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Meiotic Behavior in Intra- and Interspecific Sexual and Somatic Polyploid Hybrids of Some Tropical Species

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1. Introduction

Hybridization, as stated by the plant evolutionist G. Ledyard Stebbins, can be viewed as a reunion between differentiated genetic materials. Plant intra and interspecific hybridization is a common means of extending the range of variation beyond that displayed by the parental species. Hybridization is a strong evolutionary force which can potentially reshape the genetic composition of populations and create novel genotypes that facilitate adaptation to new environments (Stebbins, 1950).

Interspecific hybridization provides information on phylogenetic relationships between any two species giving clues with regard to evolutionary patterns. Often generation of such information is based on cross compatibility, chromosome association, and pollen fertility. Such information also helps in developing breeding strategies for introgression of genes from related species into economically useful species. As it creates genetic variation, it has great potential for plant improvement (Goodman et al., 1987; Choudhary et al., 2000; Sain et al., 2002).

For certain crops, plant breeders in the 20th century have increasingly used interspecific hybridization for gene transfer from a non-cultivated plant species to a crop variety in a related species. Goodman et al. (1987) presented a list of species in which gene transfer have been successful. Wild relatives may be sources of useful traits for the improvement of crops. From a plant breeding point of view it is desirable to document the possibility of transferring traits to a crop plant from its wild relatives through conventional sexual hybridization. Sexual exchanges between species as sources of genetic variability to improve crops have been made possible during the last century by the discovery of efficient ways to circumvent the natural barriers to genetic exchange (Goodman et al., 1987). However, inherent problems of specific introgression such as hybrid instability, infertility, non-Mendelian segregations, and low levels of intergenomic crossing-over can constitute important limitations (Stebbins, 1950). Moreover, features associated with polyploidy or ploidy dissimilarity between species may result in additional constraints for interspecific gene flow (Rieseberg et al., 2000).

After a hybrid plant has been successfully recovered, differences in the number or compatibility of parental chromosomes may cause sterility. Cytogenetic manipulations have been instrumental in obtaining stable gene transfers. Sterility may result from incomplete or unstable pairing of chromosomes during cell division. For a desired gene from the donor to be incorporated into a chromosome of the crop variety, recombination must take place. If the two species are closely related, natural pairing and recombination may occur (Goodman et al., 1987). High pairing affinity contributes so that once the barriers separating the species are overcome the gene pools of the two genera are interchangeable (Zwierzykowski et al., 1999).

Until recently, the results of interspecific hybridization could only be studied in a fairly indirect manner. One method was to analyze the phenotype of hybrids, such as the symmetry of morphological characters or the viability of pollen or seed. Alternatively, meiosis in hybrids could be studied by light microscopy and the degree of differentiation between hybridizing taxa estimated by analyses of chromosome pairing behavior and meiotic abnormalities (Rieseberg et al., 2000). Although both of these approaches have been extremely valuable, they can only provide glimpses into the complex interactions of alien genes and genomes following genetic recombination.

Cytological analyses are usually performed to evaluate the meiotic process in experimental hybrids. Species with close genetic affinity produce hybrids with regular chromosome pairing, while the hybrids of those more distantly related species have meiotic irregularities and are sterile (Marfil et al., 2006). In diploid interspecific hybrids, the meiotic analysis of chromosome association in the F_1 generation shows the genetic homology between the respective pairs of chromosomes. However, in interspecific tetraploid or hexaploid hybrids, chromosome pairing is affected by the number and similarity among genomes.

In this chapter, we will describe some examples of tropical species for which microsporogenesis was analyzed under light microscopy. Particularly, we will discuss the meiotic behavior in interspecific sexual tetraploid hybrids of *Brachiaria ruziziensis* x *B. brizantha* and in intraspecific sexual hexaploid hybrids of *B. humidicola*. The meiotic behavior of some artificial polyploids, namely somatic hybrids obtained through protoplast fusion in *Citrus* and *Passiflora* species will be discussed.

2. Some considerations about the *Brachiaria* genus

Brachiaria (Syn. *Urochloa* P. Beauv.), a genus of African origin, consists of about 100 species distributed across tropical and subtropical regions of the world, most of which are apomictic. It is the single most important grass genus for tropical pastures, widely adopted due to widespread adaptation to poor and acid soils commonly found in the tropics (Miles et al., 1996). Some *Brachiaria* species were introduced into South America during the second half of the 20th century and are currently used over millions of hectares as pastures for both beef and dairy cattle (Boddey et al. 2004). In Brazil, there are ten registered cultivars listed on the National Service for Cultivar Protection. Among them, only one (cv. Mulato II) is a hybrid. The others are derived from selection of the natural variability found in the genus. *Brachiaria brizantha* and *B. decumbens* are considered pivotal species in the genus since the first contains accessions resistant to the major insect-pest, spittlebugs, and the second, although lacking resistance to the insect, is well adapted to infertile, acid soils generally found in tropical savannas (Keller-Grein et al., 1996). The gene pools in the genus *Brachiaria*

are not yet well defined. A throrough taxonomic study of a large germplasm collection classified *Brachiaria brizantha, B. decumbens* and *B. ruziziensis* in the same taxonomic group together with four other species (Renvoize et al., 1996). These species are cross-compatible. *Brachiaria* breeding is difficult to accomplish because of differences in chromosome numbers among accessions and lack of sexuality in the most important species. The majority of accessions are polyploid, mainly tetraploid (Mendes-Bonato et al., 2002; 2006a; Utsunomiya et al., 2005; Risso-Pascotto et al., 2005; 2006; Pagliarini et al., 2008) and polyploidy in the genus is correlated with apomixis (Valle & Savidan, 1996).

The *Brachiaria* germplasm collection existing at Embrapa Beef Cattle Research Center (Campo Grande, Mato Grosso do Sul, Brazil) was collected in eastern and southeastern wild tropical African savannas in the mid-1980s by the International Center for Tropical Agriculture (CIAT, Colombia) and, after, introduced to Brazil by the Embrapa Genetic Resources & Biotechnology (Cenargen/Embrapa, Brasilia, DF, Brazil). After quarantine, the *Brachiaria* collection was transferred to Embrapa Beef Cattle to serve as the basis for the breeding program.

3. Hybridization in the genus *Brachiaria*

In the existing *Brachiaria* germplasm collected in Africa, considerable genetic variation is available and much more is presently being released using sexuality to access heterozygosity otherwise fixed by apomixis (Valle & Pagliarini, 2009). Until three decades ago, the genetic improvement of *Brachiaria* species depended entirely on selection among naturally existing germplasm. Apomixis among polyploid accessions made genetic recombination impossible to exploit (Valle & Savidan, 1996). Fully sexual genotypes have since then been found, either within the species themselves or among close relatives, but generally among diploids.

Artificial hybridization in *Brachiaria* has been tried at least since the early 1970s, when Ferguson and Crowder (1974) attempted to produce hybrids by pollinating diploid (2n = 18) sexual *B. ruziziensis* with pollen from a tetraploid (2n = 36) apomictic *B. decumbens*. This early attempt was unsuccessful, and the authors suggested duplication of the chromosome number to overcome the ploidy barrier. This indeed was the right direction in *Brachiaria* breeding when an obligate sexual tetraploid was produced by the colchicine treatment of a sexual diploid *B. ruziziensis* (Swenne et al., 1981). The Belgian material became the basis of breeding in *Brachiaria.* In Brazil, the first attempt at the interspecific hybridization using the Belgian material was undertaken in 1988 at Embrapa Beef Cattle, using tetraploid apomictic accessions of *B. brizantha* and *B. decumbens* as pollen donors (Valle & Savidan, 1996).

3.1 Interspecific hybrids

3.1.1 *Brachiaria ruziziensis* (sexual, artificial tetraploid) *x B. decumbens* (apomictic, natural tetraploid)

Ndikunama (1985) reported the first successful interspecific hybridization done in Belgium, using the artificial tetraploid sexual genotype of *B. ruziziensis* as the female parent and apomictic accessions of *B. decumbens* and *B. brizantha* as pollen donors. New attempts were done by Lutts et al. (1991, 1994). They concluded that the hybrids produced significantly

more seed when *B. ruziziensis* was pollinated by *B. decumbens* than when crossed to *B. brizantha* and the seedlings were also less vigorous. At Embrapa Beef Cattle (Brazil), several hybrids were also obtained from *B. ruziziensis* x *B. decumbens* crosses, but lack of spittlebug resistance among other undesirable phenotypic traits made them unfit as candidates for a new cultivar. Thus, efforts were not spent in the analysis of their microsporogenesis.

3.1.2 *Brachiaria ruziziensis* (sexual, artificial tetraploid) *x B. brizantha* (apomictic, natural tetraploid)

Several hybrids between *B. ruziziensis* x *B. brizantha* were selected to continue in the breeding program based on their phenotypical performance, including seed production to attend the Brazilian as well as the export market. Meiotic behavior was analyzed in 11 hybrids obtained from these crosses. The mean frequency of abnormalities in the hybrids was variable (Table 1). Only in one hybrid, the mean of abnormalities was low (18.2%) (Felismino et al., 2010). In the other hybrids, the mean of meiotic abnormalities ranged from 44.9% to 69.1% (Risso-Pascotto et al., 2005; Fuzinatto et al. 2007, Adamowski et al. 2008, Felismino et al. 2010; Felismino, 2011). The main meiotic abnormalities were those related to irregular chromosome segregation due polyploidy. Precocious migration of univalents to the poles at metaphase I (Fig. 1 c), lagging chromosomes at anaphase I, and micronuclei at telophase I (Fig. 1 d) were recorded in the first meiotic division of most hybrids. In the second division, abnormalities were the same; micronuclei at prophase II (Fig. 1 e), precocious migration of chromatids to the poles at metaphase II (Fig. 1 f), lagging chromatids at anaphase II (Fig. 1 g), that generated micronuclei at telophase II (Fig. 1 h), that, in some cases, remained in the tetrad (Fig. 1i) were recorded.

Hybrids	Mode of reproduction	Mean of meiotic abnormalities	References
Hb S05	Sexual	67.4%	Risso-Pascotto et al., 2005
Hb A14	Apomictic	65.1%	Risso-Pascotto et al., 2005
HBGC076	Sexual	53.3%	Fuzinatto et al., 2007
HBGC009	Sexual	50.9%	Fuzinatto et al., 2007
HBGC014	Sexual	46.5%	Fuzinatto et al., 2007
H34	Sexual	69.1%	Adamowski et al., 2008
H27	Sexual	56.1%	Adamowski et al., 2008
H17	Apomictic	44.9%	Adamowski et al., 2008
HBGC313	Sexual	55.6%	Felismino et al., 2010
HBGC315	Sexual	48.3%	Felismino et al., 2010
HBGC324	Sexual	18.2%	Felismino et al., 2010
HBGC325	Apomictic	46.1%	Felismino et al., 2010
Hb 331	Apomictic	64.6%	Felismino, 2011
Hb 336	Sexual	49.8%	Felismino, 2011

Table 1. Percentage of meiotic abnormalities recorded in interspecific *Brachiaria* hybrids

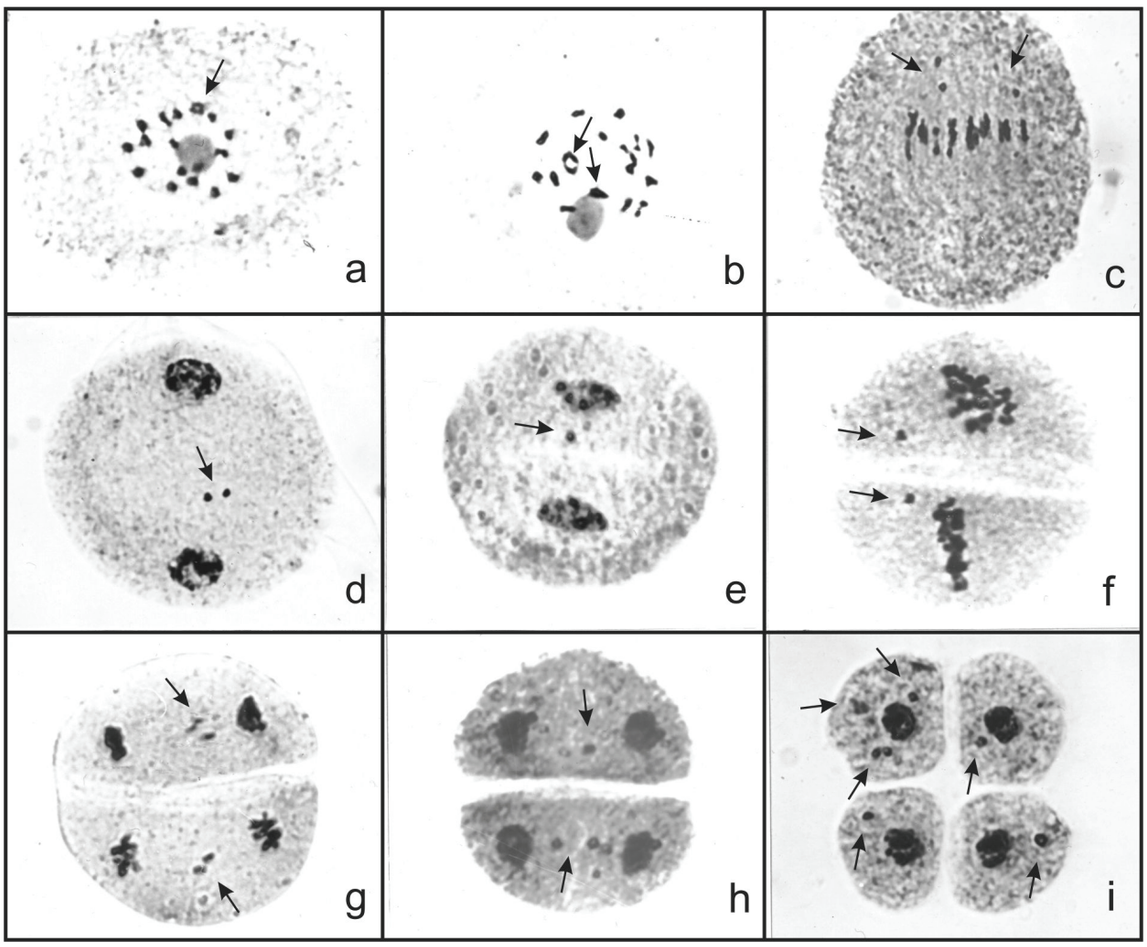

Fig. 1. Chromosome pairing and irregular chromosome segregation in interspecific *Brachiaria* hybrids. a, b) Meiocytes in diakinesis showing one (a) and two (b) quadrivalents (arrows). c) Metaphase I with precocious chromosome migration to one pole (arrows). d) Telophase I with two micronuclei (arrow). e) Early prophase II with a micronucleus (arrow). f) Metaphase II with precocious chromosome migration to one pole in both cells (arrows). g) Late anaphase II with laggard chromosomes in both cells (arrows). h) Telophase II with micronuclei in both cells (arrows). Tetrad of microspores with several micronuclei in the four microspores (i).

The meiotic behavior of micronuclei was variable among meiocytes and hybrids. In some cells, the micronuclei originated in the first meiotic division were separated by an abnormal cytokinesis, generating microcytes of different sizes and chromosome contents (Fig. 2 a – d) that entered the second division. At the end, the meiotic products depended on the number and the result of meiotic behavior of micronuclei in the first and in the second meiotic division. The end products were a tetrad with micronuclei in one, two, three or the four microspores (Fig. 2 e to g) up to a tetrad with several micronuclei in the microspores.

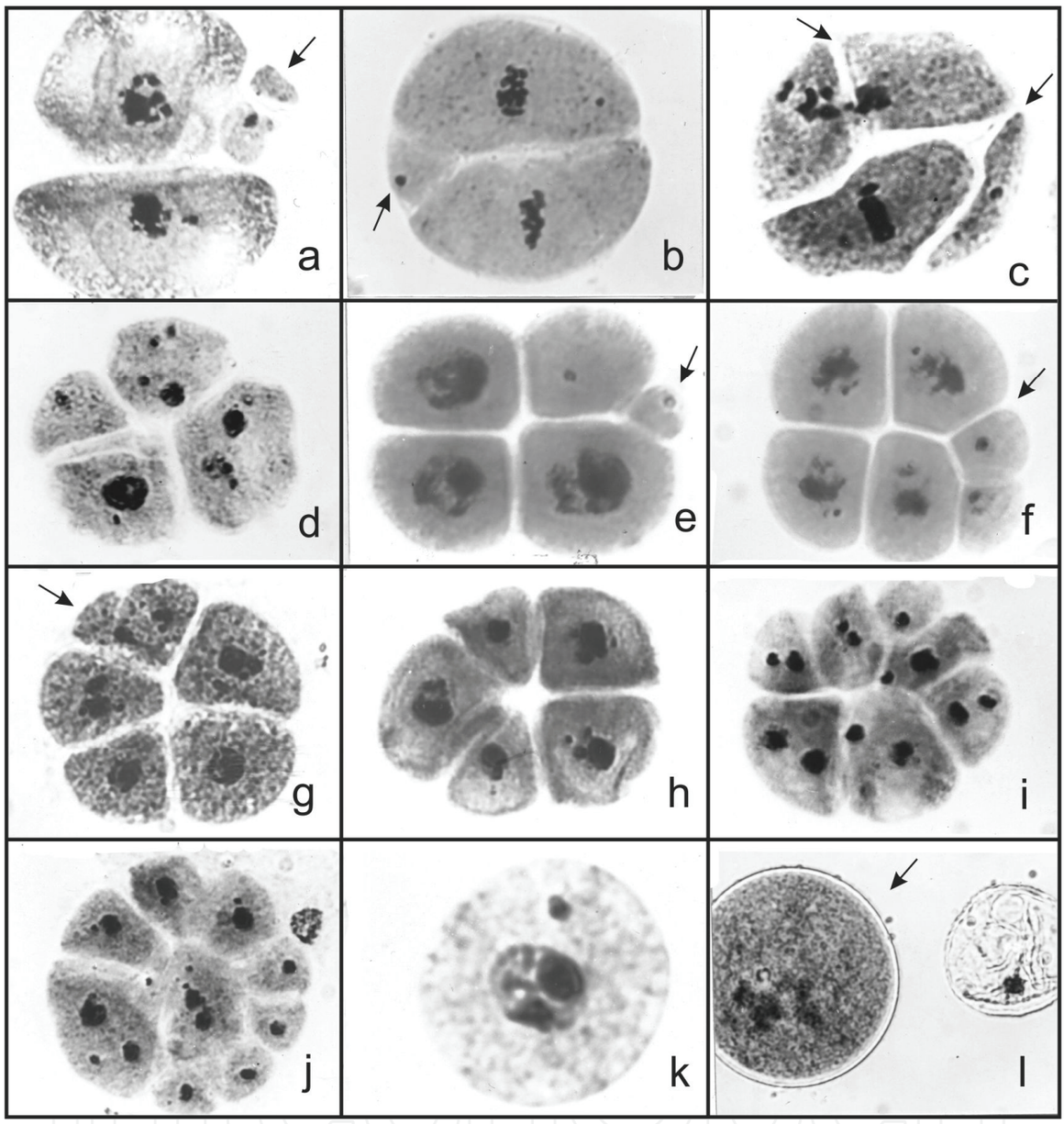

Fig. 2. Abnormal cytokinesis and the meiotic product formation in interspecific *Brachiaria* hybrids. a – c) Abnormal cytokinesis at the end of the first division leading to microcyte formation in prophase II (a) and metaphases II (b, c) (arrows) .d - g) Tetrads with micronuclei (d) and microcyte (e - g) (arrows). h) Pentad. i) Polyads with micronuclei and microcytes. k) Microspore with micronuclei (arrow). l) Viable (arrow) and sterile pollen grains.

Micronuclei were separated as microcytes with different amount of chromatin all the way to polyads with microspores and microcytes of different sizes (Fig. 2 h to j). The result was microspores of different sizes and with (Fig. 2 k) or without micronuclei. As these meiotic products are unbalanced, pollen sterility was high (Fig. 2 l).

3.1.3 *Brachiaria brizantha* (sexual, diploid) x *B. decumbens* (sexual, diploid) – tetraploidized hybrids

The first successful attempt in obtaining interspecific hybrids between *B. brizantha and B. decumbens* was recently accomplished and microsporogenesis was analyzed by Souza-Kaneshima et al. (2010). These constitute the two most widely used tropical forage species for cultivated pastures and support both beef and dairy cattle production in the tropics. Two apomictics cultivars – *B. brizantha* cv. Marandu and *B. decumbens* cv. Basilisk – cover more than 100 million hectares of cultivated pastures throughout Latin America and Southeast Asia. Artificial hybridization between two diploid (2n = 2x = 18) sexual accessions (*B. brizantha*, B105 x *B. decumbens*, D004) was performed in the greenhouse. Only three hybrids were recovered. One of them was treated with colchicine in tissue culture and two amphidiploid plants were obtained (Simioni & Valle, 2009).

The meiotic behavior was quite normal in the female (B105) and male (D004) genitors. In the diploid hybrid, genome separation was detected in a high number of cells since pachytene but chromosome association at diakinesis could not unfortunately be evaluated. At pachytene, both parental genomes were distantly positioned in the cytoplasm. The inability to share the same position inside the cell was also detected in metaphase I, when two metaphase plates were organized. In the following phases of meiosis, genome separation was not found among meiocytes, but polyads were recorded as meiotic products. These abnormalities increased the frequency of abnormal cells to 17.8% in the diploid F1 generation.

The same abnormalities recorded in the diploid hybrid were detected also in the amphidiploid hybrids CH4-8 and CH4-100, but in higher frequencies. In amphidiploids there was a predominance of bivalents at diakinesis, but one to three quadrivalents were recorded among meiocytes. Genome separation was also detected. Metaphase I with two metaphase plates and doubled anaphases I, giving rise to tetranucleated telophase I were recorded. In the second division, four cells, instead the two normal ones were observed. Polyads were also recorded among the meiotic products.

In the amphidiploid plants, however, another abnormality related to spindle organization was detected in high frequency. In the affected cells, meiosis was normal until diakinesis. From this phase on, chromosomes were chaotically dispersed in the cytoplasm because of absence of a spindle organizing center in the poles. In these cells, anaphase I did not occur and chromosomes, alone or in groups, generated several telophase nuclei of different sizes. In these cells, the first cytokinesis was abnormal, dividing the meiocyte in more than two cells. The plane of cytokinesis was apparently determined by the position of the telophase nuclei. In the second division, meiocytes with multiple spindles were abundantly recorded, generating multinucleated cells in telophase II. In both amphidiploid hybrids, meiotic products were highly abnormal, with several micronuclei and microcytes in the tetrads generating pollen grains of different sizes. The percentage of abnormal cells in the tetraploid progenies was similar, 49.2% in CH4-8 and 50.8% CH4-100.

4. Intraspecific *Brachiaria* hybrids

4.1 *Brachiaria humidicola* (sexual, natural hexaploid) x *B. humidicola* (apomictic, natural hexaploid)

Genetic variation and the means to manipulate it are the primary requirements in a breeding program. Interspecific *Brachiaria* hybrids between *B. ruziziensis* x *B. brizantha* have

shown a considerable range of meiotic abnormalities which could impair pollen viability and seed production. In interspecific tetraploid hybrids within the genus, the problem can be compounded by the issues of genome affinity (Risso-Pascotto et al., 2005; Mendes-Bonato et al., 2006 b; Fuzinatto et al., 2007; Adamowski et al., 2008; Felismino et al., 2010; Souza-Kaneshima et al., 2010). Theoretically, intraspecific hybridization should produce fewer meiotic abnormalities than interspecific hybridization, thus efforts were directed at identifying sexually compatible genitors to be crossed to the natural apomicts within the same species. The discovery of a sexually reproducing, 36-chromosome accession of *B. humidicola* (H031, BRA005100) in Burundi (Africa) by Valle & Glienke (1991) opened new opportunities for the exploitation of genetic variability within this species. *Brachiaria humidicola* is adapted to poorly drained soils (Argel & Keller-Grein, 1996) and shows desirable agronomic characteristics. This accession was crossed with an apomictic cultivar (BRS Tupi) with the same ploidy level, originating from the same African region. BRS Tupi has excellent productivity, good nutritive value, and performance under grazing, and it is being released as a new *Brachiaria* cultivar for the tropics. From among 361 progeny obtained, 50 were selected based on vigor and overall suitability for further breeding work. In the female parent (H031), meiosis was somewhat irregular, with 16.3% of abnormal tetrads, whereas the male (cv. BRS Tupi) meiosis was very regular, with only 3.1% of abnormal tetrads. Among hybrids (sexual and apomictic), the percentage of abnormal tetrads ranged from 15.8% to 98.3%. Among the hybrids, high frequencies of meiotic abnormalities were unexpected both because they were intraspecific hybrids and because both parents' meioses were relatively stable.

The frequency of abnormalities at metaphase I in the hybrids was, in general, lower than that at metaphase II. The meiotic phases more affected by irregular chromosome segregation were anaphase II and telophase II. Metaphase II was affected by chromosomes outside the plate as well as anaphase II showing a great frequency of several lagging chromosomes. This suggests that the parental genomes did not display the same meiotic rhythm in the second division, generating several micronuclei in telophase II which remained in the tetrads. Problems related to differences in the meiotic rhythm have also been frequently recorded among interspecific tetraploid *Brachiaria* hybrids (Mendes-Bonato et al., 2006 b; Adamowski et al., 2008; Souza-Kaneshima et al., 2010). In general, the absence of genome affinity can result in chromosome elimination of one of the parents by asynchrony during meiotic phases.

5. Chromosome association and gene introgression

The knowledge about the similarity of the genomes in different species provides a means by which evolutionary relationships can be assessed. In addition, it offers an important starting point in alien introgression programs. According to King et al. (1999), the similarity of the genomes of the crop to those of related species is important as this permits an estimation of the frequency of recombination that will occur in interspecific hybrids. Thus, this information allows predictions to be made of the likelihood of transferring specific target genes from one species to another.

Multivalent chromosome association at diakinesis revealed genome affinity between both parental species in the hybrids, suggesting some possibility for gene introgression. Analyses of meiocytes at diakinesis in the 11 interspecific tetraploid *Brachiaria* hybrids showed that some chromosome association can occur among the parental genomes, but in low frequency.

No more than four quadrivalents were found in these hybrids (Fig. 1 a, b), but one tetravalent was the most common multiple chromosome association (Risso-Pascotto et al., 2005; Fuzinatto et al., 2007; Adamowski et al., 2008; Felismino et al., 2010; Felismino, 2011). The occurrence of quadrivalents among *B. ruziziensis* x *B. brizantha* shows that both species are closely related and their gene pools are interchangeable. Thus, we can assume that gene introgression can occur in the hybrids, but in low frequency. Recombination was expected in these hybrids because these species belong to the same taxonomic/genomic group – group 5 (Renvoize et al., 1996; Valle & Savidan, 1996). However, the occurrence of chromosome pairing per se does not ensure that the desirable genes are being introgressed into the hybrid because of the probability of the chromosomes involved containing such genes. According to Goodman et al. (1987), even when successful, interspecific hybrids generally present other problems: (1) even after several cycles of selection, the recombination will frequently not separate tightly linked genes, and undesirable traits may be carried along with the desirable one; (2) the inheritance and expression of the desirable introgressed gene may be unpredictable in the new genetic background.

6. Genome affinity

Genome separation and genome elimination are common among interspecific hybrids. Shwarzacher et al. (1992) reported several examples among plants where the phenomenon occurred. According to Luckens et al. (2006), the formation of natural allopolyploids requires the adaptation of two nuclear genomes within a single cytoplasm, which may involve programmed genetic changes during the first generation following genome fusion.

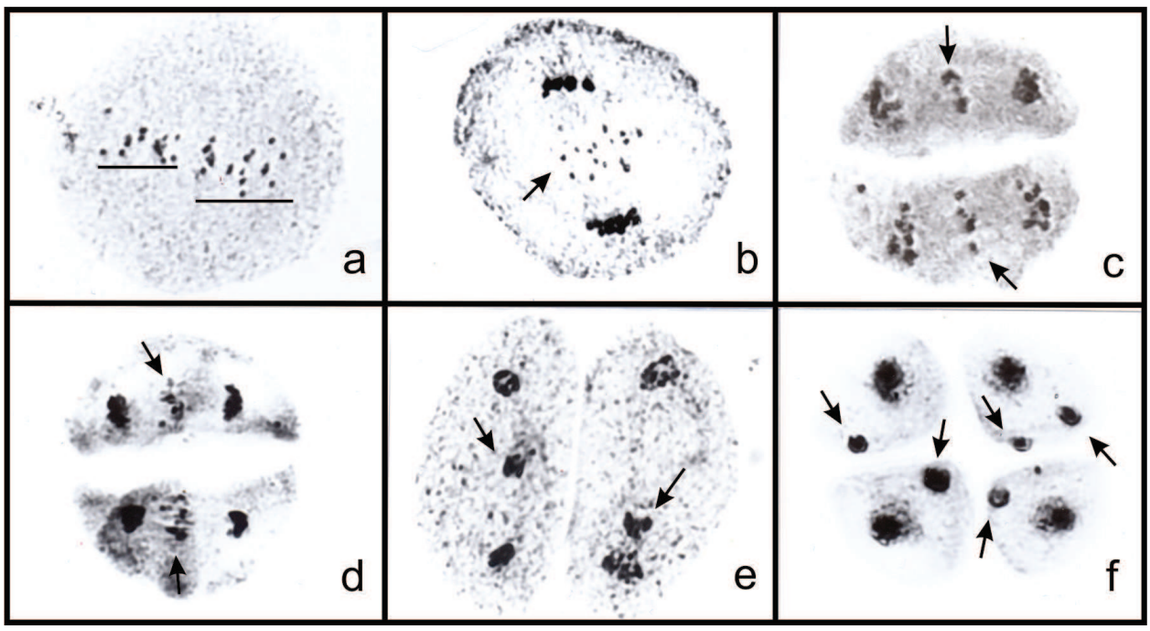

Fig. 3. Aspects of genome affinity and chromosome elimination observed in some interspecific hybrids. a) The parental genomes separated into two metaphase plates in the meiocyte. b) Meiocyte in anaphase I with a laggard genome (arrow). c, d) Meiocytes in anaphase II with a laggard genome (arrow). Meiocyte in telophase II with large micronuclei resulting from the laggard genome (arrow). f) Tetrad of microspores with large micronuclei (arrows).

Genome similarities in the genus *Brachiaria* were not studied from the cytogenetic point of view. Karyological studies in the genus were performed for a few species and accessions (Bernini & Marin-Morales, 2001), and genomes were not yet described. In the last decade, however, evaluation of the meiotic behavior in some hybrids between *B. ruziziensis* x *B. brizantha* (Risso-Pascotto et al., 2004; Mendes-Bonato et al., 2006b; Adamowski et al., 2008; Fuzinatto et al., 2007; Felismino et al., 2010; Felismino, 2011) has revealed that genomes of parental species not always show perfect affinity. Lacking of genome affinity during meiosis was revealed by asynchrony in the meiosis time (Fig. 3 b to f) (Risso-Pascotto et al., 2004; Adamowski et al., 2008) or separation of the two parental genomes into two metaphase plates (Fig. 3 a) (Mendes-Bonato et al., 2006b). The lack of genome affinity between *B. ruziziensis* and *B. brizantha* seems to be genotype-specific.

7. Artificial polyploids obtained through protoplast fusion, a case study involving *Passiflora* species

The genus *Passiflora* L. (Passifloraceae), which includes the commercial species of passionflowers, is a large and widespread genus consisting primarily of tropical species. Currently the taxonomy of the genus designates four subgenera *Astrophea* (with 57 species), *Deidamioides* (13) *Decaloba (*214) and *Passiflora* (236), in which are included the typical passion fruits (Ulmer & MacDougal, 2004). Most are vines, though some representatives are shrubs or trees. South America is the center of diversity for most of the *Passiflora* species; around 40 are indigenous to Asia and South Pacific Islands. Passion vines are evergreen climbers, grown for their edible fruits, and several species are cultivated for their unusual and beautiful flowers (Vanderplank, 1996).

Several species are important for their nutritional and pharmacological properties, but some of them are used as ornamental plants. Among all, about 50 species bear edible fruits but only the two forms of *Passiflora edulis,* i.e., the purple and the yellow ones are considered to be of value in international commerce. Although small-scale, the juice is manufactured to be exported to EU countries. The wide genetic variability, known to exist within and between *Passiflora* species can be exploited in breeding programs in order to obtain improved populations or to search for genes of interest. Commercial passion fruit varieties available are susceptible to a number of pests and diseases with considerable negative effects on production (for a review see Vieira & Carneiro, 2004; Zerbini et al., 2008). With the purpose of producing rootstocks resistant to soil-borne diseases, interspecific protoplast fusion was performed, and the hybrids between the yellow passion fruit, *P. edulis* f. *flavicarpa* and two wild species *P. amethystina* and *P. cincinnata*, all with 2n=2x=18 were obtained (Dornelas et al. 1995; Vieira & Dornelas, 1996). Briefly, all the hybrid cells were produced by PEG-mediated protoplast fusion and shoot regeneration occurred via indirect organogenesis. Selection was performed based on total protein and isoenzyme electrophoretic patterns at the callus stage (Vieira & Dornelas, 1996). After being acclimatized, the plantlets were transferred to the field where the vines grew to maturity and flourished, but did not set normal fruits, even when artificially crossed. In this section, we present the meiotic study of these somatic hybrids carried out in our laboratory (Barbosa & Vieira, 1997; Barbosa et al. 2007).

Firstly, we will discuss the meiotic behavior of the two parental diploid species, *P. edulis* f. *flavicarpa* (E) and *P. amethystina* (Am) and their four somatic hybrids denoted (E +Am) # 12, # 13, # 28 and # 35, each derived from different calli. Flower buds were collected from the vines, fixed, and slides were prepared using standard protocols. For segregation analysis,

cells were studied in the diakinesis, and pollen viability (V) was determined according to Alexander (1980), which employs malachite green and fuchsinic acid.

As expected, the meiosis of the parental species showed nine bivalents (9II). In *P. edulis* f. *flavicarpa*, one or two bivalents were associated with the nucleolus in 33% or 67% of the cells, respectively, indicating the presence of two nucleolar organizing regions (NOR's). In *P. amethystina*, two bivalents were associated with the nucleolus in all cells analyzed. Chromosome pairing in the parental species was regular, although some laggard chromosomes and anaphase bridges were observed (less than 1%). The analysis of somatic hybrids showed 4x = 36 chromosomes, with four NOR's; no aneuploids were present. Meiosis was irregular with high frequencies of bridges and laggards in both divisions (Table 2). Interestingly, clear differences in the meiotic behavior among the hybrids were observed. The hybrids (E + Am) # 12 and # 28 had more regular meiosis than (E + Am) # 13 and # 35. Meiotic figures in hybrid cells display bivalents and a few univalent or multivalent. The configurations observed at diakinesis are presented on Table 2. At least 14 II were observed in 96.7% of the cells of the hybrids (E + Am) # 12 and # 35 meanwhile, the same minimum of bivalents was detected in 100% of the cells of the hybrids (E +Am) # 13 and # 28. As expected, the two sets of homologous chromosomes pair preferentially in the hybrids, reflecting the level of homology between the parental chromosomes. Moreover, the frequencies of cells with quadrivalents were 73.3% in (E + Am) # 13, 83.3% in # 12 and, 93.3% in (E + Am) # 28 and # 35.

Hybrid # 28 shows the lowest number of bridges in anaphase cells (I and II) and laggard chromosomes. The hybrid (E + Am) # 35 differs from the other three, by the largest number of cells with univalents (33.3%) which explains the high level of laggard chromosomes and chromatids in the subsequent division stages (Table 2). The present data support that recombination of the chromosomes of the two parental species occurred in the hybrids, mainly in the hybrid # 28 and # 35, since most of their cells showed the occurrence of quadrivalents. In view of the presence of quadrivalents in all hybrid genotypes analyzed, *P. edulis* f. *flavicarpa* and *P. amethystina* may possess some chromosome homoeology. Later on, we observed that *P. edulis* f .*flavicarpa* and *P. amethystina* show very similar karyotypes (Cuco et al., 2005).

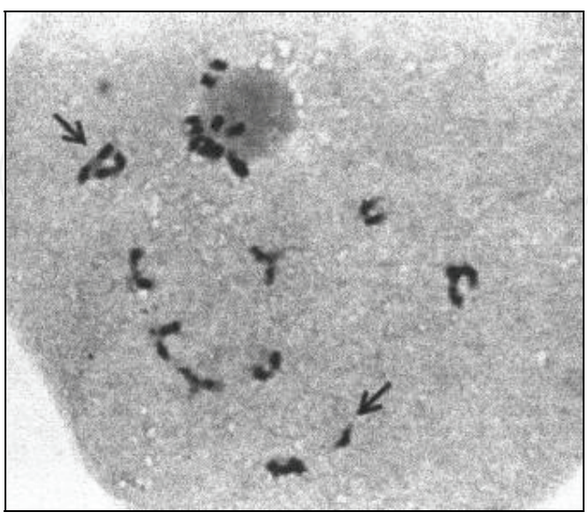

Fig. 4. Meiotic behaviour at diakinesis of the somatic hybrid (E + Am). Note the presence of bivalents, a quadrivalent (arrow) and a univalent (arrow).

Plant material	Diakinesis configurations[1]								Laggards[2]		Bridges	Laggards[2]		Chromatid Bridges
	12II +4I +2IV	13II +2I +2IV	14II +4I +1IV	14II +2IV	15II +2I +1IV	16II +1IV	17II +2I	18II	MI	AI		MII	AII	
(E)		–	–	–					0.99	0.00	0.65	0.38	0.0	0.25
(Am)		–	–	–					0.44	0.00	0.79	0.68	0.0	0.23
(E+Am) #12	3.3	0.0	3.3	26.7	10.0	40.0	10.0	6.7	1.15	0.00	2.12	2.47	0.00	7.11
(E+Am) #13	0.0	0.0	0.0	16.7	13.3	43.3	6.7	20.0	5.90	0.00	3.83	4.10	1.92	4.39
(E+Am) #28	0.0	0.0	0.0	30.0	20.0	43.3	0.0	6.7	1.15	0.00	2.09	1.15	0.00	1.37
(E+Am) #35	0.0	3.3	0.0	40.0	23.3	26.7	6.7	0.0	8.11	7.57	12.37	13.16	3.95	16.05

[1]Number of cells analyzed = 30; [2]MI and MII, first and second metaphase, respectively, AI and AII, first and second anaphase, respectively.

Table 2. Meiotic behavior (in percentages) in *P. edulis* f. *flavicarpa* (E), *P. amethystina* (Am) and their four somatic hybrids (E+Am). Modified from Barbosa & Vieira (1997).

On average, all the materials analyzed presented high levels of pollen viability, ranging from 72.9% up to 88.2%. The hybrid showing the lowest mean viability is the one that presents the higher percentage of meiotic irregularities. In contrast, the high levels of mean viability found for the parental species (>95%) and the remaining hybrid plants were related to the low percentage of meiotic irregularities observed (Table 2). The correlations between pollen viabilities and meiotic irregularities were high and negative.

Similarly, the meiotic behavior of four somatic hybrids were examined and compared with their corresponding diploid fusion parents, *P. edulis* f. *flavicarpa* (E) and *P. cincinnata* (C).The meiotic behavior revealed relatively high stability, with most of the hybrid cells showing 18 bivalents. Some instability, such as a quadrivalent configuration was also recorded which has been interpreted as an interchange that occurred in the progenitors more than as a result of *in vitro* culture or chromosome reorganization in the new genome. Even in low frequencies, the occurrence of univalents resulted in misdivision, laggard and micronucleus formation. High values of pollen viability (>70%) were found in the diploid parents as well as in the hybrid plants. As expected, the course of meiosis was regular in the parental cells. Some chromosome bridges and laggards were observed, possibly as a consequence of the formation of univalents, though they were not actually recorded. These abnormalities occurred at a very low frequency, i.e. <2% of the cells.

As expected, the meiosis of the parental species showed nine bivalents (9II), two of them were attached to the nucleolus in 67% of the cells at diakinesis. In *P. cincinnata*, this percentage was higher (73%).The four hybrid plants were 4x=36; the hybrid cells *per se* were not variable in number, i.e. no aneuploid cells occurred, at least within the sample cells analyzed.

A certain frequency of laggards and anaphase bridges in both divisions was observed in all hybrid plants (Table 3). However, differences amongst them were noted: (E+C) #07 and (E+C) #25 had a more irregular meiotic behavior than (E+C) #14 and (E+C) #26, but in the second division, the configuration changed, and the hybrids #25 and #26 had more irregular

behavior. The frequency of bivalents at diakinesis in the hybrids varied from 55% to 78.5%. These percentages should be associated with the presence of a quadrivalent, which was found in 30.0% and 32.5% of the cells of the hybrids #07 and #26, respectively, and in 17.1% and 13.3% of the cells of the hybrids #14 and #25, respectively. The disassociation of those quadrivalents should explain the laggards and the chromosome bridges observed at division II. The hybrids #07 and #25 showed higher percentages of univalents, which were also the plants that had cells with higher proportions of laggards at meiosis I and II. The occurrence of univalents varied from 4.2% in (E+C) #14 up to 15% in (E+C) #07 and #25, being correlated with other meiotic alterations and as well bivalent frequency at diakinesis. In the plant (E+C) #25 with 15% of diakinesis cells showing univalents, they appeared as laggards at metaphase I (12.3%) and underwent centromere misdivision (2.3%). Moreover, the results suggest that chromosomes that suffered longitudinal division at meiosis I underwent centromere misdivision at meiosis II (Table 3).

Plant material	**Diakinesis configurations**			**Laggards[1]**		**Bridges**	**Laggards[1]**		**Chromatid bridges**	**No. cells analyzed**
	17II +2I	16II +1IV	18II	MI	AI		MII	AII		
(E)	—	—	—	1.0	0.0	0.6	0.4	0.0	0.2	40
(C)	—	—	—	1.3	0.0	0.2	0.0	0.8	0.6	40
(E+C) #07	15.0	30.0	55.0	19.9	28.8	1.2	15.8	16.0	1.8	40
(E+C) #14	4.2	17.1	78.5	7.1	28.2	5.0	13.0	4.0	10.6	70
(E+C) #25	15.0	13.3	71.6	12.3	26.9	2.3	15.5	7.6	16.1	60
(E+C) #26	7.5	32.5	60.0	15.3	15.5	2.1	13.4	13.4	13.3	40

[1]MI and MII, first and second metaphase, respectively, AI and AII, first and second anaphase, respectively.

Table 3. Meiotic behavior (in percentages) in *P. edulis* f. *flavicarpa*(E), *P. cincinnata*(C) and their four somatic hybrids (E+C). Modified from Barbosa et al. (2007).

The frequency of microspores with micronuclei was relatively low (6.4% up to 23.2%) considering the abnormalities found at all the meiotic stages. Probably, many laggards forming bridges at anaphase I and II were included in telophase nuclei. Certainly, a fraction of these laggards was included as entire chromosomes. The frequency of laggards in metaphase II cells was somewhat the same in metaphase I (3). However, misdivision was five times more frequent at metaphase II, although laggards were found in a reduced frequency (three times less), suggesting that almost all the chromosomes that underwent longitudinal division at first division did undergo misdivision at meiosis II. Laggards in (E+C) #25 were observed in 7.6% of the anaphase II cells and did result in a micronucleus in 8.7% of the tetrad cells. Those chromosomes that went through longitudinal division at first meiosis underwent misdivision because they were attached to the centromeres, while laggards at anaphase II were eliminated.

Parental pollen viability values were 96%. In the hybrid plants, on average, all values were high, ranging from 71.6% up to 82.1%. For the meiotic analyses, one can first state that the two bivalents attached to the nucleolus of the parental cells correspond to the smallest chromosomes, i.e. 8 and 9. Our study on *Passiflora* using fluorescent *in situ* hybridization

detected positive signals corresponding to 45S rDNA sites on the secondary constrictions of chromosome 8 and satellites of chromosome 9 of *P. edulis* f. *flavicarpa* and *P. cincinnata* (Cuco et al., 2005). In addition, chromomycin A3 bandings were found in regions corresponding to 45S rDNA sites, therefore, in two chromosome pairs. No other preferentially GC-rich regions were observed in these species. The nucleolar activity was also investigated by silver staining and four silver-positive signals were detected on the smallest chromosomes pairs.

Minimal differences are also reported between the karyotypes of *P.edulis* f. *flavicarpa* and *P. cincinnata* (Cuco et al., 2005). In addition, the DNA content amongst the *Passiflora* species (at least into the subgenus *Passiflora*) is slightly variable (Souza et al. 2004; Souza et al., 2008), and *P.edulis* f. *flavicarpa* and *P. cincinnata* are very close in terms of phylogenetics (Muschner et al. 2003; Cuco et al., 2005; Padua, 2004). Based on all those features, high chromosome stability is not expected for the somatic hybrids studied here. The presence of just a few univalents in the hybrid plants is expected considering the classical taxonomy (Killip, 1938) and the modern classification proposed for the genus *Passiflora* (Ulmer & Macdougal, 2004) that indicate the proximity of these species. Univalents and the various ways they behave throughout meiosis probably contributed to the formation of the micronuclei observed in the somatic hybrid microspores. The presence of univalents as micronuclei in tetrads was frequently associated with a reduction of vigor and fertility in hybrid plants (e.g. Cao et al., 2003) as above reported for the somatic hybrids between *P. edulis* f. *flavicarpa* and *P. amethystina*. Finally, the more irregular the meiotic behavior, the lower was the pollen viability. This correlation was also abovementioned for the previous set of hybrids (E+Am).

All species used as parents are auto-incompatible and when they were artificially crossed with the somatic hybrids (theoretically, 2n=2x=18 × 2n=4x=36 → 2n=3x=27), few seeds were obtained. The hybrids were female-sterile and pollen producers. In practical terms, the levels of pollen viability and multivalent pairing in the two types of somatic hybrids suggest that these materials can be used as rootstocks due to their compatibility to *P. edulis* f. *flavicarpa* promoting resistance to soil diseases. The three *Passiflora* species investigated are closely related as they belong to the same subgenus (*Passiflora*), although *P. amethystine* belongs to the series *Lobatae*, and *P. edulis* and *P. cincinnata* are members of the series *Passiflora.* Most passionflowers are diploids, though aneuploidy and polyploidy have been reported as evolutionary mechanisms, and have symmetrical karyotypes that could be distinguished from each other by minor differences (Melo et al., 2001; Cuco et al., 2005; Souza et al., 2008).

Intriguingly, newly formed polyploids show genetic and cytological alterations. Consequently, an interest in studies aiming at understanding these genetic events involved in artificial allopolyploid formation increased in recent years, also for *Citrus*, another important fruit species (Chen et al., 2004). In the case of the interspecific allotetraploid somatic hybrids, 'Hamlin' sweet orange + 'Rough Lemon' and 'Key'lime + 'Valencia' sweet orange, meiotic analysis revealed that <20% of the meiocytes exhibited normal tetrad formation in somatic hybrid plants, and irregular chromosome behavior with univalent or multivalent pairing also occurred. Moreover, meiotic abnormalities such as chromosome bridges, chromosomes orientated away from the equatorial plate, especially lagging chromosomes, which resulted in different sizes of pollen grains, were frequently observed in both somatic hybrids. Comparing to passionflowers hybrids, pollen germinability was very low (<15%), intermediate between their corresponding *Citrus* fusion parents. Concluding,

the meiotic behavior of *Citrus* somatic hybrids provided valuable information for their practical utilization, particularly for intergenomic recombination, and increased the germplasm available for interploid crosses in *Citrus* triploid seedless breeding programs.

8. Conclusion

The meiotic analysis of polyploidy hybrids is an important element in taxonomic and evolutionary studies of plants. It also provides an important starting point in alien introgression programs and permits an estimation of the frequency of recombination that will occur in interspecific hybrids. Chromosome pairing is the most used method of assessing genomic relationships between species. This information allows predictions to be made of the likelihood of transferring specific target genes from one species to another. From accumulated results obtained through cytological studies in hybrids, it is evident that cytogenetic analyses are of prime importance in determining which genotypes can continue in the process of cultivar development and which can be successfully used in breeding programs.

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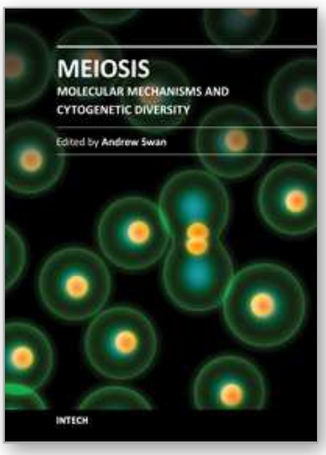

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