# *rDNA loci and heterochromatin positions support a distinct genome type for 'x = 9 species' of section* Arachis *(*Arachis, *Leguminosae)*

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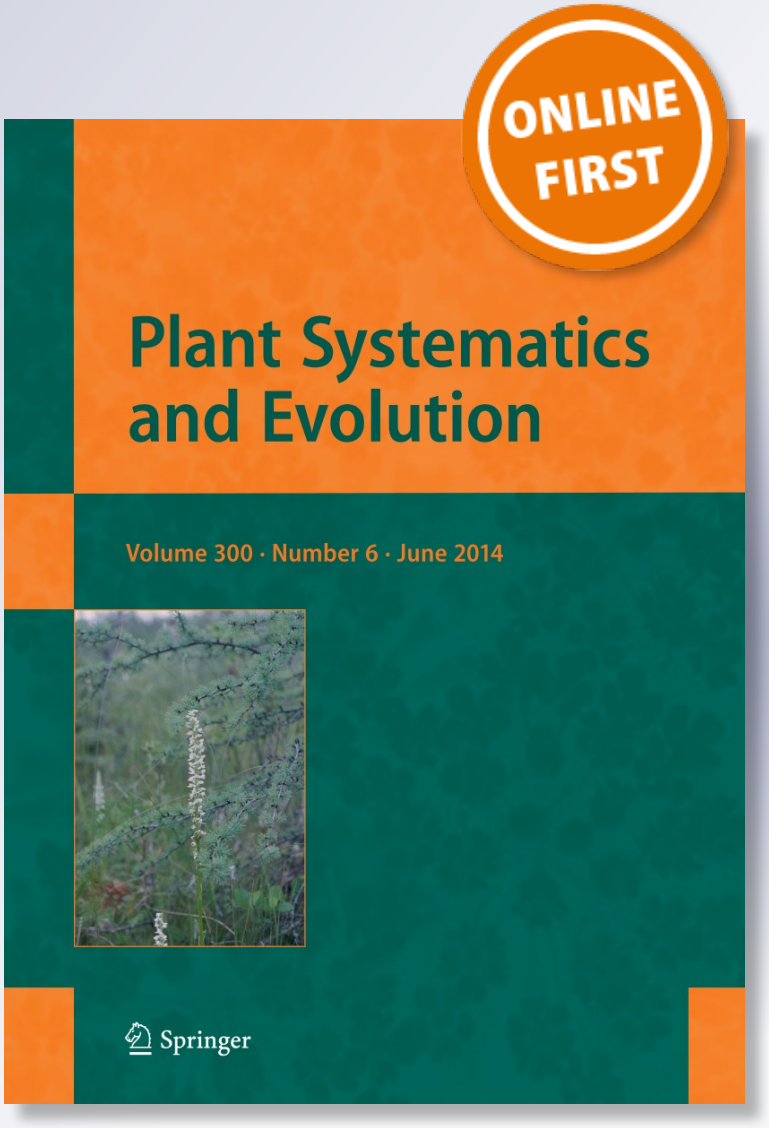


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ORIGINAL ARTICLE

# rDNA loci and heterochromatin positions support a distinct genome type for '*x* = 9 species' of section *Arachis* (*Arachis*, Leguminosae)

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**Abstract** Most species of the genus *Arachis* (Leguminosae; 80 spp.) are diploid with $x = 10$ and only four species have $x = 9$ chromosomes. Three of these $x = 9$ species belong to section *Arachis* and are morphologically and chromosomally similar. To study the homeology of the genomes of $x = 9$ species and their relation to other genomes in section *Arachis*, we applied fluorescence in situ hybridization (FISH) of 18S–26S and 5S rDNA and 4′,6-diamidino-2-phenylindole (DAPI) banding. FISH revealed for these three species one pair of 5S rDNA sites interstitially within the short arm of the metacentric pair 6 and one pair of 18S–26S rDNA sites in the proximal region of the long arm of the SAT chromosomes. Conspicuous DAPI+ bands were detected pericentromerically in all nine chromosome pairs of *A. decora* and *A. praecox* and in all but one pair of *A. palustris*. Our results suggest that all three species with $x = 9$ of section *Arachis* share the same genome type and are different from the other genome types A, B, D, F, and K described for this section. Apparently, the $x = 9$ species of section *Arachis* form a monophyletic group characterized by a genome type, that we propose to call G genome.

**Keywords** Chromosomes · DAPI+ bands · FISH Genome type · rDNA sites · $x = 9$ species of section *Arachis*

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## Introduction

The genus *Arachis* (Leguminosae), which is native to South America, has 80 formally recognized species, including the economically important peanut species *A. hypogaea* L. *Arachis* species are divided into nine sections according to morphology, geographic distribution, and cross-compatibility (Krapovickas and Gregory 1994; Valls and Simpson 2005). Chromosome numbers are currently known for 76 species (Lavia et al. 2008 and references therein). Most of them (67 spp.) are diploid with $x = 10$ ($2n = 20$), a few (4 spp.) are diploid with $x = 9$ ($2n = 18$), and the rest (5 spp.) are tetraploid with $x = 10$. Three of the diploid $x = 9$ species, *A. decora* Krapov. W.C. Gregory and Valls, *A. palustris* Krapov. W.C. Gregory and Valls, and *A. praecox* Krapov. W.C. Gregory and Valls (hereafter called $x = 9$ species), belong to section *Arachis*, whereas one species, *A. porphyrocalyx* Valls and C.E. Simpson, belongs to section *Erectoides*.

The $x = 9$ species of section *Arachis* are annual plants from Brazil (Fig. 1). *Arachis palustris* is found in the northern part of the distribution range, extending on both sides of the Tocantins River in the states of Maranhão and Tocantins between 7°22′S and 12°33′S. *Arachis decora* is distributed in the northeast of the state of Goiás and in the south of state of Tocantins, separated from *A. palustris* by approximately 230 km. The two species co-occur in the Tocantins River basin, implying that there is a geographic continuity between the two species. In contrast, the third species, *A. praecox*, is apparently known only from two populations in the state of Mato Grosso, over 1,000 km away from the other two species.

Morphologically, the three $x = 9$ species are highly similar to one another (Veiga et al. 2001). *Arachis praecox* is distinguished by its short main shoot axis, only up to

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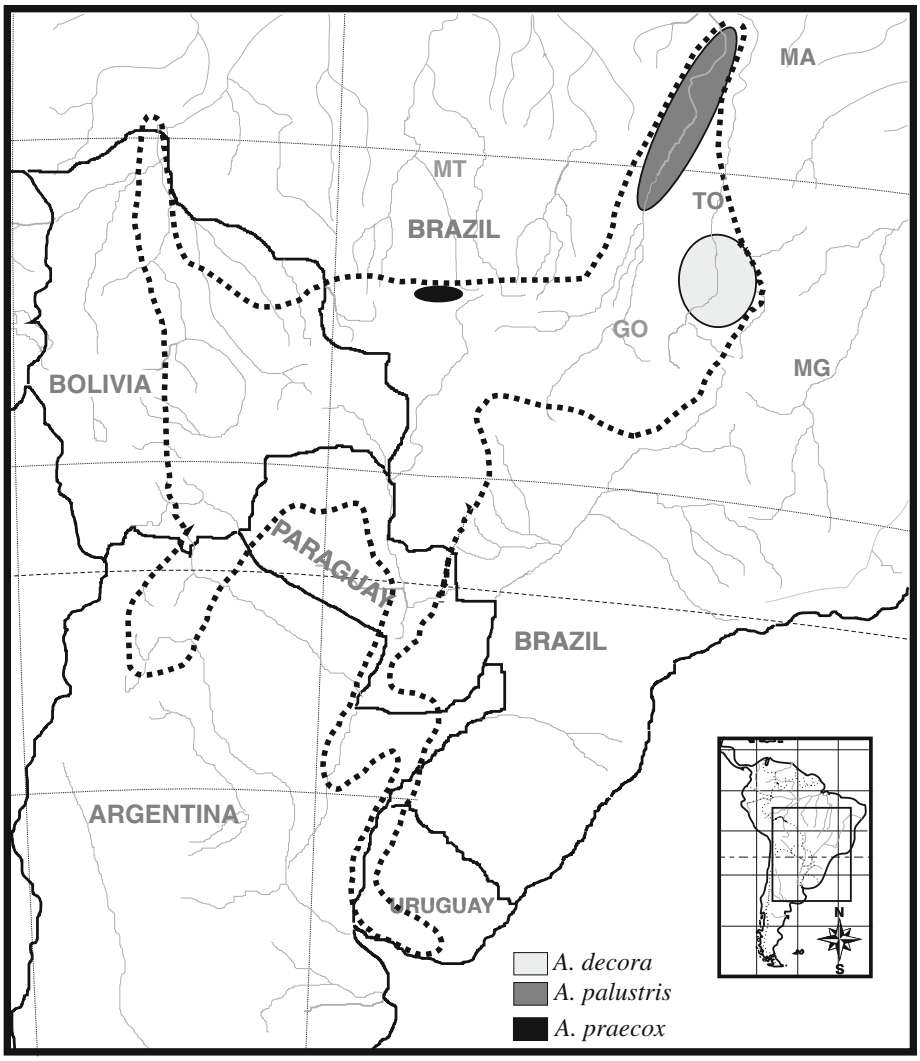


**Fig. 1** Geographic distribution of *Arachis* species with $x = 9$ of section *Arachis*. *Light gray A. decora*, *dark gray A. palustris*, *black A. praecox*. The *dashed line* indicates the distribution of the whole section *Arachis*

2–3 cm long, whereas *A. decora* and *A. palustris* have a main shoot axis of up to 15 cm long. *Arachis decora* differs from *A. palustris* only in the presence of setae on stipules (Krapovickas and Gregory 1994). Although previous studies of our lab have shown that they share the karyotype formula (16m + 2sm) with the same SAT chromosome type 3 (Lavia 1998), they are still poorly characterized chromosomally, and their genomic constitution has not been assigned to any of the known genomes in section *Arachis* (Robledo et al. 2009; Robledo and Seijo 2010).

At least five different genomes, A, B, D, F, and K, have been described in section *Arachis* (Robledo and Seijo 2008, 2010; Robledo et al. 2009). The A genome is characterized by the presence of the A chromosome pair, which is significantly smaller than the rest of the chromosomes of the complement, and the arms display a differential condensation pattern (Husted 1933, 1936; Fernández and Krapovickas 1994). The B genome, traditionally characterized by the absence of the A pair, has recently been split into the three genome types: B s.s., F, and K (Robledo and Seijo 2010). The D genome is unique to *A. glandulifera* and is characterized by an asymmetric karyotype with several subtelocentric chromosomes and the absence of the A pair (Robledo and Seijo 2008; Stalker 1991).

Analyses of molecular datasets have revealed that the three $x = 9$ species form a clade (Bechara et al. 2010; Friend et al. 2010; Moretzshon et al. 2013), but their relationship with other species of the section is still unclear. For example, analyses of microsatellites (Moretzshon et al. 2004), single-copy gene sequences (Moretzshon et al. 2013), trnT-F cpDNA marker (Tallury et al. 2005), and ITS and 5.8S of the nuclear rDNA (Bechara et al. 2010) suggest that these taxa are more closely related to some non-A genome species; whereas, RAPD (Creste et al. 2005) and AFLP (Milla et al. 2005) analyses suggest that they are genetically more similar to A genome species.

Based on current morphological, chromosomal, and molecular evidence, it is difficult to address the genome

**Table 1** List of the *Arachis* species studied and their collector and provenance

| Species | Collector[a] and Provenance[b] |
|---|---|
| *A. decora* Krapov., W.C. Gregory and Valls | VSW 9955. Brasil, GO, Mun. Campos Bellos, 20 km northeast of Campos Bellos, on the way to Aurora do Norte. 13°01′S 46°42′W. Type |
| *A. palustris* Krapov., W.C. Gregory and Valls | VPmSv 13023. Brasil, TO, Mun Filadelfia 7°25′S 43°37′W. |
| *A. praecox* Krapov., W.C. Gregory and Valls | VSGr 6416. Brasil, MT, Mun. Barra do Bugres, 71 km north of Cáceres, on the way to Barra do Bugres. Type |

[a] Gr = A. Gripp; Pm = R.N. Pittman, S = C.E. Simpson, Sv = G.P. Silva, V = J.F.M. Valls, W = W.L. Werneck

[b] GO = Goias state; MT = Mato Grosso state, TO = Tocantins state

identity of the three $x = 9$ species. Nevertheless, the fact that these species form a group within section *Arachis* suggests that they may be phylogenetically related. Indeed, we have previously proposed a single origin for these species (Lavia et al. 2008 and references therein). To determine their genomic affiliation and understand their relationship with other species of the section, detailed chromosomal analyses are necessary. Fluorescent in situ hybridization (FISH) has been successfully used to analyze homeology of genomes (Jiang and Gill 1994). For instance, 4́,6-diamidino-2-phenylindole (DAPI) banding and physical mapping of ribosomal genes by FISH in other *Arachis* species has increased the number of chromosome markers, which has allowed establishing homeologies in a large number of chromosome pairs (Ortiz et al. 2011; Seijo et al. 2004), particularly in species with A and non-A genomes of section *Arachis* (Robledo et al. 2009; Robledo and Seijo 2010).

Taking these antecedents into consideration, the goals of the present work were: (1) to describe chromosomal markers that contribute to the genomic identification of the $x = 9$ species; (2) to provide information about the homeology of genomes within section *Arachis*; and (3) to discuss the implications of our results to increase our understanding of relationships with other species of section *Arachis*. To this end, we analyzed distribution patterns of heterochromatin using DAPI and the mapping of the ribosomal gene loci using FISH.

## Materials and methods

### Plant material

Seeds of the *Arachis* species used in this study were obtained from the peanut germplasm collections of the Instituto de Botánica del Nordeste (IBONE) Corrientes, Argentina. The voucher material is deposited in the herbaria CTES and CEN. Origin and collection information of voucher specimens is listed in Table 1.

### Chromosome preparations

Actively growing root-tips (10–15 mm long) from germinating seeds were pretreated with 2 mM 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 digested in 1 % (w/v) cellulose (from *Trichoderma viridae*, Onozuka R-10; Serva, Heidelberg, Germany) plus 10 % (v/v) pectinase dissolved in 40 % glycerol (from *Aspergillus niger*; Sigma-Aldrich, St. Louis, MO, USA) in 0.01 M/L citrate buffer, pH 4.8, at 37 °C for 1 h. Subsequently, meristematic cells were removed from the root tips, squashed in 45 % acetic acid on a slide, and covered with a coverslip. After removal of the coverslip with carbon dioxide, the slides were air-dried for 1–2 days at room temperature and then stored at −20 °C until use.

### Probe labeling and fluorescent in situ hybridization

The 5S and 18S–26S rDNA loci were localized using probes pA5S, pA18S and pA26S isolated from genomic DNA of *A. hypogaea* (Robledo and Seijo 2008) and labeled by nick translation with digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany) or biotin-11-dUTP (Sigma-Aldrich). Pretreatment of slides, 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, with a stringency to allow sequences with 80–85 % identity to remain hybridized), post-hybridization washing, blocking, and indirect detection with fluorochrome-conjugated antibodies were performed according to Moscone et al. (1996). The first set of antibodies consisted of anti-biotin produced in goat (Sigma-Aldrich) and monoclonal anti-digoxigenin conjugated to fluorescein isothiocyanate (FITC) produced in mouse (Sigma-Aldrich). The second set consisted of anti-goat conjugated to tetramethyl-rhodamine isothiocyanate (TRITC) produced in rabbit (Sigma-Aldrich) and anti-mouse conjugated to FITC produced in sheep (Sigma-Aldrich). Preparations were counterstained by mounting them with Vectashield medium (Vector Laboratories, USA) containing 2 mg/mL of 4′,6-diamidino-2-phenylindole (DAPI). Counterstaining with DAPI reveals a C-banding-like pattern with major heterochromatic bands

fluorescing more intensely in *Arachis* species (Seijo et al. 2004).

### Fluorescence microscopy and image acquisition

Chromosomes were viewed with a Leica DMRX fluorescence microscope (Leica, Heerbrugg, Switzerland) and digitally photographed with a computer-assisted Leica DC 350 digital camera system. Red, green, and blue images were captured in black and white using the respective filters for TRITC, FITC, and DAPI excitations. Digital images were processed with Photoshop, version 7.0 (Adobe, San Jose, CA, USA).

### Karyotype analysis and loci mapping

For karyotype determination, we used 3–6 individuals per species and four metaphase plates per individual. Chromosome measurements were made using the computer application MicroMeasure version 3.3 (Reeves and Tear 2000). Karyotype description is based on the nomenclature by Levan et al. (1964). Chromosomes were classified in two categories according to the centromeric index (CI = short arm × 100/total length of chromosome): metacentric (m) when CI = 50–37.5, and submetacentric (sm) when CI = 37.5–25. SAT chromosomes were classified on the basis of the satellite relative size and position of the centromere (Fernández and Krapovickas 1994). The total chromosome length (TCL) was obtained by summing the average length of each chromosome in the four metaphase samples. Chromosome mean length was calculated by dividing the TCL by the number of chromosomes of the species. The karyotype asymmetry indices were estimated using the intrachromosomal (A1) and interchromosomal (A2) indexes by Romero Zarco (1986).

Data from homeologous chromosomes were combined for each species to obtain mean values of different pairs of chromosomes in the same metaphases, and, subsequently, of the same chromosome pair in different metaphases. Mean values of each species were represented as haploid complements in the ideograms. Chromosomes were ordered first by morphology and then by decreasing size. Based on the analysis of results of the FISH experiments with the 18S–26S and 5S ribosomal genes and of the DAPI staining, we constructed consensus ideograms for each of the $x = 9$ species studied.

## Results

The general karyotype features, and percentage of heterochromatin per chromosome complement and per chromosomes bearing 5S and 18S–26S rDNA for the three $x = 9$ *Arachis* species are listed in Table 2. Representative somatic metaphases are shown in Fig. 2, and the consensus ideograms for each species are illustrated in Fig. 3.

### General karyotype features

In all species, karyotypes consisted mainly of metacentric chromosomes, reflected in their karyotype formula 16m + 2 sm (Fig. 3). The mean chromosome length ranged between 2.65 µm (*A. decora*) and 3.32 µm (*A. praecox*), and the total karyotype length between 47.70 (*A. decora*) and 59.76 µm (*A. praecox*) (Table 2). A1 ranged from 0.21 (*A. decora*) to 0.25 µm (*A. praecox*), while A2 ranged between 0.11 (*A. praecox*) and 0.14 (*A. decora*). The A1 values indicate a slight variation between the length of the short and long chromosome arms in each species, whereas the A2 values indicate high similarity in chromosome sizes in each species. In other words, the two asymmetry indexes indicate that the karyotypes of the three species are symmetric (Table 2).

### Heterochromatin distribution

The counterstaining with DAPI revealed a C-banding-like pattern. Conspicuous DAPI+ pericentromeric bands were present in all chromosome pairs in *A. praecox* and *A. decora*, whereas they were present in all but one pair (pair 5) in *A. palustris* (Fig. 3). Furthermore, in *A. praecox* and *A.*

**Table 2** Karyotypic features in $x = 9$ species of section *Arachis*

| Species | Karyotype formula | Total chromosome length, µm (SE) | Chromosome mean length, µm (SE) | CI | Asymmetry indexes | | Heterochromatin mean percentage | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | $A_1$ | $A_2$ | Karyotype | Pair SAT | Pair with 5S |
| *A. decora* | 16m + 2sm | 47.70 (0.69) | 2.65 (0.09) | 43.20 (0.01) | 0.21 | 0.14 | 20.70 | 16.78 | 23.01 |
| *A. palustris* | 16m + 2sm | 48. 06 (0.81) | 2.67 (0.09) | 43.20 (0.01) | 0.23 | 0.12 | 23.03 | 17.27 | 23.03 |
| *A. praecox* | 16m + 2sm | 59.76 (0.36) | 3.32 (0.03) | 42.50 (0.01) | 0.25 | 0.11 | 24.07 | 17.61 | 30.01 |

*CI* centromeric index, $A_1$ intra-chromosomal asymmetry index, $A_2$ inter-chromosomal asymmetry index, *m* metacentric, *sm* submetacentric, *SE* standard error

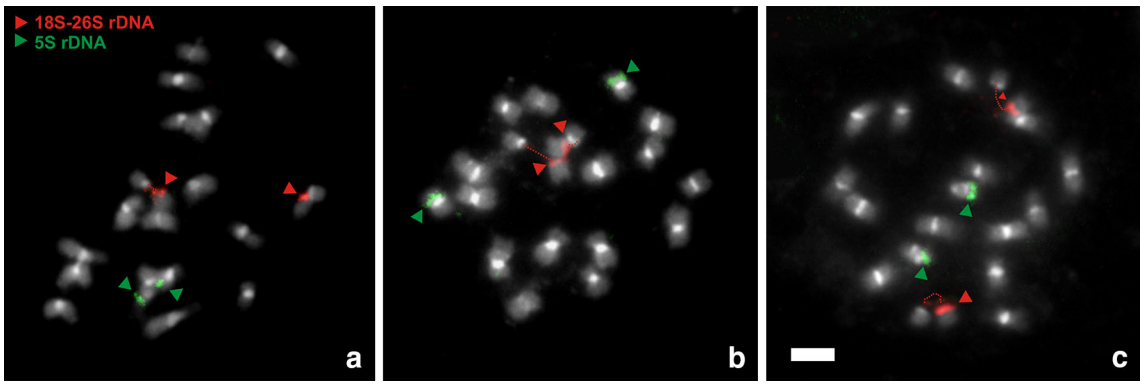


**Fig. 2** Somatic metaphases of species of section *Arachis* with $x = 9$ after double fluorescent in situ hybridization (FISH). **a** *A. decora*, **b** *A. palustris*, **c** *A. praecox*. The 5S rDNA loci are illustrated by the *green* signals and the 18S–26S rDNA loci by the *red* signals. DAPI counterstaining, in *gray* is highlighting the heterochromatin bands. The *arrows* illustrate the homologous position of rDNA signals. *Scale bar* 2 μm

*palustris*, we observed a chromosome pair with one additional weak heterochromatin band in interstitial position. In *A. praecox*, this band was 0.4 μm and was located on the long arm of pair 6, whereas *in A. palustris*, the band was shorter (0.29 μm) and was located on the long arm of pair 1.

The percentage of heterochromatin in relation to chromosome length in pair SAT was similar in the three species but in the pair with 5S rDNA signal was significantly higher in *A. praecox* (30.01 %) than in *A. palustris* (23.03 %) and *A. decora* (23.01 %) (Table 2). The total percentages of heterochromatin 24.07 and 23.03 are a clear overstatement of the resolution, taking into account the high variability of chromosome condensation and band appearance in such images as shown in Table 2.

### Chromosome mapping of the 5S and 18S–26S rRNA genes by FISH

All species had only one pair of 18S–26S rDNA sites localized in the proximal region of the long arm of SAT chromosomes. This pair is a SAT chromosome type 3 and the longest of the complements in the three species. It is submetacentric, except in *A. praecox* where it is metacentric (Fig. 3).

One pair of 5S rDNA sites was observed in interstitial position of the short arm in pair 6 in the three species. We also observed a DAPI+ interstitial band on the long arm of chromosome pair with 5S rDNA sites in *A. praecox* (Figs. 2, 3).

## Discussion

The present work is the first to analyze the distribution of heterochromatin and location of ribosomal sites in three *Arachis* species of section *Arachis* characterized by $x = 9$, unusual in the genus. We used results to identify homeologous chromosomes, define the genome type of these species, and discuss possible relationships of the $x = 9$ species with closely related $x = 10$ species with known genome types A, B, F, D, or K (Robledo et al. 2009; Robledo and Seijo 2010).

### Chromosomal patterns of heterochromatin and 5S and 18S–26S rDNA loci

The identification of heterochromatic bands and localization of rDNA loci showed that the three $x = 9$ species of section *Arachis* are genomically similar. First, they all lack the small A chromosome pair, which supports findings from a previous work that also suggest the absence of such chromosome pair (Lavia 1998). Second, all species have pericentromeric DAPI+ bands with the same brightness, position, and size in all chromosome pairs, except *A. palustris*, which lacks these bands in one chromosome pair (pair 5). Third, the three species have only one pair of 18S–26S rDNA sites in the SAT chromosomes, and one pair of 5S rDNA sites in chromosome pair 6.

The homeology observed among chromosomes supports the hypothesis that the $x = 9$ species derive from the same common ancestor, as previously suggested (Creste et al. 2005; Lavia et al. 2008; Friend et al. 2010). On the other hand, some results such as the length of chromosomes, the percentage of heterochromatin in the chromosome pair with 5S rDNA, and the similarity in external morphology and the geographic distribution of the plants suggest that *A. decora* and *A. palustris* are more similar to each other than to *A. praecox*. Such relationship pattern agrees with results from molecular studies by Creste et al. (2005), in which the $x = 9$ species form a cluster, and, within this, *A. praecox* is sister to a cluster comprising *A. decora* and *A. palustris*.

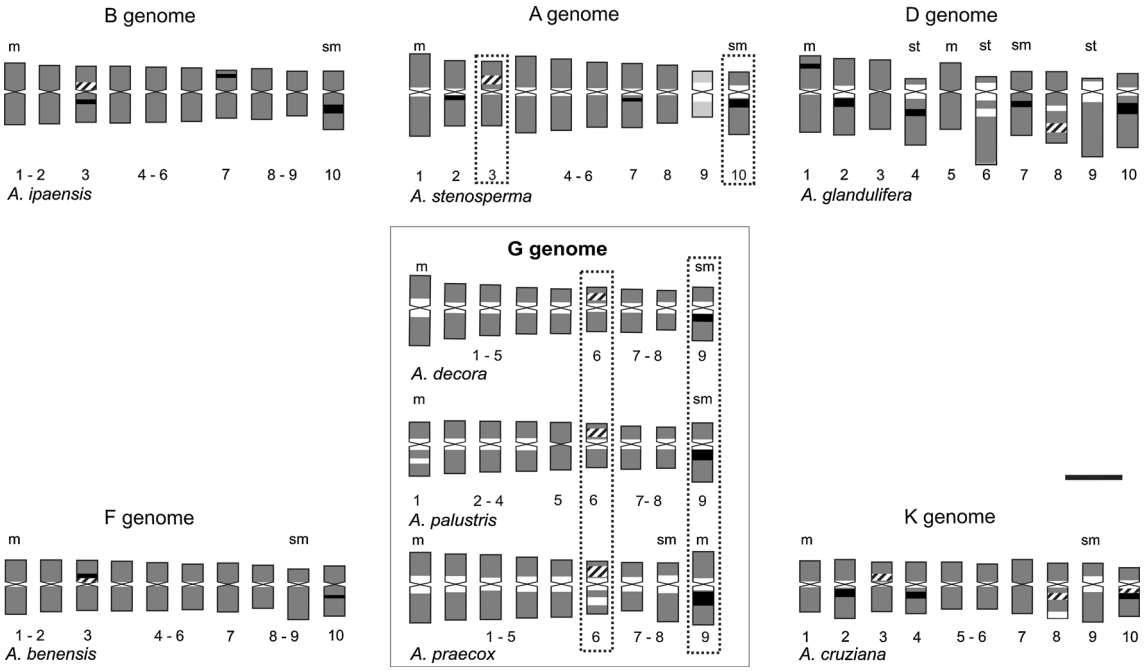


**Fig. 3** Ideograms of species belonging to section *Arachis*. Species with $x = 9$ are grouped in the G genome box, with chromosomes ordered by morphology according to decreasing size. Ideograms of $x = 10$ species (A, B, D, F and K genomes) were adapted from Robledo et al. (2009) and Robledo and Seijo (2010). The A genome is located above the G genome to facilitate comparison and homeologous pairs are highlighted with *dotted lines*. *Striped bands* illustrate 5S rDNA loci; *black bands* illustrate 18S–26S rDNA loci; *white bands* illustrate DAPI+ heterochromatic regions. Chromosomes with similar morphology are arranged in groups. *m* submetacentric, *sm* submetacentric, *st* subtelocentric. *Scale bar* 3 μm

Based on the number and localization of rDNA sites and the heterochromatic pattern of chromosome pairs carrying the landmarks analyzed, we suggest the following homeologies between $x = 9$ species and the other *Arachis* species (Fig. 3). Pair 9 carries the 18S–26S rDNA locus and corresponds to pair A10, and pair 6 with the 5S rDNA locus to pair A3 of the A genome (Seijo et al. 2004; Robledo et al. 2009; Robledo and Seijo 2010).

## Relationships with other species of section Arachis and genomic assignment

Relationships among $x = 9$ and $x = 10$ species of section *Arachis* have been studied using different approaches, ranging from crossing experiments (in which obtaining viable or non-fertile interspecific hybrids is a test of the degree of relationship between the parent species), to molecular phylogenetic analyses, and the comparative chromosome analysis presented here.

In their crossing experiments, Stalker et al. (1991) reported viable hybrids between *A. palustris* ($x = 9$) and *A. duranensis* ($x = 10$, A genome), but recent crosses by Tallury et al. (2005) and Custodio (2009) between $x = 9$ species and $x = 10$ species with A, K or B s.s. genome produced non-fertile hybrids.

After analyzing the results obtained by Stalker et al. (1991), we consider that their conclusions are doubtful, because their reported mean of bivalents (II = 9.89) is higher than expected in a hybrid with $2n = 19$. The most likely explanation for their results is that the siblings they analyzed resulted from selfing of the female progenitor *A. duranensis* with $2n = 20$. Therefore, the absence of viable hybrids between $x = 9$ species and other species of section *Arachis* suggests that $x = 9$ species are reproductively isolated.

The molecular phylogenetic relationships of the $x = 9$ species with the rest of the section are still unclear, in some studies the $x = 9$ species are more closely related to non-A genome species, whereas in others they are more genetically similar to A genome specie. To provide new insights into such relationships, in the present study, we compared the new identified genome characteristics of $x = 9$ species with previously published characteristics of the A, B, D, F, and K genomes of section *Arachis*.

A genome species have the same DAPI banding pattern as $x = 9$ species, a single pair of 5S rDNA sites in interstitial position, and an inter-chromosomal asymmetry index similar to that of $x = 9$ species (Robledo et al. 2009). However, the heterochromatic bands in $x = 9$ species are more conspicuous and are of very similar size among

chromosomes than those in A genome species. In addition, $x = 9$ species have only one pair of 18S–26S rDNA sites, while A genome species have two (*A. correntina*, *A. duranensis*, *A. schininii* and *A. villosa*) or four (*A. cardenasii*) (Robledo et al. 2009). Finally, and most importantly, $x = 9$ species lack the "A" chromosomes characteristic of the A genome species.

The B genome detected in five species (*A. ipaensis*, *A. magna*, *A. gregoryi*, *A. valida* and *A. williamsii*) is characterized by the absence of DAPI heterochromatic bands in all chromosomes of the complement (Robledo and Seijo 2010), while $x = 9$ species have heterochromatic bands in all chromosomes, indicating that they do not share the genome type.

The D genome occurs only in *A. glandulifera* and is characterized by an asymmetric karyotype (Stalker 1991; Fernández and Krapovickas 1994), only seven chromosomes with DAPI+ pericentromeric bands, and five pairs of 18S–26S rDNA sites (Robledo and Seijo 2008). The genome of $x = 9$ species displays none of these characteristics, since all $x = 9$ species have a symmetric karyotype (mainly composed of metacentric chromosomes), almost all or all chromosomes with DAPI+ pericentromeric bands, and only one pair of 18S–26S rDNA sites.

Species characterized by the F genome, *A. trinitensis* and *A. benensis* (Robledo and Seijo 2010), have one pair of each ribosomal site, as the $x = 9$ species. However, their small and faint centromeric bands occur in seven or eight of ten chromosome pairs, whereas in $x = 9$ species the bands are conspicuous and occur in all or almost all chromosomes.

Finally, the K genome characterizes three *Arachis* species*: A. batizocoi*, *A. cruziana*, and *A. krapovickasii* (Robledo and Seijo 2010). Similarly to $x = 9$ species, K genome species have conspicuous centromeric DAPI+ bands in nine of ten chromosome pairs, but differ in the number and position of the ribosomal sites. K genome species have in fact three chromosome pairs with interstitial sites of 5S rDNA, one of which co-localizes with an 18S–26S rDNA site (Robledo and Seijo 2010).

Our comparative analyses indicate that $x = 9$ species are chromosomally most closely related to A genome species, because of their similarities in centromeric heterochromatic bands, the interchromosomal asymmetry index and the same number and position of 5S rDNA sites. Yet, the observed genome characteristics of $x = 9$ species are different from any known genome type, since the basic chromosome number is different. Therefore, the three $x = 9$ species of section *Arachis* seem to have their own genome type, which we here propose as the G genome.

In conclusion, the morphological similarities, the incompatibility with other species of section *Arachis,* the reduced basic number (different from the rest of the section), and our results on the uniformity of DAPI bands and the ribosomal signals in the three $x = 9$ species of section *Arachis* suggest that these species form a monophyletic group characterized by its own G genome. It has been proposed that $x = 9$ *Arachis* species originated from non-A genome species of section *Arachis* (Bechara et al. 2010). However, our cytogenetic evidences suggest that it is possible that these species derived from an ancestor with $x = 10$ and an A genome through some cytogenetic mechanism yet to be determined.

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