Genomic affinities in *Turnera* (subseries *Turnera*, Turneraceae) inferred by in situ hybridization techniques

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Abstract: Subseries *Turnera* comprises a polyploid complex with ploidy levels ranging from diploid ($2n = 2x = 10$) to octoploid ($2n = 8x = 40$). The use of fluorescent in situ hybridization greatly improved the knowledge of the karyotypes of *Turnera* species by detecting and mapping rDNA sites. Interspecific variability in the number of sites was detected, but not in correlation with the ploidy level. A chromosome pair with a strong hybridization signal was always visible and this signal corresponded to the secondary constriction detectable by conventional techniques. Genomic in situ hybridization experiments combined with information on meiotic pairing in species and interspecific hybrids revealed that homologies detected by molecular analysis are greater than those detected by chromosome pairing. This suggests that the formation of the allopolyploids could involve species more closely related than previously assumed. Despite the molecular affinity among the genomes, the meiotic pairing is probably controlled by specific genes that restrict homeologous pairing in polyploids.

Key words: Turnera, in situ hybridization, GISH–FISH.

Résumé : La sous-série *Turnera* est un complexe d'espèces polyploïdes dont les niveaux de ploïdie vont de diploïde ($2n = 2x = 10$) à octoploïde ($2n = 2x = 40$). L'emploi de l'analyse FISH a permis de grandement améliorer la connaissance des carytoypes chez le genre *Turnera* en rendant possible la détection et la localisation des sites d'ADNr. Une variabilité interspécifique a été observée pour ce qui est du nombre de sites, mais sans que celle-ci ne soit corrélée à la ploïdie. Une paire de chromosomes affichant un signal d'hybridation très fort était toujours visible et correspondait à la constriction secondaire observée grâce aux techniques conventionnelles. Des analyses GISH combinées avec l'analyse de l'appariement méiotique chez les espèces et chez des hybrides interspécifiques ont révélé que les homologies détectées par analyse moléculaire étaient supérieures à celles détectées par appariement chromosomique. Cela suggère que la formation d'allopolyploïdes impliquerait des espèces plus fortement apparentées que ce qui avait été soupçonné auparavant. En dépit de l'affinité moléculaire entre les génomes, l'appariement méiotique est vraisemblablement contrôlé par des gènes spécifiques qui limitent les appariements homéologues chez les polyploïdes.

Mots-clés : Turnera, hybridation in situ, GISH–FISH.

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Introduction

Turnera is the largest genus in the family Turneraceae. The genus contains 120 species distributed in America and 2 species in Africa (Arbo 2005). Urban (1883) divided the genus into 9 series, *Annulares*, *Anomalae*, *Capitatae*, *Leiocarpae*, *Microphyllae*, *Papilliferae*, *Salicifoliae*, *Stenodictyae*, and *Turnera* (= *Canaligerae*).

The subseries *Turnera* is composed of 20 species distributed from southern USA to central Argentina (Arbo 1986); ploidy levels range from diploid ($2x$) to octoploid ($8x$) and the basic chromosome number is $x = 5$ (Fernández 1987; Solís Neffa and Fernández 2000). Two groups can be differentiated in this subseries according to the color of flowers, one group having yellow flowers and the other whitish blue flowers. Some authors have postulated that diploid entities with yellow flowers are closely related species sharing a common basic genome (genome A), based on the meiotic pairing behavior observed in species and interspecific hybrids (Arbo and Fernández 1987; Fernández and Arbo 1989, 1990, 1993*a*, 1993*b*). Likewise, diploid entities having whitish blue flowers have been designated as possessing the C genome (Fernández and Arbo 1996). The genome formula of species within the subseries *Turnera* has been established

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based on chromosome pairing during meiosis in artificial interspecific hybrids (Fernández and Arbo 1989, 1990, 1993*b*, 2000*a*, 2000*b*; Fernández and Solís Neffa 2004; Fernández et al. 2010).

In the present work, molecular techniques including fluorescent in situ hybridization (GISH using total genomic DNA and FISH using specific DNA sequences) were applied for the first time to characterize different taxa of the subseries *Turnera*. The number and distribution of rDNA sites were determined with FISH and genomic affinities between diploid and polyploid species were analyzed through GISH. The results obtained were combined with available information on *Turnera* species to clarify the relationships among them.

Materials and methods

Plant material

Analyses were performed on plants of 8 species of *Turnera* cultivated in the greenhouse at the Instituto de Botánica del Nordeste (Table 1). Voucher specimens were deposited in the herbarium of the Institute (CTES).

Fluorescent in situ hybridization

In situ hybridization was performed following Poggio et al. (1999*a*, 1999*b*) with minor modifications. The pTa71 plasmid, containing the 18S–5.8S–25S ribosomal sequence from *Triticum aestivum* (Gerlach and Bedbrook 1979), was used as a probe in FISH experiments. For GISH procedures, total genomic DNAs of *T. subulata* and *T. grandiflora* were used as probes. DNA was extracted from dried leaves in silica gel using the CTAB protocol of Doyle and Doyle (1990) with minor modifications. The probes were labeled with a Biotin Nick Translation Kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions.

Root tips were pretreated in 2 mmol/L 8-hydroxyquinoline (Merck) for 3 h at room temperature and fixed in ethanol : acetic acid (3:1) for 24 h. Fixed root tips were washed in 10 mmol/L citric acid – sodium citrate buffer (pH 4.6) to remove fixative. They were then transferred to an enzyme solution containing 2% cellulase Onozuka R10 (Merck) and 20% liquid pectinase (Sigma) and squashed in a drop of 45% acetic acid. Slides were selected by phase-contrast light microscopy. After removal of cover slips by freezing, the slides were air-dried.

Slide preparations were incubated in 100 µg/mL DNase-free RNase in 2× SSC (1×: 150 mmol/L NaCl plus 15 mmol/L sodium citrate) for 1 h at 37 °C in a humidified chamber and washed 3 times in 2× SSC at room temperature for 5 min. The slides were post-fixed in freshly prepared 4% (*w*/*v*) paraformaldehyde in water for 10 min, washed in 2× SSC for 15 min, dehydrated in a graded ethanol series, and air-dried. The hybridization mixture consisted of 50% (*v*/*v*) deionized formamide, 10% (*w*/*v*) dextran sulphate, and 0.1% (*w*/*v*) SDS, and 100 ng of labeled probe was added to 30 µL of hybridization mixture for each slide. The hybridization–probe mixture was denatured for 15 min at 75 °C, loaded onto the slide preparation, and covered with a plastic cover slip. The slides were placed on a thermocycler for 7 min at 75 °C (denaturation), 10 min at 45 °C, and 10 min at 37 °C. The slides were incubated over-

Table 1. Taxon, locality, collector, no. of chromosomes, ploidy level and kind of ploidy, and genome designation of all the *Turnera* species studied.

Taxon	Locality	Collector	Chr. no.	Ploidy	Genome designation
T. krapovickasii Arbo	Bolivia, Tarija, O'Connor	Ahumada 4549	10	2*x*, diploid	$C^{g}C^{g}$ (Fernández and Arbo 1989)
T. subulata Sm.	Brasil, Maranhao, Sao Luis	Arbo 2410	10	2*x*, diploid	$A^{su}A^{su}$ (Fernández and Arbo 1989)
T .grandiflora (Urb.) Arbo	Argentina, Corrientes, Paso de la Patria	Fernández 366	10	2*x*, diploid	$A^{k}A^{k}$ (Fernández and Arbo 1993*b*)
T. grandidentata (Urb.) Arbo	Paraguay, Paraguarí	Arbo 6109	20	4*x*, segmental allotetraploid	$A^{g}A^{g}A^{t}A^{t}$ (Fernández and Arbo 1990)
T. orientalis (Urb.) Arbo	Argentina, Misiones, Teyú Cuaré	Cabral 358	30	6*x*, segmental allohexaploid	$A^{o}A^{o}BBB^{o}B^{o}$ (Fernández and Arbo 1993*b*)
T. occidentalis Arbo & Shore	Perú, Cajamarca, Contumaza, Jaguey	Sagástegui A 14896	30	6*x*, segmental allohexaploid	$A^{oc}A^{oc}BBB^{o}B^{o}$ (López 2009)
T. velutina C. Presl	México, Oxaca, Santo Domingo	Koch & Fryxell 78341	30	6*x*, segmental allohexaploid	$AAA^{v}A^{v}C^{v}C^{v}$ (Fernández and Arbo 2000*b*)
T. aurelii Arbo	Argentina, Formosa	López & Vanii 111	40	8*x*, segmental alloоctoploid	$A^{a}A^{a}A^{o}A^{o}BBB^{o}B^{o}$ (Fernández and Arbo 1993*b*)

Fig. 1. In situ hybridization in *Turnera* species. (*a*–*e*) FISH: (*a*) *T. subulata*, (*b*) *T. krapovickasii*, (*c*) *T. grandidentata*, (*d*) *T. velutina*, and (*e*) *T. aurelii.* All cells were hybridized with pTa71 (wheat 45S rDNA) labeled with biotin and detected with Cy3. Arrows indicate hybridization sites. DAPI counterstaining. (*f*–*o*) GISH: (*f*–*g*) *T. krapovickasii* (A^kA^k), (*h*–*i*) *T. grandidentata* ($A^gA^gA^rA^r$), (*j*–*k*) *T. occidentalis* ($A^{oc}A^{oc}BBB^oB^o$), (*l*–*m*) *T. orientalis* ($A^oA^oBBB^oB^o$), and (*n*–*o*) *T. velutina* ($AAA^vA^vCvC^v$). (*f*–*m*) Chromosomes hybridized with genomic DNA of *T. subulata* ($A^{su}A^{su}$) labeled with biotin and detected with Cy3. (*n*–*o*) Chromosomes hybridized with genomic DNA of *T. grandiflora* (C^gC^g) labeled with biotin and detected with Cy3. DAPI counterstaining. Arrows indicate weak hybridization. Bars = 5 μm.

night at 37 °C for hybridization. Following hybridization, cover slips were carefully floated off by placing the slides in 2× SSC at 42 °C for 3 min. Slides were given a stringent wash in 20% formamide in 0.1× SSC at 42 °C for 10 min and then washed in 0.1× SSC at 42 °C for 5 min; 2× SSC at 42 °C for 5 min; and 4× SSC, 0.2% (*v*/*v*) Tween 20 at 42 °C for 5 min.

Slides were treated with 100 μL of detection buffer (2.5% BSA in 4× SSC – Tween 20) and incubated for 5 min at room temperature. To detect biotin-labeled probes, slides were incubated in a 1:40 solution of streptavidin-Cy3 conjugate (Sigma) in detection buffer for 1 h at 37 °C in a wet chamber and then washed 3 times in 4× SSC for 10 min each at room temperature.

Slides were counterstained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) in 4× SSC – Tween 20 for 10 min at room temperature and then mounted in anti-fade solution (Vector Labs). Slides were examined with a Carl Zeiss Axiophot epifluorescence microscope. A minimum of 5 cells were analyzed for each experiment. Photographs were taken using Kodak Gold 400 color print film.

Results and discussion

Detection of rDNA sites (FISH)

To analyze the number, location, and intensity of rDNA sites, the ribosomal sequence of hexaploid wheat was used as probe in FISH experiments on chromosomes or interphase nuclei from 7 taxa of subseries *Turnera* having different ploidy levels.

Turnera subulata (2*x*) showed a maximum of 4 signals; 3 of these were of high intensity and the remaining one was hardly detectable (Fig. 1*a*). The diploid *T. krapovickasii* showed a maximum of 4 signals (Fig. 1*b*), 2 of which were larger and of higher intensity than the remaining ones. The former signals corresponded to the number of satellite chromosomes and were always located in the secondary constriction region. *Turnera grandidentata* (4*x*) exhibited a maximum of 10 signals (Fig. 1*c*). The metaphase cells of the allohexaploid *T. velutina* showed a maximum of 6 signals (Fig. 1*d*). *Turnera aurelii* (8*x*) also displayed 6 well-defined signals of high intensity (Fig. 1*e*).

A mapping of rDNA sites was previously performed for the closely related species *T. occidentalis* and *T. orientalis*. Although there were differences in the locations of these sites between the two species, the pair of signals of higher intensity co-localized with the secondary constriction, which was detectable by means of conventional techniques. The other two pairs of lower intensity signals were always present (López et al. 2010).

In summary, the number of rDNA signals differed among the *Turnera* species investigated. It ranged from 4 to 10 signals, with 6 being the most frequent number (Fig. 1). Signal intensity varied within species, but in general a pair of higher intensity signals was observed, probably resulting from a larger number of rDNA copies. When the signal could be mapped, it corresponded to the satellite chromosome detectable by conventional techniques.

The use of FISH greatly enhanced the knowledge of the karyotypes of *Turnera* species by detecting and mapping rDNA sites. There was interspecific variability in the number of sites, which was not closely associated with the ploidy level. The strongest hybridization signal corresponded to the secondary constriction detectable by conventional techniques, which, in turn, would correspond to an active nucleolar organizer region.

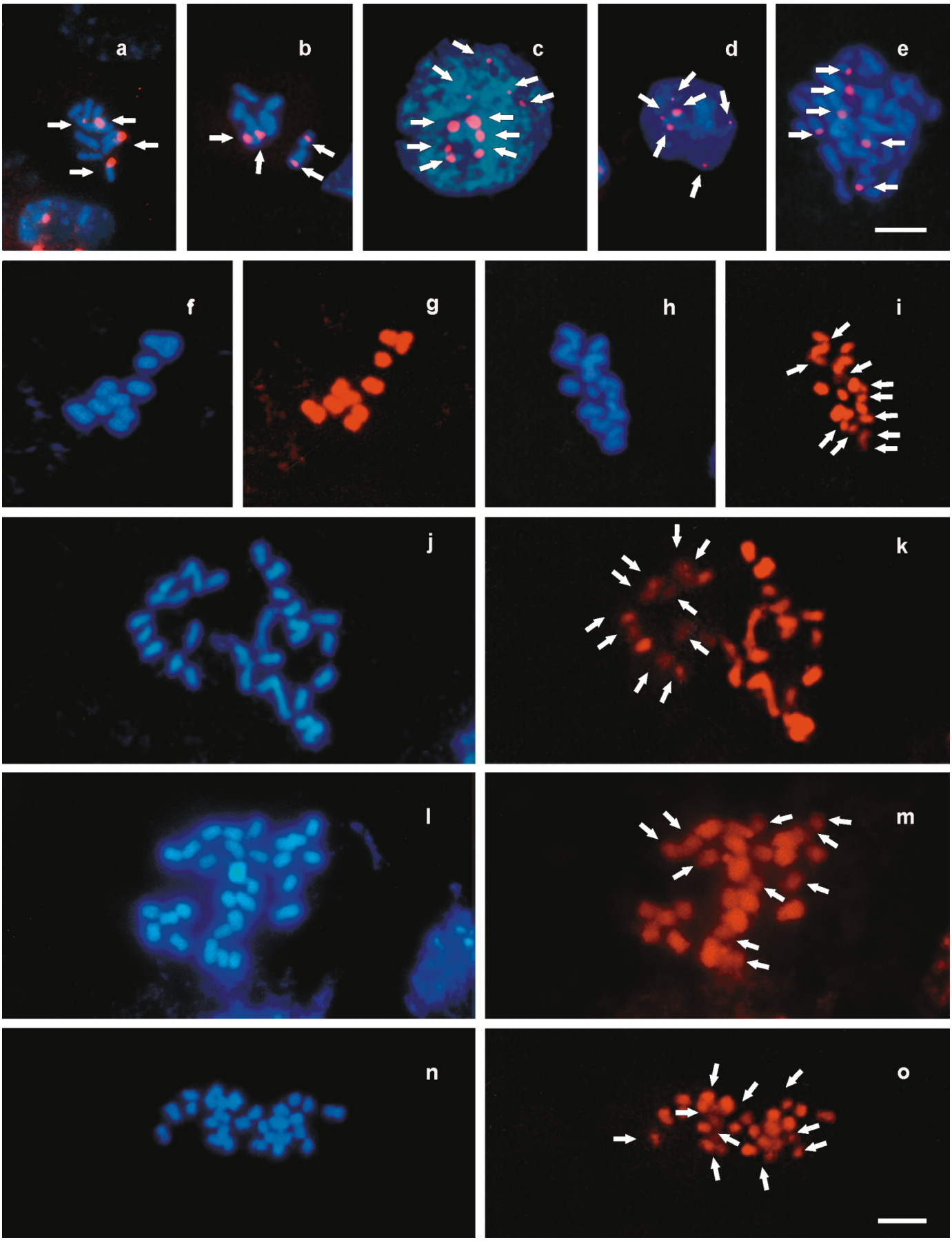
Assessment of genomic affinities using total genomic DNA probes (GISH)

In situ hybridization experiments using total genomic DNA from different species of *Turnera* as probe were performed to assess genomic affinities. These results were compared with those obtained previously from analysis of the meiotic behavior of artificial interspecific hybrids.

The in situ hybridization experiment for the diploid cytotypes of species with yellow flowers, *T. subulata* and *T. krapovickasii*, was carried out using genomic DNA of *T. subulata* as a probe on metaphase chromosomes of *T. krapovickasii*. A signal homogeneously distributed all along the chromosomes (Figs. 1*f*, 1*g*) indicated high genomic affinity between the species. This result supports the genomic formula $A^{su}A^{su}$ for *T. subulata* and A^kA^k for *T. krapovickasii* obtained from the analysis of artificial interspecific hybrids, which showed 5 bivalents as the typical meiotic configuration (Fernández and Arbo 1989).

Total genomic DNA of *T. subulata* as probe was also used to analyze the homology with *T. grandidentata* ($A^gA^gA^rA^r$). Although in situ hybridization showed a signal all along the chromosomes, it was intense in only 10 of them (Figs. 1*h*, 1*i*), indicating a higher degree of homology at the level of repeated DNA sequences with one of the *T. grandidentata* genomes. Cytogenetic studies in the artificial hybrid *T. subulata* × *T. grandidentata* ($2n = 3x = 15$) showed 5I+5II as the most frequent configuration at meiotic metaphase I, but trivalents also occurred with a frequency of 0.73 (range: 0–5) (Fernández and Arbo 1993*a*). The higher homology established by GISH with one of the *T. grandidentata* genomes corresponds with the most frequent configuration observed in the hybrids. On the other hand, the presence of hybridization signals in the second *T. grandidentata* genome may indicate homeology between the two genomes that combined to form the segmental allotetraploid, which is consistent with the observed trivalent formation.

Another series of experiments was aimed at characterizing the species *T. orientalis* and *T. occidentalis*. These were considered to be the same entity until they were separated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by Arbo (2005) based on systematic studies; their identities were further confirmed by cytogenetic studies (López et al. 2010). To deepen the analysis of this issue, these hexaploid species were hybridized with a probe consisting of genomic DNA from a diploid species, *T. subulata* (proposed A-genome donor). In both hexaploids, the hybridization signal was strong in 20 chromosomes and weak in 10 chromosomes (Figs. 1*j*–1*m*), indicating that they have a similar affinity with *T. subulata*.

The hexaploid *T. velutina*, whose proposed genomic formula is $AAA^vA^vC^vC^v$, was hybridized with total genomic DNA of *T. grandiflora* (C^gC^g). The hybridization signal was strong in 20 chromosomes and weak in 10 chromosomes (Figs. 1*n*, 1*o*). The artificial tetraploid hybrid *T. velutina* × *T. grandiflora*, whose proposed genomic formula is $AA^vC^gC^v$, showed 7 different meiotic configurations, the most frequent being 8I+6II (Fernández and Arbo 2000*b*). The number of bivalents varied from 4 to 9 and only 1 tetravalent was observed.

Some artificial interspecific hybrids that resulted from crossing *T. grandiflora* with other species of the *T. ulmifolia* complex were studied by Fernández and Arbo (1993*b*, 2000*b*). They postulated that a gene in the *T. grandiflora* genome would prevent the autosyndetic pairing between chromosomes of the other genomes involved in hybrid formation, with the C genome showing homeology with the A genome proposed for *T. velutina*.

To summarize, the results of the in situ hybridization experiments provide evidence that all the genomes in the *Turnera* complex show different degrees of homeology at the level of repeated DNA. However, the genome nomenclature will follow the convention established for the different species of the complex based on the meiotic behavior observed in the interspecific hybrids. The study of the meiotic pairing in species and interspecific hybrids combined with in situ hybridization revealed that homologies detected by molecular analysis are greater than those detected by chromosome pairing. This indicates that the species involved in the formation of the allopolyploids are more closely related than previously thought. In addition, the meiotic pairing is probably controlled by specific genes that restrict homeologous pairing regardless of the molecular affinity among the involved genomes.

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