

Occurrence of  $2n$  microspore production in diploid interspecific hybrids between the wild parental species of peanut (*Arachis hypogaea* L., Leguminosae) and its relevance in the genetic origin of the cultigen

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Abbreviations: ANOVA, analysis of variance; DAPI, 4,6-diamidino- 2-phenylindole; PCR, polymerase chain reaction; PMCs, pollen mother cells.

## ABSTRACT

Unreduced ( $2n$ ) gametes are the driving force that leads to polyploidization of plants in nature and an important tool for ploidy breeding. Peanut (*Arachis hypogaea* L., Leguminosae) seems most likely to have originated by sexual polyploidization through interspecific hybridization between the wild diploid relatives -*A. duranensis* and *A. ipaënsis*-, and subsequent binding of the unreduced gametes in the hybrid. Therefore, aiming to contribute to the understanding the event of polyploid origin of this important crop, we made diploid hybrids between *A. duranensis* and *A. ipaënsis*, and then investigated the production of

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unreduced gametes in the hybrids by analyzing the size range of pollen, the constitution of the sporads, and the microsporogenesis process. The meiotic abnormalities found, such as persistent bridges, tripolar spindles and cytomixis processes, would lead to the formation of restitution nuclei in both the first and second meiotic division. These failures in the regularity of meiosis lead to the formation of dyads and triads, and consequently to  $2n$  pollen grains. These findings constitute the first evidence of the formation of unreduced gametes in intergenomic hybrids obtained between the wild progenitors of *A. hypogaea* and enable us to propose a model of origin of the crop through sexual polyploidization.

Keywords: peanut, unreduced gametes, sexual polyploidization, genetic origin.

## INTRODUCTION

The genus *Arachis* includes 82 species (Krapovickas and Gregory, 1994; Valls and Simpson, 2005; Valls et al., 2013; Santana and Valls, 2015; Valls and Simpson, 2017), 31 of which belong to the section *Arachis*. This section includes the peanut cultigen *A. hypogaea*, which is one of the most cultivated edible legumes worldwide and an excellent source of proteins and oils. Peanut cultivation is concentrated mainly in warm areas of Asia, Africa, and America, with a worldwide production that reached 47 million tons in 2017 (FAOSTAT, <http://fao.org/faostat>). Argentina, with a production of 901,800 tons of peanuts in grain (2016/2017, Bolsa de Cereales de Córdoba, <http://www.bccba.com.ar/mani-7165.html>), is one of the main producers and exporters in the world, with 98% of the planted area concentrated in the center and south of the province of Córdoba (Ministerio de Agricultura, Ganadería y Pesca, Argentina, <https://www.argentina.gob.ar/agricultura-ganaderia-y-pesca>).

The cultivated peanut is a segmental allotetraploid species with  $2n = 40$  chromosomes (Husted, 1933, 1936; Gregory and Gregory, 1976; Bertioli et al., 2019). Based on the results

obtained through different techniques, different species have been proposed as the likely progenitors of *A. hypogaea* (Lavia et al., 2008). Currently, given the information obtained by classical cytogenetic (Fernández and Krapovickas, 1994), molecular cytogenetic (Seijo et al., 2004, 2007; Robledo et al., 2009; Robledo and Seijo, 2010), molecular analysis (Kochert et al., 1991, 1996; Grabiele et al., 2012; Moretzsohn et al., 2013; Bertioli et al., 2016, 2019), and morphological and geographic analysis (Krapovickas and Gregory, 1994), it is well accepted that the progenitors of *A. hypogaea* (AABB) are *A. duranensis* (A genome) and *A. ipaënsis* (B genome). This hypothesis has also been supported by the development of a synthetic amphidiploid by crosses between *A. ipaënsis* and *A. duranensis* and subsequent chromosome duplication, as well as by obtaining successful hybrids between the synthetic amphidiploid and *A. hypogaea* (Fávero et al., 2006).

Currently, it is largely accepted that *A. hypogaea* originated from a single allopolyploidization event (Seijo et al., 2004, 2007; Bertioli et al., 2011, 2019; Grabiele et al., 2012). Molecular studies using sequence analysis of non-coding regions of cpDNA and the non-transcribed spacer of the nuclear rDNA 5S unequivocally identified *A. duranensis* as the female parent of the AABB tetraploid *A. hypogaea* (Grabiele et al., 2012). However, after many attempts, few researchers have obtained successful crosses with *A. ipaënsis* by using *A. duranensis* as the female parent (Mallikarjuna et al., 2011; García et al., 2016; Simpson, 2017).

Allopolyploidy as a result of interspecific hybridization, accompanied by chromosome duplication, has played a key role in the evolution of many important plants, such as cereal, forage, oilseed and fiber crops (Jauhar, 2007). Allopolyploids may form either through asexual polyploidization via somatic cell chromosome doubling or through sexual polyploidization. However, the latter represents the main evolutionary route for polyploidization among plant species (Ramsey and Schemske, 1998; Jauhar, 2003, 2007; De

Storme and Geelen, 2013; Kreiner, 2017a, 2017b). In nature, sexual polyploids are highly efficient, even more than the polyploids generated by somatic chromosome duplication. In this context, the phenomenon responsible for sexual polyploidization is the production of unreduced ( $2n$ ) gametes in the parental species of polyploids or their hybrids (Jauhar, 2003). Sexual allopolyploids, depending on the parental contribution, can be formed via two pathways: either bilateral, after the union of two unreduced gametes ( $2n + 2n$ ), or unilateral, by an uneven ploidy level combination ( $2n + n$ ) (den Nijs and Peloquin, 1977). Most techniques to detect unreduced gametes focus on pollen, since it is more convenient to isolate and screen than egg cells (Dewitte et al., 2012). Large pollen has frequently been attributed to  $2n$  pollen since there is a positive correlation between DNA content and cell volume, which, in turn, influences pollen diameter (Bretagnolle and Thompson, 1995). Thus,  $2n$  pollen grains are larger than reduced grains, and plants producing  $2n$  pollen grains display bimodal pollen grain size distribution instead of a normal distribution (Ramsey and Schemske, 1998; Dewitte et al., 2012). The analysis of microsporogenesis may also provide an useful method to detect the presence and the frequency of  $2n$  gametes, since the presence of dyads and triads in the sporad stage constitutes strong evidence of unreduced pollen production (Orjeda et al., 1990; Dewitte et al., 2012).

All the above allow assuming that peanut probably originated by sexual polyploidization, through interspecific hybridization between *A. duranensis* and *A. ipaënsis*, and subsequent binding of the unreduced gametes. Therefore, as a first step to test this hypothesis and contribute to the understanding of the mode of peanut origin, in the present study, we carried out controlled reciprocal crosses between *A. duranensis* and *A. ipaënsis* and identified their hybrids<sup>2x</sup> both by analyzing the distribution patterns of DAPI+ heterochromatin by using fluorescent banding and by genotyping using microsatellite markers. As a second step, in an attempt to elucidate the contribution of  $2n$  gametes to the origin of peanut, we studied the

occurrence of unreduced gametes and estimated the frequency of their production by analyzing the size range of pollen and the constitution of the sporads. Finally, we analyzed the meiotic chromosome behavior of PMCs to detect the underlying cytological mechanisms involved in the origin of unreduced gametes.

The results obtained here, i.e. the presence of unreduced gametes in the artificially synthesized interspecific hybrids between *A. duranensis* and *A. ipaënsis*, constitute the first evidence on the genetic origin of peanut via sexual polyploidization. The results also allow unraveling, in induced diploid hybrids (AB), the evolutionary steps that could have occurred in nature at the time of the origin of tetraploid peanut.

## **MATERIALS AND METHODS**

### **PLANT MATERIAL**

The material used in the crosses is detailed in Table 1.

### **METHODS**

#### **Crosses**

Crosses were made between November 2013 and April 2014, in the greenhouse of the Instituto de Botánica del Nordeste (IBONE), Corrientes, Argentina. The technique consisted of emasculation of flowers of the female parents in bud phase (before the floral opening) between 16:00 and 19:00 h. In the morning of the following day, emasculated flowers were pollinated with pollen grains from the male parent (Fávero et al., 2006). The crosses were reciprocal, taking both species as female and male parents. The fruits were harvested during June-July 2014 and kept in the Germplasm Bank of the IBONE. In early September, the total harvested seeds were placed, conveniently separated and identified, on individual germinators with vermiculite.

### Identification of hybrids

The seedlings resulting from the germination of the fruits obtained from the reciprocal crosses between *A. duranensis* and *A. ipaënsis* were identified by fluorescent DAPI banding and microsatellite marker analysis, both of which were used for the separation of hybrids and possible self-pollinated individuals.

DAPI banding was performed due to the difference in the heterochromatin distribution patterns in the different genomes of the species involved in the crosses. Species with the A genome (*A. duranensis*) have centromeric DAPI+ bands, while those with the B genome (*A. ipaënsis*) are devoid of heterochromatic bands (Seijo et al., 2004; Robledo et al., 2009; Robledo and Seijo, 2010). If the individuals resulting from the crosses were hybrids, DAPI+ heterochromatic bands should be found in half of the chromosome complement. Root tips were treated with 2mM 8-hydroxyquinoline and subsequently fixed in absolute ethanol:glacial acetic acid solution (3:1) for 12h at 4° C (Fernández and Krapovickas, 1994 modified). The root apices were digested in 1% (w/v) cellulase (from *Trichoderma viridae*; Onozuka R-10, Serva) plus 10% (v/v) pectinase (from *Aspergillus niger*, Sigma) dissolved in 40% glycerol in 0.01 M citrate buffer (pH 4.8) for 2 h at 37 °C before squashing, and slides were air dried according to Gerber and Schweizer (1988). DAPI banding was performed according to the protocol described by Schweizer (1976).

Microsatellite markers were chosen due to their codominant nature, ease of use, and speed in providing results. DNA was extracted using the protocol described by Inglis et al. (2018). The quality and quantity of the DNA were evaluated in 0.8% agarose gel electrophoresis and using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), respectively. DNA amplification reactions were performed by multiplex PCR designed with the Multiplex Manager program (Holleley and Geerts, 2009). The selected parameters

included a maximum of three primers each marked with a different fluorescence (6-FAM, HEX and NED) by reaction and similar annealing temperatures. PCR reactions were performed using the QIAGEN multiplex PCR kit (Qiagen, Hilden, Germany), with 2X of master mix, 0.05  $\mu$ M of primers (forward and reverse), 1 ng of genomic DNA and adjusting the final volume to 5  $\mu$ L with ultra-pure water. The amplifications were performed in ABI GeneAmp PCR system 9700 thermal cyclers (Applied Biosystems, Foster City, USA) with the following cycling program: initial denaturation of 15 min at 95° C, followed by 30 cycles composed of denaturation from 30 s at 94° C, hybridization of 90 s at 58° C and extension from 90 s at 72° C, followed by a final extension of 30 min at 72° C. The amplified fragments were resolved by capillary electrophoresis on an ABI 3730 DNA Analyzer (Applied Biosystems). For a better resolution, the obtained PCR products were diluted 20x in ultra-pure water. Loading samples contained 1  $\mu$ l of PCR product diluted 20x, 0.2  $\mu$ l of Rox-labelled size standards and 8  $\mu$ l of Hi-di Formamide (Applied Biosystems), and denatured for 4 minutes at 95° C. For the allele sizing, the electropherograms were analyzed with the GeneMapper® software (Applied Biosystems).

### **Detection and analysis of unreduced gametes**

The frequency of unreduced gametes was determined by analyzing the pollen size distribution and the constitution of the sporads from all the hybrids obtained. Pollen stainability with carmine:glycerine (1:1) was used as an estimation of pollen viability (Pittenger and Frolik, 1951). Three mature flower buds for each hybrid individual were analyzed. All pollen grains found were counted, and those regularly shaped and darkly stained were considered to be viable. Pollen viability was expressed as an average percentage of the stained pollen grains/total pollen grains analyzed.

All viable pollen grains per flower bud were measured and counted using ImageJ software (Rasband, 1997-2019). Pollen size was estimated by the Em index, i.e. the distance between the aperture angle and the middle point of the opposite side (Van Campo, 1957). According to their size, viable pollen grains were classified into four categories: small (S), normal (No), large  $2n$  and large  $4n$ . Normal pollen was considered to determine the mean pollen diameter of each individual. Pollen grains showing a size 0.25 times smaller than the average size of the individuals were considered as small pollen. Unreduced pollen was detected following Darlington (1937): pollen grains showing a size 1.25 times larger than the average size of the individuals were considered as large  $2n$  grains (giant pollen), while deeply stained grains showing a size 1.5 times larger than the average size of the individuals were considered large  $4n$  grains (jumbo pollen).

To confirm that large pollen grains effectively represent  $2n$  and  $4n$  pollen production, microspores at sporad stage were screened in all hybrid plants. For this analysis, PMCs from fresh flower buds were stained with carmine:glycerine (1:1). Sporads were classified as tetrads (Te), abnormal tetrads (Te $\neq$ ), triads (Tr), dyads (Dy), monads (Mo) and polyads (Pol). Tetrads were considered normal when they had four equal-sized cells. Abnormal tetrads include tetrads with four microspores of different sizes, tetrads with collapsed microspores and tetrads with microcytes.

To evaluate the existence of significant differences for pollen size and the production of  $2n$  gametes among individuals, one-way ANOVA at a significance level of 5 % ( $\alpha = 0.05$ ) after Bartlett's test of homogeneity was performed. Also, Tukey's test at 5 % was carried out to test differences between each pair of means. All statistical analyses were performed using the INFOSTAT software version 10.0 (Di Rienzo et al., 2017).

### **Analysis of meiotic behavior**



Meiotic analysis was carried out in PMCs from young flower buds, either fresh or fixed in ethanol:acetic acid (3:1). PMCs were stained and squashed in 2% lacto-propionic orcein (Dyer, 1963). Each anther was processed individually. The meiotic behavior was evaluated from prophase I to telophase II and the frequency of each abnormality was recorded. All PMCs found were analyzed.

## RESULTS AND DISCUSSION

### Crosses

A total of 220 crosses were made. *Arachis duranensis* was used as female parent in 153 of the crosses (69.5% of the hybridizations), obtaining pegs in 40.5% of the cases, whereas *A. ipaënsis* was used as female parent in 67 of the crosses (30.5% of the hybridizations), obtaining pegs in 20.9% of the cases. Unsuccessful results in both species included pollinations that were not effective and pegs that dried due to natural causes.

All the fruits from the different hybrid progenies were collected. A total of 47 seeds were collected from the flower pots of *A. duranensis*, whereas only ten were found in pots where *A. ipaënsis* was used as the female parent. Also, in the latter, ten seedlings were found, suggesting the lack of dormancy of those seeds.

Every seed collected was put to germinate in individual cups with mineral substratum. All seeds of *A. ipaënsis* x *A. duranensis* germinated, whereas seeds from the reciprocal crosses did not. The ungerminated seeds were removed from the cups, stored and re-sown several months later. This procedure allowed 16 seeds to germinate, suggesting that they presented the physiological characteristics of dormancy.

### Identification of hybrids

The fluorescent DAPI banding technique allowed determining that six of the seedlings germinated in the pot of *A. ipaënsis* as the female parent and six of the seedlings germinated in vermiculite from *A. duranensis* x *A. ipaënsis* crosses were hybrids (Table 1), because they presented the characteristic banding pattern: DAPI+ centromeric bands on chromosomes of the A genome and absence of DAPI+ centromeric bands on chromosomes of the B genome (Fig. 1). All the hybrids analyzed showed diploid level with  $2n = 20$  chromosomes in the mitotic cells.

A total of 26 polymorphic microsatellite markers were also used for hybrid identification. The 26 loci confirmed that the six seedlings from the *A. duranensis* x *A. ipaënsis* crosses and the six seedlings from the *A. ipaënsis* x *A. duranensis* crosses were true F<sub>1</sub> hybrids. Since 13 of the markers were polymorphic between the accessions K 7988 and V 14167 of *A. duranensis*, it was also possible to identify that four of these seedlings resulted from crosses between *A. ipaënsis* x *A. duranensis* V 14167 and that two resulted from crosses between *A. ipaënsis* x *A. duranensis* K 7988. As expected, microsatellite markers proved to be efficient in the identification of hybrids. All the individuals of the progenies were undoubtedly identified as the hybrid or self-pollinated condition.

The success rate achieved was calculated as the number of hybrid individuals in relation to the number of pollinations. In the crosses using *A. ipaënsis* as the female parent, we obtained a success rate of 8.95%. Our results are slightly different from Fávero et al. (2006), despite the fact that the same accessions were used as parents in both studies. In the case of *A. duranensis* x *A. ipaënsis* crosses, the success rate could not be calculated because not all the seeds managed to germinate. However, obtaining hybrids with *A. duranensis* as a female parent has not been previously registered. Although several authors have made attempts to achieve this hybrid (Simpson, unpublished results, 1984-1986; Stalker et. al., 1991), every effort was unsuccessful. The crosses obtained in the present work, together with those

obtained by Simpson (2017), constitute the first [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup> hybrids successfully obtained.

### **Estimation of unreduced gametes based on the viability, size and morphology of pollen grains**

The pollen grains of *A. duranensis*, *A. ipaënsis* and the 12 hybrids derived from the reciprocal interspecific crosses (Table 2) were analyzed to estimate pollen viability and determine variation in their pollen grain size, in order to identify potential unreduced gamete producers.

Both parental species had high pollen viability: *A. duranensis* V 14167 97.65%, *A. duranensis* K 7988 98.55% and *A. ipaënsis* 98.75%. By contrast, the hybrids had very low viability, ranging from 1.45% to 3.96% in [*A. ipaënsis* x *A. duranensis*]<sup>2x</sup> and from 1.27% to 3.11% in [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup> (Table 2, Fig. S1). In the genus *Arachis*, pollen viability of interspecific hybrids has traditionally been used to infer the genomic relationships between the species involved in the crosses. Hybrids between species that have the same genome type generally show values of pollen viability between 25% and 70% (Smartt et al., 1978; Stalker et al., 1991; Krapovickas and Gregory, 1994; Tallury et al., 2005; Burow et al., 2009), whereas crossings between species with different genome types usually show values below 10% (Stalker et al., 1991; Krapovickas and Gregory, 1994; Burow et al., 2009). As expected, the intergenomic hybrids obtained between *A. duranensis* (AA) and *A. ipaënsis* (BB) showed values of pollen viability lower than 4% (Table 2), data that support their hybrid condition.

A significant difference in the size of viable pollen grains was detected between both progenitor species (F=128.2, p= 0.0001), *A. duranensis* having a pollen grain larger than *A. ipaënsis* (Fig. S1). The average pollen size of *A. duranensis* was  $34.87 \pm 1.90 \mu\text{m}$ , varying

from 31.36 to 39.20  $\mu\text{m}$ , whereas that of *A. ipaënsis* was  $32.18 \pm 1.69 \mu\text{m}$ , varying from 27.44 to 35.28  $\mu\text{m}$ .

In the hybrid individuals, the size of viable pollen grains showed significant variation ( $F=139.50$ ,  $p<0.0001$ ) and, so, they were classified as small, normal, large  $2n$  (giant pollen) or large  $4n$  pollen (jumbo pollen) (Fig. 2A-2C). Hybrid individuals also showed a significant difference in the size of normal viable pollen ( $F=4.53$ ,  $p=0.0354$ ) depending on the pollen donor species, with [*A. ipaënsis* x *A. duranensis*]<sup>2x</sup> hybrids having a larger pollen size. The average size of normal pollen of [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup> hybrids was  $34.95 \pm 2.94 \mu\text{m}$ , varying from 29.40 to 39.20  $\mu\text{m}$ , whereas that of [*A. ipaënsis* x *A. duranensis*]<sup>2x</sup> hybrids was  $36.10 \pm 2.80 \mu\text{m}$ , varying from 29.40 to 41.16  $\mu\text{m}$ . Among the large pollen grains found, those with a size equal to or higher than 43.68 and 45.12  $\mu\text{m}$  were classified as  $2n$  pollen or giant pollen grains in [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup> and [*A. ipaënsis* x *A. duranensis*]<sup>2x</sup> hybrids, respectively (Fig. 2B), whereas those with a size equal to or higher than 52.45  $\mu\text{m}$  and 54.15  $\mu\text{m}$  were classified as  $4n$  pollen or jumbo pollen grains in [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup> and [*A. ipaënsis* x *A. duranensis*]<sup>2x</sup> hybrids, respectively (Fig. 2C). All hybrid individuals analyzed produced giant pollen grains, while 80% produced jumbo pollen grains (Table 2). Usually, the presence of large pollen grains results in a bimodal size distribution, as opposed to a normal size distribution (Tondini et al., 1993). Concerning that, the analysis of the distribution of pollen grain sizes of the parental species showed an expected normal distribution of the data (Fig. 3). In contrast, the frequencies of the pollen grain sizes of the F1 hybrids were found to have a bimodal distribution, attributed to the high production of normal and large grains in [*A. ipaënsis* x *A. duranensis*]<sup>2x</sup> hybrids, and due to the abundant presence of small and large grains in [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup> hybrids (Fig. 3).

Regarding the shape, normal pollen grains were elliptical in both the parental species and hybrids, while the large pollen grains detected in hybrids were spherical shaped.

Following the criteria of Orjeda et al. (1990), Kovalsky and Solís Neffa (2012) and Dewitte et al. (2012), three facts suggest that the giant pollen grains detected in the hybrids correspond to  $2n$  gametes. The first evidence is the presence of triads and dyads in the hybrids, indicating that unreduced gametes certainly occurred and that  $2n$  pollen formation was expected (see below). The second evidence, i.e. the bimodal distribution of pollen size in hybrids, is in accordance with the description for other species with  $2n$  pollen grains. Lastly, besides pollen size, the spherical instead of ellipsoidal pollen shape detected in the giant pollen grains of the hybrids has been associated with the level of ploidy, simplifying the determination of  $2n$  pollen. In this sense, Akutsu et al. (2007) observed that by omitting the first or second meiotic division, the restitution nuclei consequently combines tetrads and the resulting  $2n$  pollen (dyad) is a semi-sphere, which looks round under the microscope.

The finding of  $2n$  and  $4n$  pollen in the [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup> and [*A. ipaënsis* x *A. duranensis*]<sup>2x</sup> interspecific hybrids is the first evidence of the production of unreduced gametes in *Arachis* hybrids involving the wild parental species of peanut. In this sense, the importance of the finding lies in the fact that all artificially developed hybrids produced  $2n$  pollen grains (Table 2, Figs. 2B and 2C). The ANOVA test showed that the production frequency of  $2n$  gametes did not differ statistically between the F<sub>1</sub> hybrids (F=1.55, p=0.17), but three of them (IpaDur1, IpaDur2 and DurIpa11) showed the highest frequency (between 0.82% and 1.35%). Another relevant result is that, although all F<sub>1</sub> hybrids showed low pollen viability (less than 4%), in the three aforementioned individuals  $2n$  pollen represented 34.73%, 34.1%, and 26.37% of the viable grains (Fig. 4), respectively. By contrast, in the rest of the F<sub>1</sub> hybrids,  $2n$  pollen represented between 1.24% and 21.16% of the viable grains. Therefore, although pollen viability of the hybrids was low, in some of them,  $2n$  gametes represented a significant proportion of viable grains, thus with a high probability of participating in the fertilization. These findings suggest that the three F<sub>1</sub> hybrids with the

highest production of  $2n$  gametes could be used as diploid parents in the genetic improvement of peanut and for the development of new ones, as well as to provide a bridge to transfer desirable genes from wild diploid parents to the gene pool of peanut.

### **Estimation of unreduced gametes derived from sporads**

The sporad constitution of the 12 hybrids derived from reciprocal interspecific crosses was analyzed to ascertain if the giant pollen grains found in the hybrids corresponded to unreduced gametes, according to the proposed criteria aforementioned (Orjeda et al., 1990; Kovalsky and Solís Neffa, 2012; Dewitte et al., 2012). This analysis revealed, in addition to the expected normal tetrads, the presence of triads (up to 15.98%), dyads (up to 26.52%) and monads (up to 2.50%) (Table 3, Fig. 2D-2G). The presence of triads and dyads indicates the production of one and two  $2n$  microspores, respectively, and that the production of  $2n$  pollen is expected. These data are in agreement with previous studies with hybrids, such as *Begonia* (Dewitte et al., 2010a) and *Brassica* (Mason et al., 2011). In addition, the presence of monads indicates that  $4n$  microspores also occurred, based on the assumption that each monad results in one jumbo pollen grain (Kovalsky and Solís Neffa, 2012). Among sporads with unreduced microspores, only the frequency of production of dyads differed statistically ( $F=23.95$ ,  $p<0.0001$ ) between  $F_1$  hybrids (Table 3), being the individuals DurIpa 9 (37.29%) and IpaDur 2 (20.83%) the highest producers.

Additionally, abnormal tetrads (tetrads with four microspores of different sizes, tetrads with collapsed microspores and tetrads with microcytes) and polyads were found (Table 3, Fig. S2), a fact that likely leads to the production of abnormal gametes (see below).

### **Cytological mechanisms involved in the formation of $2n$ gametes**

To identify possible mechanisms for the production of  $2n$  gametes, a detailed analysis of the meiosis of the hybrids was performed. This analysis revealed a large number of meiotic

abnormalities at different stages (Table 4), which can be grouped into two major categories: those that would produce aneuploid gametes (Fig. S2) and those that would originate unreduced gametes (Fig. 5).

The origin of aneuploid gametes has been related to out-of-plate and nucleus chromosomes, laggard chromosomes, chromosomes with precocious migration, multipolar spindles and cytomixis (Risso-Pascotto et al., 2005; Wang et al., 2010; Ortiz et al., 2011; Guan et al., 2012; Mursalimov and Deineko, 2018). In our analysis, one tripolar spindle in anaphase I (6.95%), and two tripolar spindles in anaphase II (up to 11.11%) were found. Although these types of spindle alterations are rarely observed in plants (De Storme and Geelen, 2013), we assume that the presence of these alterations would also contribute to the formation of aneuploid gametes. Multipolar spindles have been reported in several plant species (Risso-Pascotto et al., 2005; Wang et al., 2010; Reis et al., 2016) and are correlated with abnormal cytokinesis, which leads to the formation of polyads with unbalanced microspores (Tilquin et al., 1984; Risso-Pascotto et al., 2005), such as telophases II with five or six nuclei and the hexads detected in the hybrids. On the other hand, out-of-plate chromosomes were detected in metaphase I (up to 51.02%), anaphase I (up to 18.18%), metaphase II (up to 25%) and anaphase II (up to 7.80%). Meiotic abnormalities related to irregular chromosome segregation, such as precocious chromosome migration to the poles in metaphase I (up to 38.09%) and metaphase II (up to 3.85%), and laggard chromosomes at anaphase I and II (up to 24.65%) were also detected. All the aforementioned meiotic abnormalities could alter the regular chromosome segregation, and the chromosomes that ultimately cannot be incorporated into the telophase I and II nuclei, lead to the origin of micronuclei (Ortiz et al., 2011; Pagliarini, 2000; Seijo and Solis Neffa, 2006; Wang et al., 2015). In our analysis, one micronucleus was observed at anaphase I, telophase I and metaphase II (up to 12.5%), and one-two micronuclei were observed at anaphase II and

telophase II (up to 8.33%). The frequency of micronuclei recorded was lower than that expected from the number of PMCs that presented segregation and spindle abnormalities (Table 4). This suggests that a large proportion of the chromosomes irregularly segregated should have been included at least into one of the main nuclei. However, those inclusions did not guarantee the restitution of the euploid chromosome number of the main nuclei. In this sense, the counting at anaphase I, prophase II, metaphase II, anaphase II and telophase II revealed PMCs with plates and nuclei with an unbalanced number of chromosomes (Table 4). Finally, cytomixis was observed in all the divisional stages of PMCs, a fact that would contribute to the abnormal meiotic behavior, since the transfer of different amounts of chromatin results in unbalanced complements of the PMCs involved (Guan et al., 2012; Qiu et al., 2017). Therefore, all the aforementioned meiotic abnormalities would lead to the formation of microcytes, as well as of sporads with more than four microspores (pentads and hexads) or sporads with four microspores with different sizes (Table 3). The ultimate consequence of these irregularities is the high number of unviable pollen grains detected in the hybrids (Table 2). This would explain the great diversity of pollen grain sizes found, and would also be the main cause of the low pollen viability of the hybrids analyzed.

Concerning the origin of unreduced gametes, detailed cytological studies in several plant species have revealed that these phenomena may be the result of a plethora of cellular failures. According to the review of De Storme and Geelen (2013), these are generally subdivided into three main classes: (1) alterations in meiotic spindle dynamics, (2) defects in meiotic cell plate formation, and (3) omission of meiosis I or II. The process of formation of unreduced gametes has also been associated both with chromosomal bridges (Bajer, 1964) and with the phenomenon of cytomixis, although this has not yet been confirmed experimentally (Dewitte et al., 2012; Mursalimov and Deineko, 2015, 2018).



The meiotic irregularities linked to the formation of unreduced gametes found in the hybrids here analyzed are individually described below.

### ***Chromosome bridges***

The formation of bridges was observed in up to 64.10% of the PMCs in anaphase I (Fig. 5A), and could be preserved during telophase I (Fig. 5B), like those detected in up to 33.34% of the PMCs. During the meiotic division process, the chromatin continuity of the bridges may not be interrupted, which is why the presence of residual bridges is noticed during meiosis II. This was detected in up to 2.75% of the PMCs in prophase II, 33.90% in metaphase II (Fig. 5C) and 14.27% in anaphase II (Fig. 5D). Likewise, bridges can be generated during meiosis II, as those found in up to 75% of the PMCs in anaphase II (Fig. 5E). Finally, bridge formation may occur in the second meiotic division of cells showing residual bridges of the first division, as observed in up to 15.91% of the PMCs in anaphase II (Fig. 5F).

It has been determined that the persistence of some bridges in telophase I and II can bring the poles closer together and thus establish a chromatin continuity between them, promoting the formation of the nuclear membrane around the nuclei involved (Seijo, unpublished results, 2002). Zhang et al. (2017) proposed that chromosome stickiness in thick chromosomal bridges restricts the separation during telophase I. This phenomenon would explain the formation of the restitution nuclei detected in the individuals analyzed in telophase I and in later meiotic phases. This nuclear restitution mechanism has already been cited in different genera such as *Begonia* (Dewitte et al., 2010b) and *Arachis* (Ortiz et al., 2011) and, in both cases, the results showed that this mechanism is involved in the origin of unreduced gametes.

### ***Tripolar spindles***

In several plant species, it has been proposed that the aberrations in the formation of the meiotic spindle constitute one of the main causes of the nuclear restitution in the microsporogenesis process (Bretagnolle and Thompson, 1995; Zhang and Kang, 2010). One of the spindle defects leading to meiotic non-reduction are tripolar spindles (Rim and Beuselinck, 1996). In our analysis, one tripolar spindle was detected in up to 25% of the PMCs in anaphase II (Fig. 5G). In addition, in meiosis II, this phenomenon was found associated with a residual bridge in up to 1.29% of PMCs.

According to Zhang and Kang (2010), the presence of tripolar and fused spindles may explain the formation of triads and dyads. Likewise, some PMCs can exhibit absence of cytokinesis, producing high frequencies of this type of sporads (Zhang et al., 2017). In tripolar spindles, unilateral defects in metaphase II spindle orientation lead to a rejoining of chromatids at one pole and normal separation at the other pole, producing triads with one  $2n$  spore and two  $n$  spores. Due to the aforementioned, these irregularities are currently the main mechanisms involved in the formation of unreduced gametes (De Storme and Geelen, 2013). In this work, the tripolar spindles detected in anaphase II of individuals 2, 3 and 6 would explain the presence of telophases II with a nucleus of restitution ( $2n$ ) and two  $n$  nuclei. Consequently, this is consistent with the finding of triads that will produce  $2n$  and  $n$  pollen grains.

### ***Cytomixis***

In this work, the migration of chromatin from one of the PMCs to another was observed in all meiotic phases (Table 4, Fig. 5H), showing the highest percentages during meiotic prophase, up to 100% of the PMCs in prophase I and 66.67% in prophase II. Cytomixis has been proposed as one of the mechanisms of unreduced gamete formation (Dewitte et al., 2012; Mursalimov and Deineko, 2015), as observed in *Dactylis* (Falistocco et al., 1995), *Sorghum*

*bicolor* (Ghaffari, 2006), *Arachis pintoi* (Lavia et al., 2011), *Triticum* and *Secale* (Sidorchuck et al., 2016), *Lippia alba* (Reis et al., 2016) and *Lilium* (Zhang et al., 2017).

It has been proposed that the mechanism of formation of unreduced gametes by cytomixis is the development of binucleated meiocytes, formed when an entire nucleus migrates to the recipient cell (Sidorchuck et al., 2016; Mursalimov and Deineko, 2018). These nuclei show no signs of damage in the zygo-pachytene phase and progressively continue meiotic division (Mursalimov and Deineko, 2015), being observable until the nuclear membrane disappears in metaphase I, after which, they are undetectable. Consequently, it is likely that a single meiotic spindle will form for both nuclei, leading to the formation of unreduced pollen. Although this has not been experimentally confirmed, it has been demonstrated that, in plants with a high cytomixis rate, the pollen produced is more than twice the size of normal pollen (Mursalimov and Deineko, 2018). This allows us to assume that cytomixis could also be a possible cause of the generation of unreduced gametes in the hybrids analyzed.

### ***Restitution nuclei***

Our analysis suggests that the high frequency of irregularities such as bridge persistence, tripolar spindle formation and cytomixis are the meiotic mechanisms that would origin restitution nuclei. All hybrid individuals presented evidence of nuclear restitution in both meiotic divisions. The analysis of PMCs showed that nuclear restitution began in the first meiotic division (see above) as restitution nuclei were found in up to 35% of the PMCs in telophase I (Fig. 5I), and then observed as a single nucleus in prophase II (Fig. 5J) and as a single plate in metaphase II (Fig. 5K), in up to 57.14% and 56.14% of the PMCs, respectively. At the end of meiotic division, during anaphase II (Fig. 5L) and telophase II (Fig. 5M), these unreduced nuclei may either undergo meiotic division and give rise to two  $2n$  nuclei (up to 48.68% of the PMCs) or suffer a second nuclear restitution process, giving

rise to  $4n$  restitution nuclei, as those found in the present study (up to 48.49% of the PMCs, Fig. 5N). In this sense, Dewitte et al. (2010b) showed that *Begonia* 'Orococo' mainly produces first division restitution dyads and  $2n$  pollen grains, as a consequence of an omitted meiosis I. In addition, it has been proposed that, if a complete loss of meiotic cytokinesis occurs, tetranuclear spores are formed, developing  $4n$  gametes (De Storme and Geelen, 2013). Consequently, these mechanisms would explain the finding of both dyads and monads, as well as the formation of  $2n$  and  $4n$  pollen grains.

We also found that the process of nuclear restitution could originate in the second meiotic division, because one nucleus of restitution and two haploid nuclei were found in up to 22.22% of the PMCs in anaphase II (Fig. 5O) and 14.47% in telophase II (Fig. 5P). This can occur if the chromosomes located on one of the metaphase II plates segregate towards the poles, while the chromosomes on the other plate undergo nuclear restitution (see above), resulting in the formation of triads, and therefore in  $2n$  and  $n$  pollen grains. Several authors proposed that second division restitution, i.e. incomplete separation of nuclei during meiosis II, gives rise to  $2n$  pollen grains (Bretagnolle and Thompson, 1995; Dewitte et al., 2010b) as observed in this work.

An important fact to point out is the high production of  $2n$  restitution nuclei found in the [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup> hybrid. This phenomenon occurred in up to 57.14% of the PMCs in prophase II, 56.14% in metaphase II, 22.22% in anaphase II and 48.68% in telophase II. Although the rate of production of dyads and triads and the formation of  $2n$  pollen grains of this hybrid were not high, it should be noted that we were able to analyze the meiosis of a single individual, which, taking into account the records of sporads and pollen of other hybrids of the same cross, could indicate that they may have high rates of production of restitution nuclei. These results suggest that hybrids in which *A. duranensis* was used as the female parent produced a greater number of restitution nuclei.

In summary, the meiotic abnormalities found in the meiosis of intergenomic hybrids between *A. duranensis* and *A. ipaënsis* (persistent bridges, tripolar spindles and cytomixis) are direct evidences of the formation of restitution nuclei in both the first and second meiotic division. These failures in the regularity of meiosis lead to the formation of monads, dyads and triads, and consequently to the formation of  $2n$  and  $4n$  gametes.

### **Origin of *A. hypogaea***

Several authors have studied the origin of *A. hypogaea* for many years. Based on the morphological characteristics of this species, Krapovickas and Gregory (1994) identified six botanical varieties: two belonging to the subsp. *hypogaea* (*hypogaea* and *hirsuta*) and four belonging to the subsp. *fastigiata* (*fastigiata*, *vulgaris*, *aequatoriana* and *peruviana*). The objective of elucidating the origin of peanut is to find out which are its wild progenitors, what type of polyploid is, whether it was a single event of polyploid origin, where and when the event occurred, and how this event happened. Currently, cytogenetic, phylogenetic and molecular evidences indicate that the wild species involved in the origin of peanut are *A. duranensis* and *A. ipaënsis* (Kochert et al., 1996; Seijo et al., 2007; Robledo et al., 2009; Grabiele et al., 2012; Moretzsohn et al., 2013; Bertioli et al., 2016). Recent research has also established that *A. hypogaea* is a segmental allotetraploid, and that the six botanical varieties of the crop, as well as the immediate wild antecessor *A. monticola*, have suffered a single event of polyploid origin, involving *A. duranensis* as the female parent and *A. ipaënsis* as the male parent (Seijo et al., 2007, 2018; Grabiele et al., 2012; Bertioli et al., 2019). It has been hypothesized that the probable center of origin of *A. hypogaea* was the south of Bolivia and northwest of Argentina, which is the current distribution area of the diploid species involved in its origin (Krapovickas and Gregory, 1994; Simpson, 2001; Dillehay et al., 2007; Grabiele et al., 2012). Furthermore, analysis of nucleotide divergence has determined that, about 9,400

years ago, *A. ipaënsis* was carried by humans into the area of *A. duranensis*, enabling their hybridization and formation of *A. hypogaea* (Bertioli et al., 2016).

Despite the great efforts made in elucidating all the aforementioned questions, there are no accurate data on how the event of polyploidization of the crop occurred. The model of origin proposed suggests that it was an event of hybridization between wild species followed by a chromosomal duplication or fusion of unreduced gametes, giving rise to a wild tetraploid with two complements of each genome, A and B. After the origin of this wild allotetraploid, *A. hypogaea* arose by domestication. In this context, the ability of [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup> hybrids to produce 2n pollen constitutes relevant evidence to support the fact that the most probable mechanism for the origin of peanut was sexual polyploidization. In this sense, this crop could have originated as a result of the production of a diploid AB hybrid through natural hybridization between *A. duranensis* (AA) and *A. ipaënsis* (BB). This amphiploid would have the ability to produce polyploid individuals either by unilateral sexual polyploidization through the union of a normal gamete (*n*) with an unreduced one (*2n*), triploid bridge, or by bilateral sexual polyploidization through the formation and union of two *2n* gametes.

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#### SUPPLEMENTAL MATERIAL

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**Figure S1.** Pollen grain size and viability of parental species and hybrids.

**Figure S2.** Meiotic abnormalities involved in the origin of aneuploid gametes.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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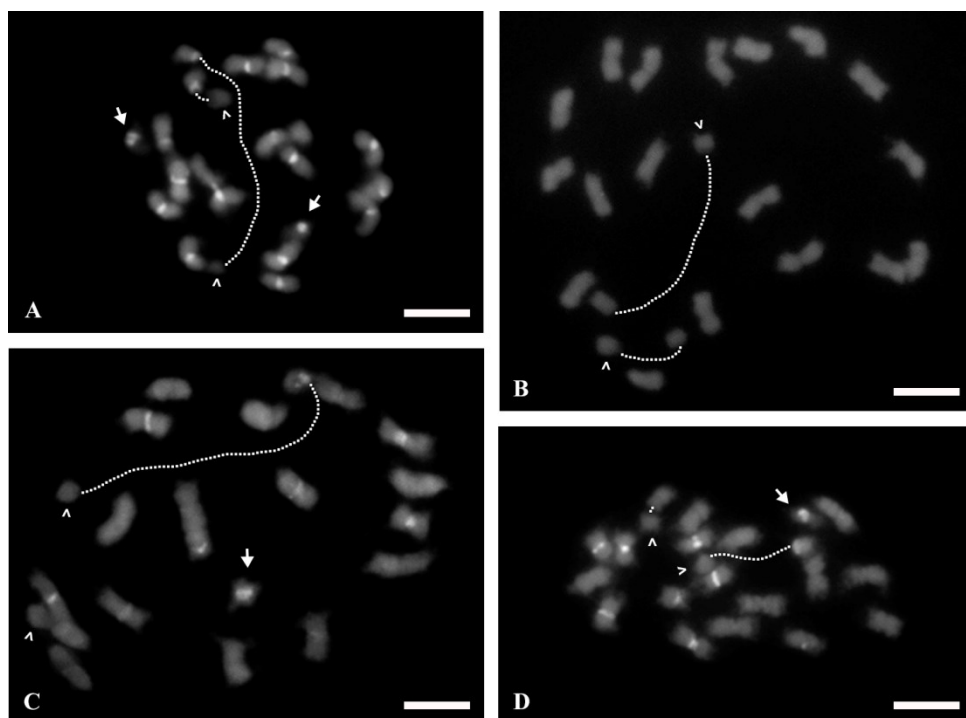
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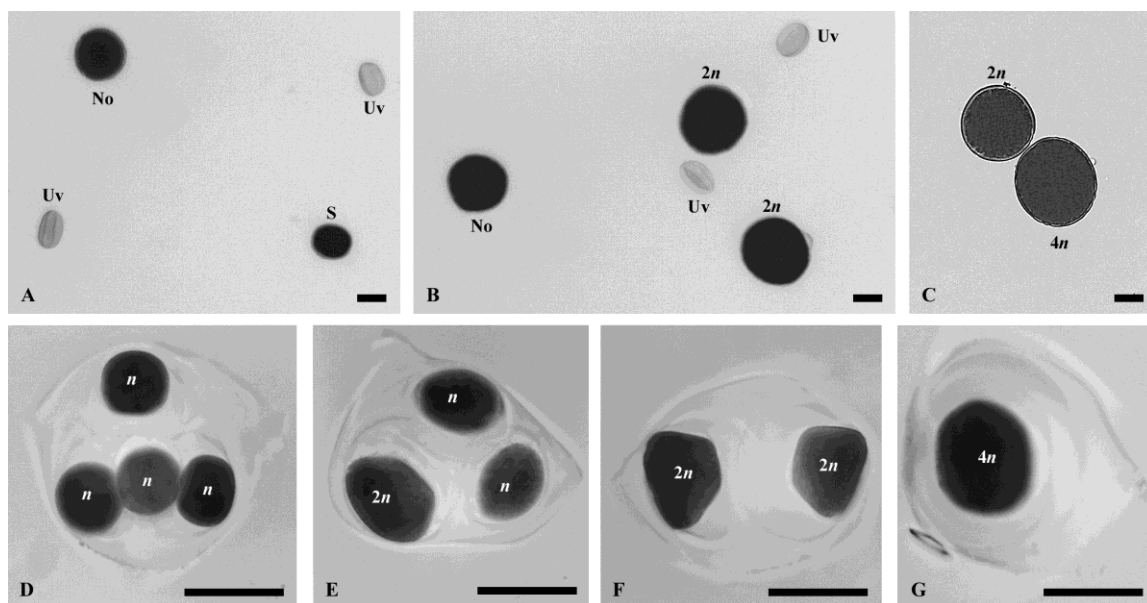
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## FIGURE LEGENDS

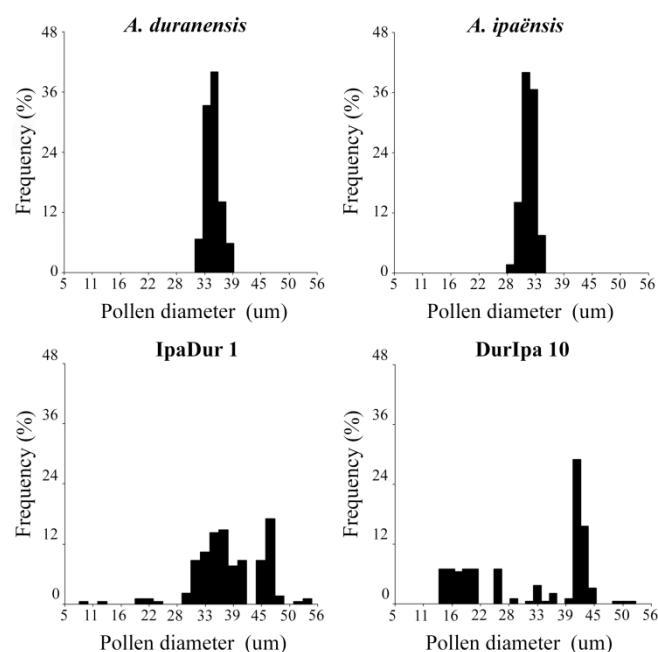




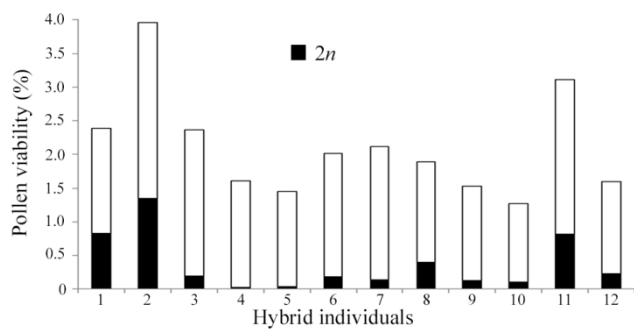
**Figure 1.** Somatic metaphases of *Arachis* diploids after DAPI staining. *Arachis duranensis* (AA) with DAPI<sup>+</sup> centromeric bands in all chromosomes (A). *Arachis ipaënsis* (BB) with chromosomes devoid of bands (B). [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup> hybrid (C) and [*A. ipaënsis* x *A. duranensis*]<sup>2x</sup> hybrid (D), showing half of the chromosomes (those belonging to the A genome) with conspicuous centromeric bands and half of the chromosomes (those of the B genome) without centromeric bands. White arrows indicate “A chromosomes” and white arrowheads indicate the satellites. Scale bar = 5µm.



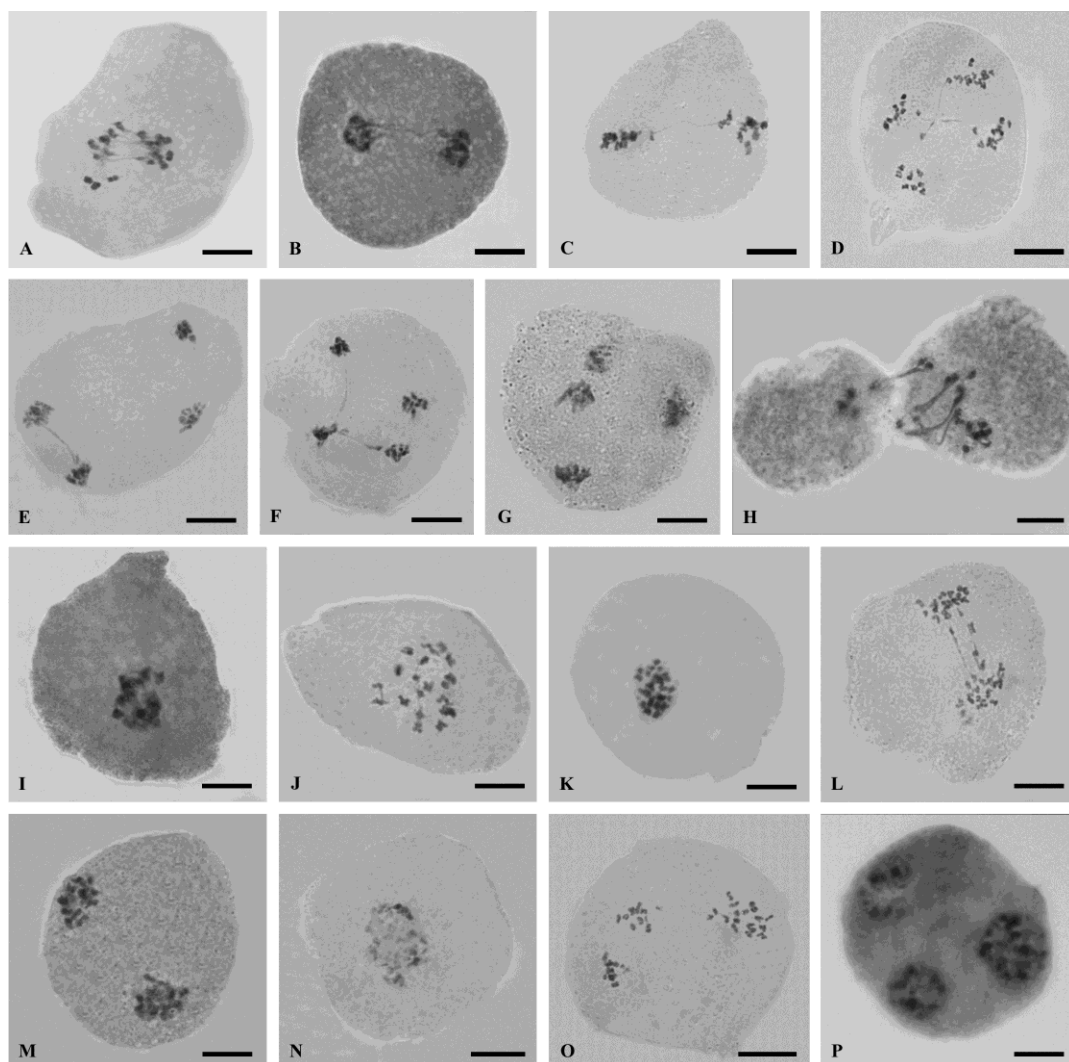
**Figure 2.** Variation in pollen size and sporad constitution in the hybrids. Small and normal viable pollen grains (A). Size difference between normal and 2n pollen grains (B) and size difference between 2n and 4n pollen grains (C). Normal tetrad (D). Triad with one 2n microspore (E). Dyad with two 2n microspores (F). Monad with one 4n microspore (G). S, small; No, normal; Uv, unviable. Scale bar = 20  $\mu$ m.



**Figure 3.** Frequency distribution of pollen grain size in the parental species (*A. duranensis* and *A. ipaënsis*) and the hybrid individuals (IpaDur 1 and DurIpa 10).



**Figure 4.** Percentages of  $2n$  pollen grains in relation to viable grains in the 12  $F_1$  hybrid individuals analyzed. Individuals 1-6 are [*A. ipaënsis* x *A. duranensis*] $^{2x}$  and 7-12 are [*A. duranensis* x *A. ipaënsis*] $^{2x}$ .



**Figure 5.** Meiotic abnormalities involved in the origin of unreduced gametes. Anaphase I (**A**) and telophase I (**B**) with chromosome bridges. Metaphase II (**C**) and anaphase II (**D**) with

residual bridges. Anaphase II with chromosome bridges (E). Anaphase II with chromosome and residual bridges (F). Anaphase II with tripolar spindle (G). Cytomixis in Metaphase I showing chromosome migration (H). Telophase I (I), prophase II (J) and metaphase II (K) with one restitution nucleus. Anaphase II (L) and telophase II (M) with two  $2n$  nuclei. Telophase II with a  $4n$  restitution nucleus (N). Anaphase II (O) and telophase II (P) with one restitution nucleus and two  $n$  nuclei. Scale bar = 10  $\mu\text{m}$ .

## TABLES

**Table 1.** Accessions and species used for crosses and induced F1 hybrids obtained in this study. Chromosome number ( $2n$ ), genome type (G).

Species	$2n$	G	Origin and collection number
<i>A. duranensis</i> Krapov. & W.C.Greg.	20	AA	Argentina, Prov. Salta, Dept. Capital. 24°45'S; 65°26'W. VEvNv 14167.
	20	AA	Argentina, Prov. Salta, Dept. Gral José de San Martín. 22°15'S; 63°43'W. K 7988.
<i>A. ipaënsis</i> Krapov. & W.C.Greg	20	BB	Bolivia, Dept. Tarija, Prov. Gran Chaco, Ipa. 21°00'S; 63°24'W. KGBPScS 30076
[ <i>A. ipaënsis</i> K30076 x <i>A. duranensis</i> K 7988] <sup>2x</sup>	20	BA	IpaDur 1, 2
[ <i>A. ipaënsis</i> K30076 x <i>A. duranensis</i> V14167] <sup>2x</sup>	20	BA	IpaDur 3, 4, 5, 6
[ <i>A. duranensis</i> V14167 x <i>A. ipaënsis</i> K30076] <sup>2x</sup>	20	AB	DurIpa 7, 8, 9, 10, 11, 12


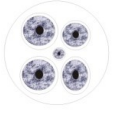

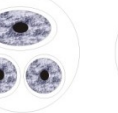
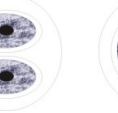

Abbreviations: B= D. Banks; Ev= A. Echeverry; G= W.C. Gregory; K= A. Krapovickas; Nv= L. Novara; P= J. Pietrarelli; S= C.E. Simpson; Sc= A. Schinini; V= J.F.M Valls. Prov.= province; Dept.= department.

**Table 2.** Types of pollen grains found and pollen viability evaluated in *A. duranensis*, *A. ipaënsis* and the 12 F<sub>1</sub> hybrids obtained.

Individuals	n	Unviable Pollen Grains (%)	Types of Viable Pollen Grains (%)				Viability (%)
			S	No	2n	4n	
<i>A. duranensis</i> V 14167	2000	2.35	-	-	-	-	97.65
<i>A. duranensis</i> K 7988	2000	1.45	-	-	-	-	98.55
<i>A. ipaënsis</i> K 30076	2000	1.25	-	-	-	-	98.75
<b>IpaDur</b>							
1	7623	97.61	0.09	1.39	0.83	0.08	2.39
2	5716	96.04	0.12	2.47	1.35	0.02	3.96
3	6150	97.63	0.21	1.95	0.19	0.02	2.37
4	7082	98.39	1.41	0.15	0.02	0.03	1.61
5	7314	98.55	1.24	0.17	0.04	0.00	1.45
6	6857	97.99	1.28	0.48	0.18	0.07	2.01
<b>DurIpa</b>							
7	7164	97.89	1.48	0.34	0.14	0.15	2.11
8	6511	98.11	1.00	0.34	0.40	0.15	1.89
9	6036	98.47	1.09	0.31	0.13	0.00	1.53
10	8333	98.73	0.92	0.21	0.10	0.04	1.27
11	6900	96.89	1.18	0.86	0.82	0.25	3.11
12	6823	98.40	0.79	0.45	0.23	0.13	1.60

Abbreviations: IpaDur = [*A. ipaënsis* x *A. duranensis*]<sup>2x</sup>, DurIpa = [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup>, n= number of pollen grains analyzed, S = small size pollen grain, No= reduced normal pollen grain, 2n = giant pollen grain, 4n= jumbo pollen grain.

**Table 3.** Analysis of the constitution of sporads in the 12 hybrid individuals studied.

Hybrid individuals	n	Types of sporads (%)					
		Te	Te ≠	Pol	Tr	Dy	Mo
							
<b>IpaDur</b>							
1	280	31.43	56.78	0.00	6.43	2.86 <sup>ab</sup>	2.50
2	48	41.67	27.08	0.00	10.42	20.83 <sup>c</sup>	0.00
3	313	71.56	25.88	0.64	1.92	0.00 <sup>a</sup>	0.00
4	650	51.38	40.00	5.85	2.42	0.31 <sup>ab</sup>	0.00
5	625	51.68	41.44	1.76	4.16	0.96 <sup>ab</sup>	0.00
6	554	54.69	34.12	8.12	2.53	0.54 <sup>ab</sup>	0.00
<b>DurIpa</b>							
7	409	51.83	33.26	2.69	11.25	0.97 <sup>ab</sup>	0.00
8	363	30.85	36.33	2.47	17.37	12.13 <sup>bc</sup>	0.55
9	724	33.70	16.85	4.28	7.05	37.29 <sup>d</sup>	0.83
10	713	38.85	38.15	16.27	6.31	0.42 <sup>ab</sup>	0.00

<b>11</b>	1111	58.86	28.44	5.31	2.79	4.51 <sup>ab</sup>	0.09
<b>12</b>	1045	61.81	31.68	2.96	3.16	0.39 <sup>ab</sup>	0.00

**F=1.62 F=23.95 F= 0.49**  
**p= 0.1704 p=<0.0001 p= 0.8896**

Abbreviations: IpaDur, [*A. ipaënsis* x *A. duranensis*]<sup>2x</sup>; DurIpa, [*A. duranensis* x *A. ipaënsis*]<sup>2x</sup>; Te, tetrad; Te ≠, abnormal tetrads; Pol, polyad; Tr, triad; Dy, Dyad; Mo, monad.

**Table 4:** Meiotic abnormalities recorded in five hybrid individuals studied.

Phases	Abnormalities	Hybrid individuals									
		IpaDur						DurIpa			
		1		2		3		6		7	
	n	%	n	%	n	%	n	%	n	%	
<b>PI</b>	Cytomixis	(188)	19.68	(2)	100	-	-	(215)	46.05	-	-
<b>MI</b>	Cell with only one univalent 1-7	(72)	-	(49)	-	(4)	25.00	(63)	-	-	-
	Chromosomes with precocious migration 1-7	-	-	-	-	-	-	-	38.09	-	-
	Out-of-plate chromosomes	-	25.00	-	51.02	-	25.00	-	25.40	-	-
	Cytomixis	-	8.34	-	4.09	-	-	-	12.66	-	-
<b>AI</b>	1-5 Laggard chromosomes	(22)	-	(72)	6.94	(39)	20.52	(70)	12.86	-	-
	1-5 Out-of-plate chromosomes	-	18.18	-	11.11	-	-	-	11.43	-	-
	Unbalanced plates	-	68.18	-	8.33	-	2.56	-	10.00	-	-
	1 Micronucleus	-	-	-	2.78	-	-	-	-	-	-
	1 Bivalent with precocious migration	-	-	-	6.94	-	-	-	-	-	-
	1 Tripolar spindle	-	-	-	-	-	2.56	-	8.57	-	-

			1-5							
			Chromosome bridges	4.55	23.6	64.1	52.8			-
					1	0	5			
			Cytomixis	-	22.2	-	-			-
					2					
<b>TI</b>			1		12.5	9.68				-
			Micronucleus	-	0	(31)	(6)			-
				(40)						
			1-4 Out-of-nucleus chromosomes	-	5.00	-	-			-
			1		12.5	19.3	33.3			-
			Chromosome bridge	-	0	5	4			
			1 Nucleus of restitution ( $4n$ )	-	35.0	3.23	-			-
					0					
			Cytomixis	-	-	32.2	-			-
						6				
<b>PII</b>			Unbalanced nuclei	(3)	-	(109)	0.92	(40)	60.0	(7)
					-				0	
			1 Residual bridge	-	-	2.75	-			-
			1 Nucleus of restitution ( $4n$ )	-	-	4.59	-			57.1
										4
			Cytomixis	66.6	-	7.34	40.0			-
				7			0			
<b>MII</b>			Unbalanced plates	-	(8)	50.0	25.4	(26)	23.0	(57)
						0	2		8	
			1-5 Out-of-plate chromosomes	-	25.0	16.9	-			-
					0	5				
			Chromosome s with precocious migration	-	-	-	3.85			-
			1		-	-	7.69			-
			Micronucleus	-	-	-				
			1-4 Residual bridges	-	12.5	33.9	7.69			10.5
					0	0				3
			1 Nucleus of restitution ( $4n$ )	-	-	3.39	15.3		8	56.1
										4
			Cytomixis	-	-	-	15.3		9	-

<b>AII</b>	1-6 Laggard chromosome s	-	(4)	-	(77)	24.6 5	(44)	-	(10 )	-	
	1-4 Out-of-plate chromosome s	-		-		7.80		6.82		-	
	Unbalanced plates	-		-		6.49		-		30.0 0	
	1-2 Micronuclei	-		-		3.89		-		-	
	1-4 Out-of-plate chromosome s + 1	-		-		1.29		-		-	
	chromosome bridge + 1 residual bridge	-		-				-		-	
	2 Tripolar spindles	-		-		1.29		6.82		10.0 0	
	1-2 Chromosome bridges	-			75.0 0	9.08		6.82		-	
	1 Tripolar spindle	-			25.0 0	3.89		6.82		-	
	1-2 Residual bridges	-		-		14.2 7		-		-	
	1 Nucleus of restitution (2n) + 2 nuclei (n)	-		-		1.29		-		20.0 0	
	1 Tripolar spindle + 1 residual bridge	-		-		1.29		-		-	
	1-2 Chromosome bridges +1 residual bridge	-		-				-	15.9 1	-	
	2 Nucleus of restitution (2n)	-		-						1.00	
	Cytomixis	-		-				-	11.3 6	-	
	<b>III</b>	1-2 Micronuclei	(33)	-	-	(12)	8.33	(182 )	2.75	(76 )	-
		5-6 nuclei		-	-		-		0.55		11.8



Unbalanced nuclei	-	-	-	24.1	4					
				8	9.22					
1 Nucleus of restitution ( $4n$ )	48.4	-	25.0	3.85	9.22					
	9		0							
2 nuclei of restitution ( $2n$ )	27.2	-	16.6	4.95	48.6					
	7		7		8					
1 Nucleus of restitution ( $2n$ ) and 2 nuclei ( $n$ )	12.1	-	-	9.89	14.4					
	2				7					
Cytomixis	-	-	-	20.8	1.31					
				8						
<b>TOTAL</b>	<b>318</b>	<b>35.2</b>	<b>176</b>	<b>71.0</b>	<b>331</b>	<b>55.8</b>	<b>646</b>	<b>65.1</b>	<b>150</b>	<b>89.3</b>
<b>L</b>		<b>2</b>		<b>2</b>		<b>9</b>		<b>7</b>		<b>3</b>

Abbreviations: n= absolute values of PMCs analyzed, P= prophase, M= metaphase, A= anaphase, T= telophase, I= first meiotic division, II= second meiotic division.