Study of the Evolution of Cultivated Peanut through Crossability Studies among *Arachis ipaënsis*, *A. duranensis*, and *A. hypogaea*

Alessandra P. Fávero, Charles E. Simpson,* José F. M. Valls, and Natal A. Vello

ABSTRACT

Genus *Arachis* L. includes 80 described species, of which 31 belong to section *Arachis*, including the two diploid species *A. ipaënsis* Krapov. and W.C. Gregory and *A. duranensis* Krapov. and W.C. Gregory, considered the putative B and A genome parents of the cultivated peanut. This work contributes to the study of evolution of the peanut (*Arachis hypogaea* L.), based on the successful hybridization between *A. ipaënsis* and *A. duranensis*, chromosome doubling of the hybrid, and crosses between the synthetic amphidiploid and representatives of the diversity of the crop. Diploid hybrids between *A. ipaënsis* and *A. duranensis*, confirmed by molecular markers, had pollen stains of 0.98%. Colchicine-induced tetraploids were confirmed by mitotic chromosome counts. Progeny from these amphidiploid plants had a 97.74% pollen stain and significant differences among structure sizes measured in diploid and tetraploid flowers. Hybrid individuals [*A. hypogaea* × (*A. ipaënsis* × *A. duranensis*)^{4x}] were produced from crosses involving all six botanical varieties of *A. hypogaea*. These hybrids indicate the evolutionary similarity between the wild species and the cultigen. The successful hybridization between diploid species *A. ipaënsis* and *A. duranensis* and between *A. hypogaea* and the synthetic amphidiploid support the theory that these two diploids are the parents of the cultivated peanut. Resulting materials are of great importance to peanut breeding.

THE GENUS *Arachis* includes 80 species (Valls and Simpson, 1994), 69 described by Krapovickas and Gregory (1994) and 11 described by Valls and Simpson (2005), 31 of which belong to the section *Arachis*. In this section, diploid species have annual or perennial behavior and show variable degrees of affinity to the complementary genomes that compose *A. hypogaea* and *A. monticola* Krapov and Rigoni, the two tetraploid species of the section. Husted (1933, 1936) observed the presence of two quite distinct chromosome pairs in the cultivated peanut: a pair he called A, with different staining behavior and much smaller than the remaining chromosomes, and another pair, with a secondary constriction, he called B. In spite of the observation by Husted (1936) and Smartt et al. (1978) of occasional meiotic tetravalents, the predominant bivalent pairing pattern of the chromosomes indicates the allotetraploid (or segmental allotetraploid) nature of *A. hypogaea* (Gregory and Gregory, 1976).

Alessandra P. Fávero and José F. M. Valls, Embrapa Genetic Resources and Biotechnology, SAIN Parque Estação Biológica, CP 02372, 70.770-900, Brasília, DF, Brazil; Charles E. Simpson, Texas Agric. Exp. Stn., Texas A&M University, 1229 U.S. Hwy. 281 North, Stephenville, TX 76401-0004; Natal A. Vello, Department of Genetics, Escola Superior de Agricultura "Luiz de Queiroz", ESALQ/USP, C.P. 9, 13418-900, Piracicaba, SP, Brazil. J.F.M.V. received a CNPq Fellowship. Received 17 Oct. 2005. *Corresponding author (c-simpson@tamu.edu).

Gregory and Gregory (1976) proposed that the peanut could have developed through the crossing between an annual and a perennial species, both of the section *Arachis*. They suggested that detailed karyotype studies followed by experimental interspecific crossings should be made for parental identification. Re-creation of *A. monticola* or *A. hypogaea* was considered by them a real possibility of considerable interest. Smartt et al. (1978) suggested that *A. hypogaea* probably developed from the hybridization of two diploid wild species with different genomes.

The perennial species of the section *Arachis* have 20 chromosomes, including Husted's A pair and show more similarity and better crossability to each other than those that do not have the A pair, generally being classified as the A genome species (Stalker, 1989; Stalker et al., 1991). The same applies to the annual *A. duranensis* and *A. stenosperma* Krapov. and W.C. Gregory. Section *Arachis* species without the small pair are all annual and much more heterogeneous, including a group of three with $2n = 18$ chromosomes (Lavia, 1998; Peñaloza and Valls, 1997), one with six subtelocentric pairs (Fernández and Krapovickas, 1994), considered quite distant from *A. hypogaea* and classified by Stalker (1991) as a D genome species, and a third still heterogeneous group, with 20 metacentric or submetacentric chromosomes (or a rare subtelocentric pair), which includes the most probable non-A progenitor of *A. hypogaea*, *A. ipaënsis* (Fernández and Krapovickas, 1994). Since 1976, collection has accumulated species and germplasm accessions of section *Arachis* that cross with the peanut and are not associated to the A genome. Attempts of producing artificial AABB hybrids including *A. ipaënsis*, apparently the closest species to *A. hypogaea* on the basis of the karyotype and molecular markers, have consistently failed (Singh and Smartt, 1998). On the other hand, there is growing evidence of strong similarity between *A. ipaënsis* and *A. magna* Krapov, W.C. Gregory and C.E. Simpson (Simpson et al., 2001), and other species, such as *A. williamsii* Krapov. and W.C. Gregory, can also be included in this alliance, which increases the number of accessions available with the possibility of sharing the same B genome of the cultivated peanut.

Gregory and Gregory (1976) initially suggested *A. duranensis* and *A. cardenasii* Krapov. and W.C. Gregory as possible parents of the cultivated peanut. Based on cytological characterization and crossability studies, Smartt et al. (1978) suggested that several wild species of *Arachis* with A chromosomes could be potential donors of the A genome, *A. cardenasii* being the main candidate, while *A. batizocoi* Krapov. and W.C. Gregory was considered the possible donor of the B genome. For a long time, *A. batizocoi* had been the only species of section *Arachis* with available germplasm, that produced hybrids

with *A. hypogaea*, but did not show the small A chromosome pair. In line with that, Gregory et al. (1980) warned that new collections were being made, so that other species could arise as possible progenitor candidates of *A. hypogaea*.

Based on seed protein data, Krishna and Mitra (1988) supported the indication of *A. batizocoi* and *A. duranensis* or *A. cardenasii* being the progenitors of *A. hypogaea*. Singh (1986) suggested that the parents were *A. duranensis* and *A. batizocoi,* but in 1988 he accepted the possibility of biphyletic origin, suggesting that *A. hypogaea* subsp. *fastigiata* Waldron evolved from a diploid species such as *A. batizocoi* and *A. duranensis* while *A. hypogaea* subsp. *hypogaea* evolved from diploid species such as *A. batizocoi* and *A. villosa* Benth. Hybrids between *A. hypogaea* and amphidiploids involving *A. batizocoi* and *A. duranensis* showed high pollen fertility and good fruit production, as well as a high association in bivalents, however, meiosis was not totally normal.

Contradicting the above information, Stalker and Dalmacio (1986) discarded the hypothesis that *A. cardenasii* and *A. batizocoi* could be the ancestors, based on cytological characteristics. Hilu and Stalker (1995), based on RAPD markers, stated that *A. duranensis* is likely the donor of the A genome of *A. hypogaea* but also discounted *A. batizocoi* as the donor of the B genome. Later on, Singh et al. (2002), stated that *A. batizocoi* could not be a parent of *A. hypogaea,* based on the relationship of repetitive ribosomal DNA polymorphism units of 77 accessions of wild species and *A. hypogaea.*

Kochert et al. (1991) using RFLP markers, suggested that the parents of *A. hypogaea* are *A. ipaënsis* and *A. duranensis.* Krapovickas and Gregory (1994), Fernández and Krapovickas (1994), and Seijo et al. (2004) also support this possibility. Fernández and Krapovickas (1994) demonstrate that the so-called B chromosome pair is present in every diploid *Arachis* species, including those also showing the small A pair. Therefore, the presence alone of a chromosome with a secondary constriction in a section *Arachis* species will not automatically qualify such species as a potential B genome donor. Fernández and Krapovickas (1994) suggest that the presence of a single pair with a secondary constriction in *A. hypogaea* is a consequence of the amphiplastic inhibition of one of such pairs brought in by both diploid parent species. Kochert et al. (1996) considered that *A. duranensis* was the female parent of the original hybridization event, while stressing a large amount of RFLP variability was found among the accessions of *A. duranensis.* Accessions they considered the most similar to the A genome of *A. hypogaea* were identified as accessions mostly concentrated in the Salta Province of Argentina.

Paik-Ro et al. (1992), using RFLP markers in 14 accessions of *A. hypogaea*, seven of *A. monticola*, four of *A. batizocoi*, four of *A. cardenasii*, five of *A. duranensis*, and four of *A. glandulifera*, suggested that *A. duranensis* is the closest diploid species to *A. hypogaea*, mainly the accession PI 468201 (also from Salta), that had similarity in more than 11 RFLP probes from a total of 13 probes. However, *A. ipaënsis* was not considered in the analysis. A closer affinity of *A. ipaënsis* than that of *A. duranensis*

to the *A. hypogaea/A. monticola* accessions or introgression lines has been documented by Gimenes et al. (2002), using AFLP and RFLP markers. Most probably, each of the two species is showing its closest relationship to a different genome of the tetraploid *A. hypogaea*, and there is an appreciable agreement in the literature as concerns their complementary importance to the origin of the cultivated peanut.

Krapovickas and Gregory (1994) state that *A. monticola*, a wild tetraploid species that crosses with *A. hypogaea*, generating fertile descendents, could be a parent of the cultivated peanut or a derivative. However, they believe that the most likely parental species of *A. hypogaea* would be *A. ipaënsis* and *A. duranensis*, and they do not discard the possibility of a polyphyletic origin. A possible area for the origin of the peanut would be in the southeast of Bolivia and northwest of Argentina, where natural populations of *A. ipaënsis*, *A. duranensis*, *A. batizocoi*, and *A. monticola* could come together, corroborating the hypothesis that two wild sympatric species, carrying the A and B genomes, were crossed by bee pollination, generating a sterile hybrid that would be naturally chromosome doubled. Those fertile hybrids would have been domesticated by the native people of the area.

Later on, using RAPD and ISSR markers in a study of genetic diversity, Raina et al. (2001) observed that *A. villosa*, *A. ipaënsis*, *A. monticola*, and *A. hypogaea* form a group in the dendrogram, while *A. duranensis* formed a distinct group with *A. cardenasii*. Raina and Mukai (1999a, 1999b) considered *A. ipaënsis* and *A. villosa* Benth. to be the most probable progenitors of *A. hypogaea*, based on the observation of 18S-5,8S-26S, and 5S ribosomal loci for fluorescent in situ hybridization (FISH) and for genomic in situ hybridization (GISH). It is remarkable that their findings would discard the possibility of *A. duranensis* being one of the parents of the cultivated peanut. However, a more comprehensive FISH study by Seijo et al. (2004) reestablishes *A. duranensis* as the most probable donor of the A genome, although *A. villosa* is only discarded on geographic and morphological grounds.

Stalker et al. (1991) tried to hybridize *A. ipaënsis* and *A. hypogaea*, without success. Singh and Smartt (1998) took this as an indication that perhaps *A. ipaënsis* would not be the donor of the B genome of *A. hypogaea*. They suggest that one should apply more probes to enhance the covering of the genome before defending this hypothesis.

Singh and Smartt (1998) suggested that until a fertile synthetic amphidiploid is produced between *A. duranensis* and *A. ipaënsis* and it is crossed with *A. hypogaea* to produce a fertile hybrid, hypotheses on the probable parents of *A. hypogaea* would not be confirmed. They suggested that *A. batizocoi* would continue being the probable donor of the B genome, because of the cytogenetic similarity with *A. hypogaea* and stated that the re-creation of *A. hypogaea* could not be made in an exact way, due to the long time between the present and the origin of the species. It is important to note the proven usefulness of *A. batizocoi* in breeding programs, as a component of amphidiploids that produce fertile hybrids with *A. hypogaea,*

therefore allowing the introgression of resistance genes from wild relatives into the cultigen (Simpson and Starr, 2001). The status of *A. batizocoi* as a putative progenitor of *A. hypogaea* is denied by the results obtained by Seijo et al. (2004), based on the physical mapping of the 5S and 18S-25S rRNA genes by FISH.

The objective of the present work is to report the crossability between *A. ipaënsis* and *A. duranensis*, and the accomplishment of tetraploidization through colchicine use, followed by successful crosses of the synthetic amphidiploid with several accessions of *A. hypogaea*. This work was conducted to study the evolution of the cultivated peanut through interspecific crossings, assuming the principal candidates to parents of *A. hypogaea* to be *A. ipaënsis* and *A. duranensis*.

MATERIALS AND METHODS

The experiment was conducted at the Department of Genetics of the School of Agriculture Luiz de Queiroz, ESALQ/USP, Piracicaba, São Paulo State, and at Embrapa Genetic Resources and Biotechnology, Brasilia, Federal District, Brazil. Accessions involved are shown in Table 1 and were supplied by Embrapa Genetic Resources and Biotechnology and by the Agronomic Institute of Campinas (IAC), São Paulo State, Brazil.

Crosses

From April 2000 to June 2001, crosses were made between species of section *Arachis* considered to have distinct genomes. An accession of *A. duranensis* (A genome) from the city of Salta, Argentina, was used as male parent and the only available accession of *A. ipaënsis* (B or non-A genome) as female parent (Table 1).

The hybridization technique consisted of emasculation of flowers of the female parents in bud phase between 1600 and 1900 h. In the morning of the following day, emasculated flowers were pollinated from 0700 to 0800 h, using pollen of the male parent.

Between August and October 2001, hybrids were identified by molecular analysis. Microsatellite marker technique was used for the separation of hybrid and possible self-pollinated individuals. This technique was chosen due to its codominant nature, easiness, and speed in providing results.

Molecular Characterization of the Hybrid between *A. ipaënsis* and *A. duranensis*

Leaves of each plant of the progeny were collected individually and DNA was extracted from leaves according to the adapted protocol of Murray and Thompson (1980). This stage of the research was done in the Laboratory of Plant Cellular and Molecular Biology of the Department of Genetics of the Faculty of Agriculture Luiz de Queiroz, ESALQ/USP. The amount of DNA was quantified by the use of agarose gels (1.2%) with 80 V for 1 h and diluted to the new concentration (2.5 ng mL^{-1}). DNA amplification reaction (polymerase chain reaction [PCR]) had a final volume of 13 mL, and the reagents were mixed in the cocktail form, separately from DNA. Each reaction contained PCR buffer (10mM Tris-HCl pH 8.3 and 50 mM KCl), 1.5 mM MgCl$_2$, 2.5 mM dNTPs, 5 pmol primers pair, 5 U mL^{-1} Taq DNA polymerase, 10 mg mL^{-1} BSA (bovine serum albumine), and 2.5 ng mL^{-1} DNA. Sterile Milli-Q water was added to complete the volume to 13 mL in the reaction. Mineral oil (50 mL) was applied to avoid evaporation of the cocktail. The PCR program consisted of the reaction: (i) 5 min at 94°C; (ii) 29 cycles with three stages (1 min at 94°C, 1 min at 56°C, and 1 min at 72°C); (iii) 7 min at 72°C. Amplified products were separated in 4% (w/v) agarose gel, using TBE buffer pH 8.0 (0.09 of Tris, 0.09 M boric acid, and 2 mM EDTA), at a constant 90 V cm^{-1}. The gels were stained with 10 mL of ethidium bromide (10 mg mL^{-1}) diluted in 100 mL of TBE and documented under ultraviolet light (GelDoc 2000, Biorad, Hercules, CA). The primers used were A1–041, A1–558, and LEC-1.

Pollen Viability

Estimation of pollen viability was made in eight flowers per hybrid combination; anthers were macerated on a slide and pollen staining was done with 2% acetic carmine. Stained pollen grains were counted to estimate pollen viability.

Amphidiploid Production

Cuttings approximately 20 cm long were taken from growing plants, transferred to assay tubes with the growing tips submerged in 0.2% colchicine, closed with PVC plastic film, and submitted to controlled conditions of fluorescent white light and controlled temperature from 28 to 30°C for 8 h. After treatment, cuttings were washed in running water for 20 min, cut at an angle in the oldest internode. Rooting hormone,

Table 1. Species of *Arachis*, germplasm accessions, country of origin, and Brazilian accession codes used in the crossing experiment.

Species	Accessions†	Brazilian accession code (BRA)	Agricultural type	Country of origin‡
A. duranensis Krapov. and W. C. Gregory	VNvEv 14167	036200	–	ARG
A. hypogaea L. subsp. *fastigiata* Waldron var. *fastigiata*	cv. BR1	033383	valencia	BRA
A. hypogaea§	cv. IAC-Caiapó	037371	virginia	BRA
A. hypogaea subsp. *fastigiata* var. *vulgaris* C. Harz	cv. Tatuí	001147	spanish	BRA
A. hypogaea subsp. *fastigiata* var. *hirsuta* Köhler	Mf 1538	037397		ECU
A. hypogaea subsp. *hypogaea* var. *hypogaea*	Cv IAC-Runner	037389	virginia	BRA
A. hypogaea subsp. *fastigiata* var. *aequatoriana* Krapovickas and W.C. Gregory	Mf 1678	037435		ECU
A. hypogaea subsp. *fastigiata* var. *fastigiata*	cv. IAC-Tatu-ST	011606	valencia	BRA
A. hypogaea subsp. *fastigiata* var. *peruviana* Krapov. and W.C. Gregory	Mf 1560	037401		ECU
A. hypogaea subsp. *hypogaea* var. *hypogaea*	VGaRoSv 12548	030708	virginia	BRA
A. hypogaea subsp. *hypogaea*¶	VGaRoSv 12549	030716		BRA
A. ipaënsis Krapov. and W.C. Gregory	KGBPScS 30076#	036234		BOL

† Mf, Manfredi Experiment Station, Argentina; B, D.J. Banks; Ev, A. Echeverry; G, W.C. Gregory; Ga, M.L. Galgaro; K, A. Krapovickas; Nv, L. Novara; P, J.R. Pietrarelli; Ro, D.M.S. Rocha; S, C.E. Simpson; Sc, A. Schinini; V, J.F. M. Valls.
‡ ARG, Argentina; BOL, Bolivia; BRA, Brazil; ECU, Ecuador.
§ Pedigree possibly including distinct botanical varieties.
¶ Very distinct morphological type not yet classified in a formal variety (Freitas and Valls, 2001).
# KGBPScS 30076 is only accession listed with a PI number assigned (PI 468322).

Table 2. Morphological structure averages of diploid (2x) and tetraploid (4x) flowers from the A. ipaënsis and A. duranensis hybrid.

Morphological structures	KGBPScS 30076 × VNvEv 14167		
	2x	4x	P > t
	cm		
Standard length	1.15	1.35	0.0001
Standard width	1.31	1.59	0.0037
Wing length	0.55	0.71	0.0003
Wing width	0.67	0.76	0.0028
Lower lip length	0.58	0.71	0.0028
Upper lip length	0.54	0.59	0.1132
Hypanthium length	4.04	5.31	0.0103

indole butyric acid, was applied and cuttings were transferred to the screenhouse in plastic cups with vegetable substratum. Cups were conditioned for approximately 20 d in trays covered with transparent plastic bags to maintain high humidity.

Chromosome Counting of Synthetic Amphidiploid Cuttings

In January of 2002, the detached leaf protocol of Moraes and Salgado (1984) was adapted for rooting petioles (Fávero et al., 2004). New, totally expanded leaves of the colchicine-treated cuttings were cut and the petioles were treated with indole butyric acid and immediately transferred to cups with vegetable substratum. After, two or more weeks, roots grew and were collected between 1000 and 1400 h.

Chromosome counting was done at the Laboratory of Cytology of the Department of Genetics of ESALQ/USP. Methodologies of root tips treatment and cytological preparations were adapted from Aguiar-Perecin and Vosa (1985) and Silvarolla and Aguiar-Perecin (1994).

After observation good preparations were selected and cover slips were removed in 45% acetic acid and mounted with Canadian balsam. Photomicrographs of the chromosomes were obtained with a Zeiss photomicroscope (Thornwood, NY), using Kodak Technical Pan (ISO 25) film (Rochester, NY).

Morphological Characterization

Diploid and tetraploid flower structures were measured for evaluation of morphological differences. A digital caliper was used to measure standard length and width, wing length and width, lower and upper lip length, and hypanthium length; t test was used for average comparisons (Table 2).

Crosses between A. hypogaea and the Synthetic Amphidiploid

Some colchicine-treated cuttings of the A. ipaënsis × A. duranensis hybrid were identified to have tetraploid cells.

Crosses were made between A. hypogaea and this material at Embrapa Genetic Resources and Biotechnology in screenhouse conditions from October 2002 to March 2003. Accessions of A. hypogaea were used as female parents including germplasm accessions and commercial cultivars, representing distinct agricultural types and all botanical varieties (Table 3). Fruits, harvested from March to May, were dried for 1 wk, then seeds were removed and placed to germinate.

A second complementary round of crosses, involving additional accessions of A. hypogaea was undertaken in 2003 through 2004, following the same procedures.

Molecular Characterization of Progenies of A. hypogaea and Synthetic Amphidiploid Hybridization

Molecular marker studies were done in the Laboratory of Plant Genetic Characterization of Embrapa Genetic Resources and Biotechnology. In March 2003, leaves were removed from the individual progenies originating from crosses between A. hypogaea and the synthetic amphidiploids for DNA extraction and identification of hybrids using microsatellite markers. Protocol used was adapted from Ferreira and Grattapaglia (1995). Quantification stages, PCR reactions and electrophoresis conditions were the same as before. Primer used was Lec-1. Polyacrylamide gel electrophoresis with silver staining was used for distinction among individuals based on microsatellite marker polymorphism. (Bassam et al., 1991).

RESULTS AND DISCUSSION

Diploid Crosses and AB Hybrids Obtained

Seeds of different hybrid progenies were collected between May 2001 and 2004. In the diploid crosses A. ipaënsis was used as the female parent because of unsuccessful attempts of introgression by one junior author (C.E. Simpson, unpublished data, 1984–1986) using A. duranensis as the female parent. Also, a factor in the decision was that Simpson and Starr (2001) were successful in introgression by using a non-A genome parent as the female.

Microsatellite markers proved to be efficient in the identification of hybrids. All individuals of the progenies could be identified without doubt as to the hybrid or self-pollinated condition.

Twenty-four pollinations were made, resulting in five hybrid plants, with a percentage of success of 20.83%. A diploid cell of the hybrid with a chromosome A is shown in Fig. 1. From experience of evaluating Arachis chromosomes, it is known that the other small bodies shown in Fig. 1 are chromosome satellites. Stained pollen was 0.98%.
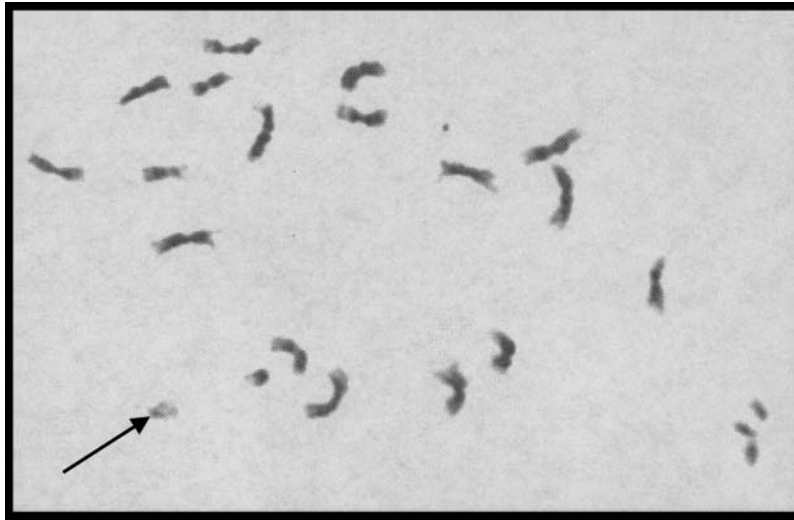
Table 3. Cross combinations between accessions of Arachis hypogaea and synthetic amphidiploid (A. ipaënsis × A duranensis), number of pollinations, number of hybrid plants obtained, and success percentage (number of pollinations compared with number of hybrids obtained).

A. hypogaea accessions		Wild species accessions	No. of pollinations	No. of hybrid plants	Success percentage	No. of F₂ seeds
BR 1	×	(K 30076 × V 14167)	290	34	12	47
IAC-Caiapó	×	(K 30076 × V 14167)	53	16	30	169
IAC-Runner	×	(K 30076 × V 14167)	62	21	34	210
IAC-Tatu-ST	×	(K 30076 × V 14167)	251	13	5	16
Mf 1560	×	(K 30076 × V 14167)	21	1	5	3
Mf 1538	×	(K 30076 × V 14167)	68	11	16	107
Mf 1678	×	(K 30076 × V 14167)	102	3†	–	31
Tatuí	×	(K 30076 × V 14167)	231	7†	–	16
V 12548	×	(K 30076 × V 14167)	77	4	5	54
V 12549	×	(K 30076 × V 14167)	51	2	4	20

† Hybrid plants resulting from precocious germination in hybridization pots. More seed may be available.

Fig. 1. KGBPScS 30076 × VNvEv 14167 diploid mitotic cell. Black arrow shows the small A chromosome; other small bodies are satellites.

Colchicine Treatment and Tetraploid Plant Obtained

The hybrid combination KGBPScS 30076 × VNvEv 14167 (*A. ipaënsis × A. duranensis*) had cells duplicated after 8 h of colchicine treatment. As treated tissues are somatic, it was common for chimerical plants to occur. These chimerical plants produced seeds. From these seeds, it was possible to obtain tetraploid plants. Plants originated from these seeds showed flowers with 97.74% stained pollen.

Measurement data of morphological structures of diploid and tetraploid flowers are shown in Table 2. There were significant differences for all the floral structures measured, except for the upper lip length (Table 2).

Arachis hypogaea and Synthetic Amphidiploid Hybridizations

Figure 2 shows it is possible to distinguish with microsatellite markers between hybrid individuals and self-pollinated progeny. Polyacrilamide gel was used for progeny distinction of a family of *A. hypogaea* cv. BR-1 × [*A. ipaënsis* (KGBPScS 30076) × *A. duranensis* (VNvEv 14167)]^{4x}. Individuals of the progeny indicated by the black arrows were considered as hybrid because they had all bands of both parents. A total of 13 microsatellites were used, but only three were polymorphic in the diploids and just one, Lec-1, was polymorphic in the amphiploids (Fig. 2). It is not clear why all three were not polymorphic.

Table 3 shows crosses made among accessions of *A. hypogaea* and the synthetic amphidiploid, the number of hybrids obtained, and the percentage of success.

An important diagnostic morphological marker is the yellow flower color in the tetraploid hybrids. All *A. hypogaea* accessions used in the work had orange flowers as did *A. ipaënsis*. *Arachis duranensis* (VNvEv 14167) with yellow flowers was always used as male parent, and the diploid hybrids consistently showed yellow flowers. Another remarkable morphological characteristic is the significant increase in the number of trichomes on the edges of leaves and on stems of hybrids.

The hybrids obtained from the cross between *A. hypogaea* and the amphidiploid *A. ipaënsis × A. duranensis* indicate that these species are closely related to *A. hypogaea*. In evolutionary studies of the cultivated peanut, the possibility of successful hybrids involving *A. hypogaea*, *A. ipaënsis*, and *A. duranensis* was a goal of primary importance to the validation of several studies in molecular, morphological, and cytogenetic characterization previously published (Kochert et al., 1991, 1996; Krapovickas and Gregory, 1994; Fernández and Krapovickas, 1994; Gimenes et al., 2002; Seijo et al., 2004), where different authors agreed that *A. ipaënsis* and *A. duranensis* would be the most probable progenitors of the cultivated peanut.

Singh and Smartt (1998) stated that, since a fertile hybrid had not been obtained from *A. ipaënsis × A. duranensis* and crossed to the cultivated peanut, it was not possible to confirm this hypothesis of the origin of *A. hypogaea*. As the result of our work, hybrids were obtained from crosses between six different botanical
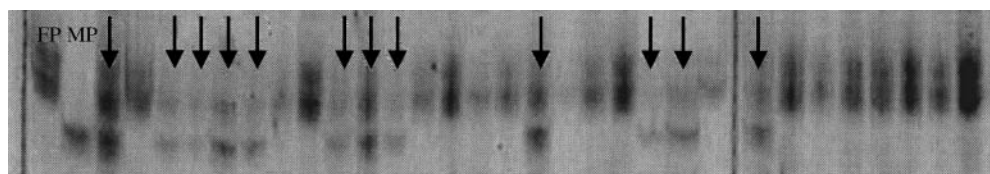

Fig. 2. Polyacrylamide gel with microsatellite (SSR) primer Lec-1. In sequence, family of *A. hypogaea* cv. BR-1 as female parent (FP), the synthetic amphidiploid *A. ipaënsis* (KGBPScS 30076) × *A. duranensis* (VNvEv 14167) as male parent (MP) and the progeny. Black arrows show hybrid individuals of the progeny.

varieties of *A. hypogaea* and a fertile synthetic amphi-diploid *A. ipaënsis* × *A. duranensis*. All tetraploid hybrid F$_1$ plants produced pegs and seeds, documenting the fertility of the F$_1$ interspecific hybrids (Table 3).

Further studies of chromosome pairing are underway, but the production of the several F$_2$ progenies at least raises *A. ipaënsis* to the same status attributed by Singh and Smartt (1998) to *A. batizocoi*, additionally emphasizing the closest association of *A. ipaënsis* to *A. hypogaea*, based on a broad array of investigative approaches.

The fact that fertile hybrids were obtained between the synthetic *A. ipaënsis* × *A. duranensis* amphidiploid and representatives of both subspecies and even more described varieties of *A. hypogaea* (Krapovickas and Gregory, 1994) than those used by Singh (1988) documents their high potential for the crop improvement, especially when recent studies based on more accurate techniques (Ferguson et al., 2004; Moretzsohn et al., 2004) start to unveil the obvious genetic diversity, previously not well understood, of *Arachis* germplasm resources.

REFERENCES

Aguiar-Perecin, M.L.R. de, and C. Vosa. 1985. C-banding in maize. II. Identification of somatic chromosomes. J. Hered. 54:37–42.

Bassam, B.J., G. Caetano-Anolles, and P.M. Gresshoff. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. 196:80–83.

Fávero, A.P., S.M. Cuco, M.R.L. de Aguiar-Perecin, J.F.M. Valls, and N.A. Vello. 2004. Rooting in leaf petioles of *Arachis* for cytological analysis. Cytologia 69:215–219.

Ferguson, M.E., P.J. Bramel, and S. Chandra. 2004. Gene diversity among botanical varieties in peanut (*Arachis hypogaea*). Crop Sci. 44:1847–1854.

Fernández, A., and A. Krapovickas. 1994. Cromosomas y evolución en *Arachis* (Leguminosae). (In Spanish, with English abstract.) Bonplandia 8:187–220.

Ferreira, M.E., and D. Grattapaglia. 1995. Introdução ao Uso de Marcadores Moleculares em Análise Genética. 2nd ed. Embrapa-CENARGEN, Brasília.

Freitas, F.O., and J.F.M. Valls. 2001. Nota sobre a ocorrência de um tipo distinto de amendoim no parque indígena do Xingú e arredores e suas implicações etnobotânicas. *In* Simpósio Internacional de Recursos Genéticos para a América Latina e Caribe, Londrina, PR.

Gimenes, M.A., C.R. Lopes, M.L. Galgaro, J.F.M. Valls, and G. Kochert. 2002. RFLP analysis of genetic variation in species of section *Arachis*, genus *Arachis* (Leguminosae). Euphytica 123:421–429.

Gregory, W.C., and M.P. Gregory. 1976. Groundnut. p. 151–154. *In* N.W. Simmonds (ed.) Evolution of crop plants. Longman Group Ltd., London.

Gregory, W.C., A. Krapovickas, and M.P. Gregory. 1980. Structure, variation, evolution and classification in *Arachis*. p. 469–481. *In* R.J. Summerfield and A.H. Bunting (ed.) Advances in Legume Science (2). Royal Botanic Gardens, Kew, Surrey, UK.

Hilu, K.W., and H.T. Stalker. 1995. Genetic relationships between peanut and wild species of *Arachis* sect. *Arachis* (Fabaceae): Evidence from RAPDs. Plant Syst. Evol. 198:167–178.

Husted, L. 1933. Cytological studies of the peanut *Arachis*. 1. Chromosome number and morphology. Cytologia 5:109–117.

Husted, L. 1936. Cytological studies of the peanut *Arachis*. 2. Chromosome number, morphology and behavior, and their application to the problem of the origin of the cultivated forms. Cytologia 7:396–423.

Kochert, G., T. Halward, W.D. Branch, and C.E. Simpson. 1991. RFLP variability in peanut (*Arachis hypogaea* L.) cultivars and wild species. Theor. Appl. Genet. 81:565–570.

Kochert, G., H.M. Stalker, M. Gimenes, L. Galgaro, C.R. Lopes, and K. Moore. 1996. RFLP and cytogenetic evidence on the origin and evolution of allotetraploid domesticated peanut, *Arachis hypogaea* (Leguminosae). Am. J. Bot. 83:1282–1291.

Krapovickas, A., and W.C. Gregory. 1994. Taxonomia del género *Arachis* (Leguminosae). (In Spanish, with English abstract.) Bonplandia 8:1–186.

Krishna, T.G., and R. Mitra. 1988. The probable genome donors to *Arachis hypogaea* L. based on arachin seed storage protein. Euphytica 37:47–52.

Lavia, G.I. 1998. Karyotypes of *Arachis palustris* and *A. praecox* (Section *Arachis*), two species with basic chromosome number x = 9. Cytologia 63:177–181.

Moraes, S. de A., and C.L. Salgado. 1984. Utilização da técnica de folhas destacadas de amendoim (*Arachis hypogaea* L.) para inoculações com *Cercospora arachidicola* Hori e *Cercospora personata* (Bert. & Curt.) Ell. & Ev. (In Portuguese, with English abstract.) Summa Phytopath. 8:39–55.

Moretzsohn, M.C., M.S. Hopkins, S.E. Mitchell, S. Kresovich, J.F.M. Valls, and M.E. Ferreira. 2004. Genetic diversity of peanut (*Arachis hypogaea* L.) and its wild relatives based on the analysis of hypervariable regions of the genome. BMC Plant Biol. 4:11.

Murray, M.G., and W.F. Thompson. 1980. Rapid isolation of high molecular-weight plant DNA. Nucleic Acids Res. 8:4321–4325.

Paik-Ro, O.G., R.L. Smith, and D.A. Knauft. 1992. Restriction fragment length polymorphism of six peanut species within the *Arachis* section. Theor. Appl. Genet. 84:201–208.

Peñaloza, A.P.S., and J.F.M. Valls. 1997. Contagem do número cromossômico em acessos de *Arachis decora* (Leguminosae). *In* Simpósio Latino-Americano de Recursos Genéticos Vegetais 1:39. IAC, Campinas.

Raina, S.N., and Y. Mukai. 1999a. Detection of a variable number of 18S–5.8S–26S and 5S ribosomal DNA *loci* by fluorescent *in situ* hybridization in diploid and tetraploid *Arachis* species. Genome 42:52–59.

Raina, S.N., and Y. Mukai. 1999b. Genomic *in situ* hybridization in *Arachis* (Fabaceae) identifies the diploid wild progenitors of cultivated (*A. hypogaea*) and related wild (*A. monticola*) peanut species. Plant Syst. Evol. 214:251–262.

Raina, S.N., V. Rani, T. Kojima, Y. Ogihara, K.P. Singh, and R.M. Devarumath. 2001. RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. Genome 44:763–772.

Seijo, J.G., G.I. Lavia, A. Fernández, A. Krapovickas, D. Ducasse, and E.A. Moscone. 2004. Physical mapping of the 5S and 18S–25S rRNA genes by fish as evidence that *Arachis duranensis* and *A. ipaënsis* are the wild diploid progenitors of *A. hypogaea*. Am. J. Bot. 91:1294–1303.

Silvarolla, M.B., and M.L.R. de Aguiar-Perecin. 1994. Evaluation of chromosome number stability in two sugarcane varieties. Braz. J. Genet. 17:237–242.

Simpson, C.E., and J.L. Starr. 2001. Registration of 'COAN' peanut. Crop Sci. 41:918.

Simpson, C.E., J.F.M. Valls, and A. Krapovickas. 2001. History of *Arachis* including evidence of *A. hypogaea* L. progenitors. Peanut Sci. 28:78–80.

Singh, A.K. 1986. Utilization of wild relatives in the genetic improvement of *Arachis* hypogaea L. 8. Synthetic amphidiploids and their importance in interspecific breeding. Theor. Appl. Genet. 72:433–439.

Singh, A.K. 1988. Putative genome donors of *Arachis hypogaea* (Fabaceae), evidence from crosses with synthetic amphidiploid. Plant Syst. Evol. 160:143–151.

Singh, A.K., and J. Smartt. 1998. The genome donors of the groundnut/peanut (*Arachis hypogaea* L.) revisited. Genet. Res. Crop Evol. 45:113–118.

Singh, K.P., A. Singh, S.N. Raina, A.K. Singh, and Y. Ogihara. 2002. Ribosomal DNA repeat unit polymorphism and heritability in peanut (*Arachis hypogaea* L.) accessions and related wild species. Euphytica 123:211–220.

Smartt, J., W.C. Gregory, and M.P. Gregory. 1978. The genomes of *Arachis hypogaea*. 1. Cytogenetic studies of putative genome donors. Euphytica 27:665–675.

Stalker, H.T. 1989. Utilizing wild Species for crop improvement. p. 139–154. *In* H.T. Stalker and C. Chapman (ed.) IBPGR Training Courses: Lecture Series, 2. Scientific Management of Germplasm:

Characterization, Evaluation and Enhancement. Int. Board for Plant Genet. Resour., Rome.

Stalker, H.T. 1991. A new species in section *Arachis* of peanuts with a D genome. Am. J. Bot. 78:630–637.

Stalker, H.T., and R.D. Dalmacio. 1986. Karyotype analysis and relationships among varieties of *Arachis hypogaea* L. Cytologia 51:617–629.

Stalker, H.T., J.S. Dhesi, D.C. Parry, and J.H. Hahn. 1991. Cytological and interfertility relationships of *Arachis* Section *Arachis*. Am. J. Bot. 78:238–246.

Valls, J.F.M., and C.E. Simpson. 1994. Taxonomy, natural distribution, and attributes of *Arachis*. p. 1–18. *In* P.C. Kerridge and B. Hardy (ed.) Biology and Agronomy of Forage *Arachis*. Centro Intern. de Agri. Trop., Cali.

Valls, J.F.M., and C.E. Simpson. 2005. New species of *Arachis* from Brazil, Paraguay, and Bolivia. Bonplandia 14(1&2):35–64.