggest that the nucleolar dominance is a genome-specific phenomenon in the *Arachis* AABB tetraploids.

## CONCLUSIONS

The comparative analysis of the recreated allopolyploid, with those occurring spontaneously in prehistorical times giving rise to *A. monticola* and the cultivated peanut,

allowed the investigation of the impact of allopolyploidy on the macroscale chromosome structure. In contrast with the data from other crops, such as *Avena*, (Hayasaki et al., 2000), *Nicotiana* (Kenton et al., 1993, Moscone et al., 1996), *Triticum* (Mukai et al., 1993), and other plant groups (Lim et al., 2008, Madlung et al., 2005), our results demonstrated that the karyotype macrostructure and DNA content of the parental species remained without detectable changes in the new and the meso-AABB polyploids of *Arachis*. However, genome-dependent amphiplasty was evident in all of them, with a clear dominance of the A genome NORs.

The facts that natural and synthetic allotetraploids displayed conserved macrostructure of the chromosome complements (this study) and low or null activity of repetitive elements (Nielen et al., 2009, 2011; Samoluk et al., 2015b) suggest that the huge amount of morphological variability observed in the >100 local races of cultivated peanut may be viewed as a consequence of few mutations in the low copy fraction, intergenomic recombination (undetectable by cytogenetic analysis) (Leal-Bertioli et al., 2015), gene conversion or remodeling of the transcriptome and the epigenetic landscape during allopolyploidization (Wang et al., 2004; Madlung et al., 2005; Chen, 2007; Jackson and Chen, 2010; Soltis et al., 2014), and further evolution rather than due to macrostructural chromosomal instability.

The large-scale chromosome stability of the parental genomes detected in the natural and synthetic AABB polyploid genomes may have a high impact on peanut breeding. The conserved collinearity between diploid and polyploid genomes may allow recombination between the homologous complements favoring the introgression of wild alleles in most of the extension of the A and B chromosome complement. However, the A genome dominance in the expression of the rDNA loci observed in the present study suggests that biases in the expression of the desirable characters may occur in the AABB *Arachis* polyploids.

## Conflict of Interest

The authors declare that there is no conflict of interest.

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