

## Meiotic behavior of interspecific hybrids between artificially tetraploidized sexual *Brachiaria ruziziensis* and tetraploid apomictic *B. brizantha* (Poaceae)

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**ABSTRACT:** The meiotic behavior of four interspecific promising hybrids was evaluated by conventional cytological methods. The female genitors were two artificially tetraploidized sexual accessions of *B. ruziziensis* (R41 and R44,  $2n = 4\chi = 36$ ), which were crossed to an agronomically superior natural tetraploid apomictic genotype of *B. brizantha* (B140 - BRA003395). Three of them (HBGC313, HBGC 315, and HBGC324) were sexual and one (HBGC325) apomictic. Analyses of some cells in diakinesis revealed multivalent chromosome configurations, suggesting that genetic recombination and introgression of some genes could be present. The four hybrids had different types of meiotic abnormalities at various frequencies. Abnormalities related to irregular chromosome segregation due to polyploidy were common among these hybrids, and characterized by precocious chromosome migration to the poles, laggard chromosomes, both generating micronuclei in telophases and tetrads and, as a consequence, unbalanced gametes. One abnormality genotype-specific, related to spindle orientation (a putative *divergent spindle* mutation), was recorded for the first time in two of the hybrids, HBGC313 and HBGC325. The sexual hybrid HBGC324 had the lower rate of abnormalities, and it could be used as a female genitor in future crosses in the breeding program. The abnormalities present in these hybrids may impact fertility and affect seed production. Based on the results, HBGC324 is the single hybrid recommended to the breeding program. Hybrids must produce a good amount of viable seeds, besides good overall dry matter production and nutritive value, in order to be widely utilized and adopted in production systems. Due to pseudogamy, the desirable superior apomictic hybrids need viable pollen grains to fertilize the secondary nucleus of the embryo sac and thus ensure normal and vigorous endosperm development and plenty of seed set.

Key words: *Brachiaria*, Brazil, cytogenetics, forage grasses, meiosis

## Comportamento meiótico de híbridos interespecíficos entre tetraplóides artificiais de *Brachiaria ruziziensis* e tetraplóides apomíticos de *B. brizantha* (Poaceae)

**RESUMO:** O comportamento meiótico de quatro híbridos interespecíficos promissores de *Brachiaria* foi avaliado por metodologia citogenética convencional. Os genitores femininos eram dois acessos sexuais tetraploidizados artificialmente de *B. ruziziensis* (R41 e R44,  $2n = 4\chi = 36$ ), os quais foram cruzados com um genótipo tetraplóide natural apomítico agronomicamente superior de *B. brizantha* (B140 - BRA003395). Três deles (HBGC313, HBGC 315 e HBGC324) eram sexuais e um (HBGC325) apomítico. A análise de algumas células em diacinese revelou a presença de configurações cromossômicas multivalentes, sugerindo que recombinação genética e introgressão de alguns genes poderão estar presentes. Os quatro híbridos apresentaram frequências e tipos variados de anormalidades meióticas. Anormalidades relacionadas à segregação irregular de cromossomos devido à poliploidia foram comuns entre os híbridos e caracterizadas pela presença de cromossomos em ascensão para os pólos e cromossomos retardatários, ambos levando à formação de micronúcleos em telófases e tétrades e, como conseqüência, gametas desbalanceados. Uma anormalidade genótipo-específica, relacionada à orientação do fuso (uma possível mutação *fuso divergente*) foi observada pela primeira vez em dois dos híbridos, HBGC313 e HBGC325. O híbrido sexual HBGC324 apresentou menor frequência de anormalidades meióticas, podendo ser usado como genitor feminino em futuros cruzamentos no programa de melhoramento. A frequência e os tipos de anormalidades observadas podem afetar a fertilidade e a produção de sementes. Com base nos resultados, o híbrido HBGC324 é recomendado para o programa de melhoramento. Híbridos devem produzir grande quantidade de sementes viáveis, além de matéria seca com alto valor nutritivo para serem amplamente utilizados na produção de pastagens. Devido à pseudogamia, os híbridos apomíticos superiores necessitam de grãos de pólen viáveis para fertilizar o núcleo secundário do saco embrionário assegurando o desenvolvimento vigoroso do endosperma e a produção de sementes.

Palavras-chave: *Brachiaria*, Brasil, citogenética, meiose, forrageira

## Introduction

In the last decades, Brazil became the largest beef cattle producer and exporter of beef products. Tropical humid climate and extensive almost flat terrain provide the natural conditions for year-round beef production on cultivated pastures. Brachiariagrass alone accounts for 85% of the cultivated pastures in Brazil, covering over 80 million hectares and sustaining the largest commercial herd in the world – about 205 million of heads. The genus *Brachiaria* has great success as a forage grass in the South American savannas due to its physiological tolerance to the typical low-fertility acid soils of the tropics (Rao et al., 1996). The most widely grown species of forage grass in the Brazilian savannas and throughout the tropics are *B. decumbens* cv. Basilisk and *B. brizantha* cv. Marandu. However, the lack of options to diversity pastures is a constraint since cv. Basilisk lacks resistance to a ubiquitous family of sucking insects, the spittlebugs, and cv. Marandu, while quite resistant, requires higher soil fertility (Miles et al., 1996) and is unsuitable for poorly-drained soils, especially in the Amazon region where large areas of dying pastures are reported (Barbosa, 2006).

Pasture renovation and intensification of beef production demand new improved cultivars, which can be obtained by selection from native germplasm or generated in breeding programs. The brachiariagrass breeding programs were delayed because the most important accessions and cultivars are polyploid ( $2n = 4x = 36$ ) and apomictic (Valle and Savidan, 1996). *Brachiaria* breeding became effective only when a suitable sexual germplasm was artificially developed in 1980s. *Brachiaria ruziziensis* (ruzigrass) is a natural, sexual diploid species. Accessions of this species were tetraploidized with colchicine in Belgium (Swenne et al., 1981; Gobbe et al., 1981) and maintained the sexuality necessary for hybridization with apomictic natural tetraploid accessions. These materials have allowed apomixis to be exploited in the breeding of *Brachiaria*.

The main objective of the *Brachiaria* program in tropical America is to use the sexuality of the tetraploid ruzigrass to release the genetic diversity locked in the natural tetraploid apomictics such as *B. brizantha* (palisadegrass), and *B. decumbens* (signalgrass), to produce novel apomictic hybrid cultivars (Valle and Savidan, 1996; Miles and Valle, 1996; Miles et al., 2004). In 1988, the Embrapa Beef Cattle Center initiated an extensive program based on interspecific hybridization with the objective of determining the inheritance of apomixis and thus manipulating this character for the development of new improved hybrids. Firstly, the crosses were preferentially done between sexual accessions of *B. ruziziensis* and natural apomictic *B. brizantha* cv. Marandu or *B. decumbens* cv. Basilisk. Afterwards, other accessions of superior agronomic performance and resistant to spittlebugs, such as *B. brizantha* B140 (BRA003395), were crossed as pollen donors. Hundreds of hybrids were ob-

tained from the crosses and 88 selected based on the leafiness and regrowth ability after cuts are under agronomic evaluation. However, besides resistance to spittlebugs and adaptation to low fertility soils, a new cultivar needs to have high seed production to meet the internal market demand and also for export.

Cultivars commercially available are direct selections from the natural genetic variability (Miles et al., 2004), except for one interspecific hybrid (cv. 'Mulato'), which produces seeds with viability rate of 10%, thus compromising the commercial interests of the seed industry and impairing wide adoption. Among several factors that could compromise seed viability, Hopkinson et al. (1996) cite interspecific hybridization *per se*, and the polyploid nature of genotypes, which impair seed viability by affecting pollen fertility. Previous cytogenetical analyses performed on some *Brachiaria* hybrids produced by Embrapa Beef Cattle (Risso-Pascotto et al., 2005a; Fuzinato et al., 2007; Adamovski et al., 2008) revealed different types and varying frequencies of meiotic abnormalities, thus indicating the possibility of selecting genotypes for higher pollen viability and, probably, higher seed production. This paper describes the meiotic behavior of four promising hybrids between *B. ruziziensis* and *B. brizantha*; also, based on the types and frequencies of abnormalities, it gives an indication about the hybrid that is more stable to ensure greater pollen fertility and success in the hybridizations.

## Material and Methods

### Plant Material

Cytological studies were carried out on four interspecific hybrids between *B. ruziziensis* and *B. brizantha* obtained at Embrapa Beef Cattle, Campo Grande, State of Mato Grosso do Sul, Brazil (20°25' S, 54°46' W, 552 m altitude). Crosses were manually performed, using pollen from cut inflorescences of the apomictic genitor collected in petri dishes into flowers of the sexual genitors in vases, in the greenhouse. Seeds obtained were individually germinated in Styrofoam trays, and then transplanted to the field as a progeny test. The genitors were two artificially tetraploidized sexual accessions of *B. ruziziensis* (R41 and R44,  $2n = 4x = 36$ ), (Swenne et al., 1981; Gobbe et al., 1981) and the natural tetraploid apomictic genotype: *B. brizantha* (B140 - BRA003395,  $2n = 4x = 36$ ). Two hybrids (HBGC313 and HBGC315) are full-sibs, and the hybrids HBGC324 and HBGC325 are half-sibs to the former two.

Table 1 shows the hybrids, their genitors, and mode of reproduction previously determined by analysis of the embryo-sac types using interference contrast microscopy on methylsalicylate-cleared ovaries (Young et al., 1979). The hybrid nature of these plants was easily identified by their intermediate, morphology between the two parental types: B140 is a tall, erect plant with large, almost glabrous leaves and inflorescences with narrow rachis and one row of spiklets whereas R44 and R41 are typical *B. ruziziensis* plants, short with stoloniferous growth,

Table 1 - Description of the *Brachiaria* hybrids, genitors, and mode of reproduction.

Hybrid	Female genitor	Male genitor	Plant	Mode of reproduction*
HBGC313	R41	B140 (BRA003395)	13	Sexual
HBGC315	R41	B140 (BRA003395)	18	Sexual
HBGC324	R44	B140 (BRA003395)	4	Sexual
HBGC325	R44	B140 (BRA003395)	5	Apomictic

\*Determined by microscope analysis of the type of the embryo sacs within cleared ovaries

hairy decumbent leaves and inflorescences with wide rachis and two rows of spiklets. These hybrids are very vigorous with excellent forage phenotypes (medium growth with a high percentage of leaves) and thus were included in a small plot agronomical evaluation

### Cytological Analyses

Inflorescences in ideal stage for meiotic studies were collected from individual plants growing in the field, fixed in ethanol 95%, chloroform and propionic acid (6:3:2, v/v) during 24 h and stored at 4°C. Anthers containing microsporocytes were isolated from the flowers and slides containing cells spreads were obtained after squashing and stained with 0.5% propionic carmine. A total of 2457, 3147, 3051, and 2374 pollen mother cells (PMCs) were analyzed in the hybrid HBGC313, HBGC315, HBGC324, and HBGC325, respectively. Images were obtained using Kodak Imagemark - HQ, ISO 25 black and white film.

## Results and Discussion

Gene introgression is possible through meiotic recombination. Analyses of diakinesis organization in the four hybrids revealed multivalent chromosome configurations ranging from one to three quadrivalents in some meocytes (Figure 1 a, b). However, in the majority of meocytes, chromosomes paired as 18 bivalents of the same genome. Chromosome pairing in hybrids is used as a method of assessing the genomic relationship between species (Alonso and Kimber, 1981) and also providing an important starting point in alien introgression programs (Gale and Miller, 1987).

The degree of differentiation between hybridizing taxa can be estimated not only by analyses of chromosome pairing, but also by meiotic abnormalities (Rieseberg et al., 2000). Despite the phylogenetic proximity between *B. ruziziensis* and *B. brizantha* (Renvoize et al., 1996), the four hybrids presented different frequencies and types of meiotic abnormalities (Table 2). Although some abnormalities were common among hybrids, others were genotype-specific. Irregular chromosome segregation was recorded among the four hybrids at different frequencies. These were characterized by precocious chromosome migration to the poles in metaphase I (Figure 1 c, d) and metaphase II (Figure 1 g); laggard chromosomes in anaphase I (Figure 1 e) and anaphase II, leading to micronuclei formation in telophase I (Figure 1 f), telophase II (Figure 1 h) and in tet-

rads (Figure 1 i - k), or generating microcytes in tetrads (Figure 1 k, l). The HBGC324 was the hybrid that showed the lower rate of irregularities; only 18.2% of the meocytes analyzed (3051) showed meiotic abnormalities. Irregular chromosome segregation is the most common meiotic abnormality recorded in *Brachiaria* hybrids (Lutts et al., 1991; Risso-Pascotto et al., 2005a; Fuzinatto et al., 2007; Adamowski et al., 2008) and such abnormalities are consequences of the interspecies hybridization and polyploidy.

Other meiotic abnormalities here found, such as chromosome stickiness, abnormal cytokinesis, and absence of cytokinesis are also reported for other *Brachiaria* hybrids (Risso-Pascotto et al., 2005a, Fuzinatto et al., 2007) and among accessions of different species of *Brachiaria* (Mendes-Bonato et al., 2001a,b; 2002a,b; Risso-Pascotto et al., 2005a,b; Utsunomiya et al., 2005), suggesting that the genes controlling these characters are in the gene pool of the genus. One abnormality, however, was recorded for the first time during the present study in two of the hybrids, HBGC313 and HBGC325. Similar abnormality had been widely reported in maize (Clark, 1940; Staiger and Cande, 1990; Shamina et al., 2000) and it was designated *divergent spindle (dv)*, a putative mutation that affects the orientation of spindle fibers (Figure 2).

In normal microsporocytes, spindle converges to form focused poles, whilst bivalents occupy a small portion in the center of the metaphase plate and the telophase nuclei are spherical (Figure 1). In the microsporocytes here analyzed showing to be affected by the putative *dv* mutation, bivalents were regularly co-oriented at metaphase plate, although distantly positioned and spread over the equatorial plate (Figure 2 a). Since the spindle fiber did not converge into focused poles in anaphase I, the segregated chromosomes remained parallel and failed to converge at the poles (Figure 2 b). As a consequence, telophase I nuclei were fusiform (Figure 2 c), the genome was grouped into two (Figures 2 d, e) or more micronuclei of different sizes formed in each pole. After this stage, the first cytokinesis occurred and divided the meocyte into as many cells as the number of telophase nuclei and/or micronuclei formed. The second meiosis progressed normally and the number of chromosomes in each metaphase plate was variable depending on the size of telophase I micronuclei (Figures 2 f - i). Sister-chromatids normally segregated in anaphase II, giving rise to tetrads, hexads (Figure 2 j) or octads (Figures 2 l, k), depending on the number of nuclei and/or micronuclei formed in the first division. The



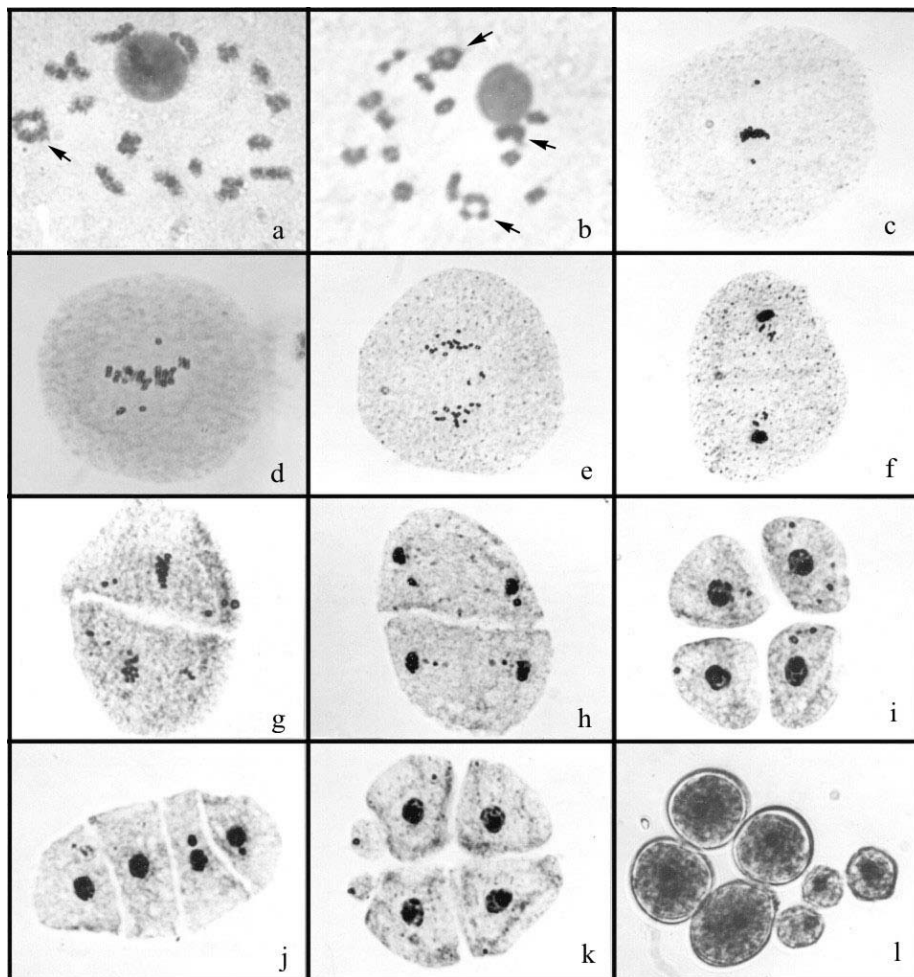


Figure 1 - Aspects of irregular chromosome segregation in the four interspecific hybrids. a, b) Diakinesis with 16 bivalents and one quadrivalent (a), and 12 bivalents and 3 quadrivalents (b), (arrows). (1000X). c, d) Metaphase I with precocious chromosome migration to the poles. e) Anaphase I with laggard chromosomes. f) Telophase I with micronuclei. g) Metaphase II with precocious chromosome migration to the poles. h) Telophase II with micronuclei. i) Isobilateral tetrad with micronuclei in all microspores. j) Linear tetrad with micronuclei. k) Tetrad with microcytes and micronuclei. l) Pollen grains with different sizes. (400X).

percentage of affected cells by *dv* varied between the two hybrids and HBGC325 showed to be more affected than HBGC313 (Table 2).

The abnormalities caused by this putative gene (*dv*) in addition to the irregular chromosome segregation due to polyploidy present in *Brachiaria* hybrids caused enough irregularities that constrain the use of those hybrids in the breeding program. In HBGC313, the rate of abnormal meiocytes was 55.6%, whilst in the HBGC325 was 46.1%. Hybrid HBGC315 also showed a high rate of abnormalities (48.3%) although characterized only by irregular chromosome segregation. The more stable hybrid analyzed was the HBGC324 with only 18.2% of abnormal cells. This hybrid reproduces sexually and therefore, it could be used in crosses with other apomictic genotypes to produce new hybrids.

Crosses between sexual and apomictic plants segregated 1:1 for the reproductive mode (Valle and Savidan, 1996; Savidan, 2000), suggesting a monogenic inheritance

of apomixis in *Brachiaria* and similarly determined for *Panicum maximum* (Savidan, 2000). Among the four selected promising hybrids, only HBGC325 is apomictic, and it can be used for seed production since megasporogenesis is dependent of male meiosis (Araújo et al., 2000). These plants, nonetheless, are pseudogamous (Alves et al., 2001), thus microsporogenesis will be affected by abnormalities, and pollen fertility may decrease.

At the moment, the *Brachiaria* breeding program aims at producing apomictic cultivars since selected traits will breed true, i.e., will not segregate, and it will contribute to establish homogeneous improved pastures. Nevertheless, superior sexual hybrids need to be included in the program, acting as female genitors in polycross blocks in order to pyramidize desirable alleles, introgress desirable genes and also to broaden the genetic base of the program. Among the four hybrids analyzed, the sexual HBGC313 and the apomictic HBGC325 should be discarded because the high frequency of the putative muta-

Table 2 - Frequencies of meiotic abnormalities in the four *Brachiaria* hybrids.

Stage*	Abnormalities	HBGC313 R41 x B140/13		HBGC315 R41 x B140/18		HBGC324 R44 x B140/4		HBGC325 R44 x B140/5	
		Sexual		Sexual		Sexual		Apomictic	
		No. of PMCs** Analyzed	No. of abnormal PMCs	No. of PMCs analyzed	No. of abnormal PMCs	No. of PMCs analyzed	No. of abnormal PMCs	No. of PMCs analyzed	No. of abnormal PMCs
MI	Precocious migration	486	140	652	134	753	54	776	83
AI	Laggard chromosomes	169	110	257	152	278	62	209	38
	Chromosome stickiness		8		-		-		-
	Divergent spindle		16		-		-		82
TI	Micronuclei	460	254	396	222	515	47	394	55
	Chromosome stickiness		14		-		12		-
	Abnormal cytokinesis		-		-		19		-
	Divergent spindle		5		-		-		70
PII	Micronuclei	449	267	235	138	305	57	482	35
	Chromosome stickiness		13		-		-		-
	Absence of cytokinesis		8		-		-		14
	Abnormal cytokinesis		-		-		19		-
	Divergent spindle		19		-		-		341
M II	Precocious migration	177	58	187	47	311	50	216	10
	Abnormal cytokinesis		-		-		9		-
	Divergent spindle		14		-		-		192
AII	Laggard chromosomes	28	11	69	48	93	34	26	2
	Divergent spindle		4		-		-		22
TII	Micronuclei	151	88	203	109	94	21	34	3
	Chromosome stickiness		2		-		-		26
	Divergent spindle		14		-		-		-
T	Micronuclei	537	281	1148	670	702	142	237	86
	Microcytes		16		1		12		13
	Polyads		24		-		18		23
Total (%)		2457	1366 (55.6)	3147	1521 (48.3)	3051	556 (18.2)	2374	1095 (46.1)

\* MI: metaphase I; AI: anaphase I; TI: telophase I; PII: prophase II; MII: metaphase II; AII: anaphase II; TII: telophase II; T: tetrad. \*\* PMCs: pollen mother cells.

tion *dv* which seriously compromises pollen fertility. From the two remaining sexual hybrids, only the HBGC324 should be kept in the program since it has high meiotic stability among cells.

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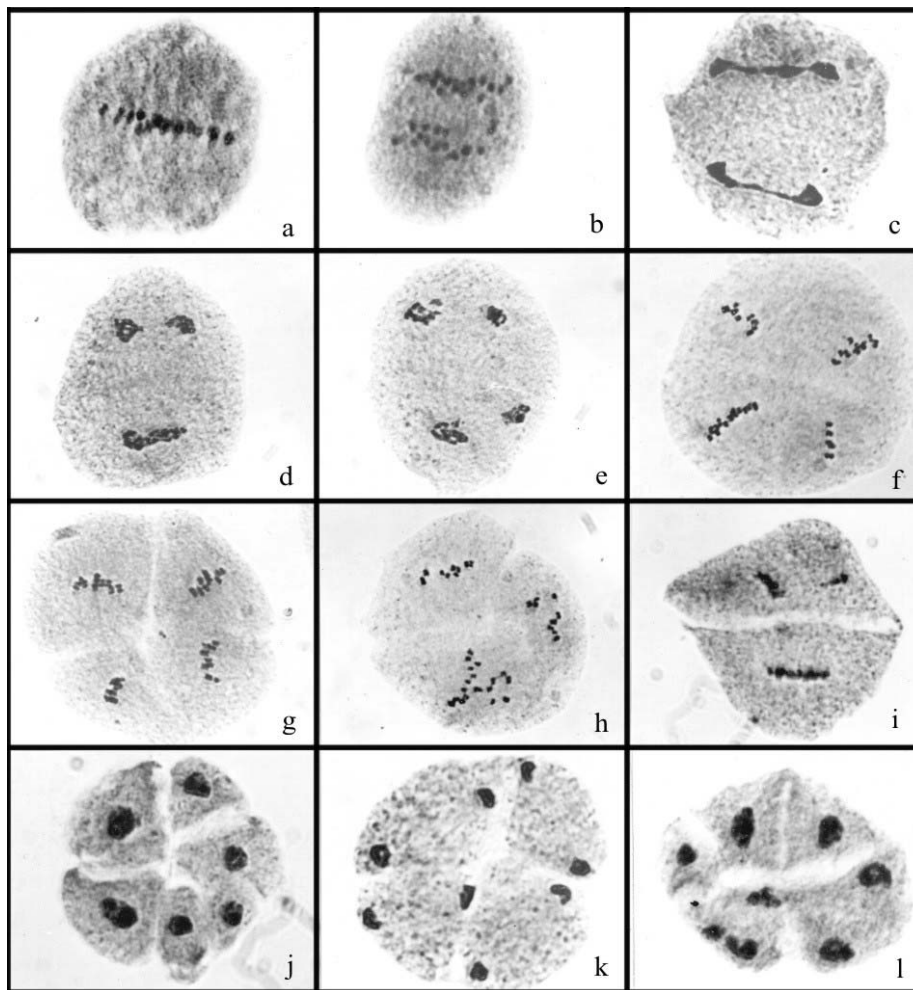


Figure 2 - Some aspects of the phenotypic expression of divergent spindle in HBGC313 and HBGC325. a) Metaphase I with a broad metaphase plate. b) Anaphase I with non-convergent chromosomes to the poles. c) Telophase I with two elongated nuclei. d, e) Telophase I with bipartite nuclei. f, g) Metaphase II with four metaphase plates with different numbers of chromosomes in each one resulting from bipartition of both telophase I nuclei. h, i) Metaphase II with three metaphase plates with different numbers of chromosomes resulting from bipartition of one telophase I nucleus. j, k) Telophase II with eight nuclei resulting from bipartition of both telophase I nuclei. l) Telophase II with six nuclei resulting from bipartition of one telophase I nucleus. (400X).

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