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ARTICLE

Genomic affinities revealed by GISH suggests intergenomic restructuring between parental genomes of the paleopolyploid genus *Zea*

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Abstract: The present work compares the molecular affinities, revealed by GISH, with the analysis of meiotic pairing in intra- and interspecific hybrids between species of *Zea* obtained in previous works. The joint analysis of these data provided evidence about the evolutionary relationships among the species from the paleopolyploid genus *Zea* (maize and teosintes). GISH and meiotic pairing of intraspecific hybrids revealed high genomic affinity between maize (*Zea mays* subsp. *mays*) and both *Zea mays* subsp. *parviglumis* and *Zea mays* subsp. *mexicana*. On the other hand, when *Zea mays* subsp. *huehuetenanguensis* DNA was probed on maize chromosomes, a lower affinity was detected, and the pattern of hybridization suggested intergenomical restructuring between the parental genomes of maize. When DNA from *Zea luxurians* was used as probe, homogeneous hybridization signals were observed through all maize chromosomes. Lower genomic affinity was observed when DNA from *Zea diploperennis* was probed on maize chromosomes, especially at knob regions. Maize chromosomes hybridized with *Zea perennis* DNA showed hybridization signals on four chromosome pairs: two chromosome pairs presented hybridization signal in only one chromosomal arm, whereas four chromosome pairs did not show any hybridization. These results are in agreement with previous GISH studies, which have identified the genomic source of the chromosomes involved in the meiotic configurations of *Z. perennis* × maize hybrids. These findings allow postulating that maize has a parental genome not shared with *Z. perennis*, and the existence of intergenomic restructuring between the parental genomes of maize. Moreover, the absence of hybridization signals in all maize knobs indicate that these heterochromatic regions were lost during the *Z. perennis* genome evolution.

Key words: GISH, intergenomic restructuring, maize, teosintes, *Zea*.

Résumé : Le présent travail visait à comparer les affinités moléculaires, révélées par des analyses GISH, ainsi que l'appariement méiotique chez des hybrides intra- et interspécifiques au sein d'espèces du genre *Zea*, lesquels hybrides avaient été obtenus antérieurement. Une analyse conjointe de ces données a fourni des informations au sujet des relations évolutives au sein de ces espèces appartenant au genre paléopolyploïde *Zea* (maïs et téosintes). Les marquages GISH et l'appariement méiotique chez ces hybrides intraspécifiques ont révélé une grande affinité génomique entre le maïs (*Zea mays* subsp. *mays*) et tant le *Zea mays* subsp. *parviglumis* que le *Zea mays* subsp. *mexicana*. Par ailleurs, quand l'ADN du *Zea mays* subsp. *huehuetenanguensis* a été employé comme sonde sur les chromosomes du maïs, une plus faible affinité a été détectée et les résultats de l'hybridation suggéraient la présence de remaniements intergénomiques entre les génomes parentaux du maïs. Lorsque l'ADN du *Zea luxurians* a été employé comme sonde, des signaux d'hybridation homogènes ont été observés sur tous les chromosomes du maïs. Une affinité génomique plus faible a été notée lorsque l'ADN du *Zea diploperennis* a été employé comme sonde sur les chromosomes du maïs, particulièrement au niveau des grands chromomères. Les chromosomes du maïs hybridés avec l'ADN du *Zea perennis* ont affiché un signal sur quatre paires chromosomiques, deux paires de chromosomes présentaient du signal seulement sur un bras chromosomique, tandis que quatre paires de chromosomes n'affichaient aucun signal. Ces résultats sont conformes aux observations GISH rapportées antérieurement, lesquelles avaient permis d'identifier la source génomique des chromosomes impliqués dans les configurations méiotiques observées chez des hybrides *Z. perennis* × maïs. Ces résultats permettent de poser l'hypothèse que le maïs contient un génome parental qui n'est pas partagé avec le *Z. perennis*, et qu'il se serait produit des remaniements intergénomiques entre les génomes parentaux du maïs. De plus, l'absence de signaux

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d'hybridation à l'endroit de tous les grands chromomères du maïs suggère que ces régions hétérochromatiques auraient été perdues au cours de l'évolution du génome du *Z. perennis*. [Traduit par la Rédaction]

Mots-clés : GISH, remaniement intergénomique, maïs, *Zea*, téosintes.

Introduction

The meiotic association of homologous or homoeologous chromosomes reveals the relative affinity between genomes in hybrids and polyploid species and detects chromosomal rearrangements acting as reproductive isolation mechanisms (Sybenga 1975). Crosses between different species of *Zea* have been performed to assess the genomic affinities of these species by meiotic pairing analysis of their hybrids. These studies provided cytogenetic evidences to reveal the cryptic polyploid nature of the genus (paleopolyploidy) and concluded that maize and its wild relatives, with $2n = 20$ chromosomes, are allotetraploids with a basic chromosome number of five ($x = 5$). On the other hand, *Zea perennis* ($2n = 40$) is, in all probability, an amphioctoploid (Naranjo et al. 1990, 1994; Poggio et al. 1990, 1999*a*, 2000*a*, 2000*b*, 2005; González et al. 2004, 2006; González and Poggio 2011). One of the most convincing evidence supporting the amphiploid hypothesis was obtained through treatments with dilute concentrations of colchicine (Naranjo et al. 1990, 1994; Poggio et al. 1990, 2000*b*, 2005). These treatments, which promote intergenomic pairing in allopolyploids (Poggio et al. 1990), joint to the analysis of the meiotic behavior of species and hybrids, supported the polyploid condition of the genus *Zea* and the existence of two parental homoeologous genomes, arbitrarily named A and B. On these bases, the genomic formulae for all species of *Zea* were proposed, being AxAxBxBx for $2n = 20$ species, and $ApApApApBp_1Bp_1Bp_2Bp_2$ for *Z. perennis* ($2n = 40$) (Naranjo et al. 1990, 1994; Poggio et al. 1990, 2000*b*, 2005). Molecular analysis provided compelling evidence that maize is a segmental allopolyploid, having undergone extensive chromosomal rearrangement (Moore et al. 1995; Gaut and Doebley 1997; Soltis and Soltis 1999; White and Doebley 1998; Swigonova et al. 2004; Wei et al. 2007; Schnable et al. 2011).

Genomic in situ hybridization (GISH) is a valuable tool for discriminating between closely related genomes in plants, as the chromosomes and (or) genomes may be distinguished on the basis of divergent dispersed repetitive sequences (Bennett 1995; Chester et al. 2010; Heslop-Harrison and Schwarzacher 2011). In *Zea*, GISH provided evidence about the evolutionary relationships among maize and some of its allied species (Poggio et al. 1999*a*, 1999*b*, 2000*a*, 2000*b*, 2005; González et al. 2004, 2006).

In this work, the results of comparative GISH experiments, using the genomic DNA of wild related species of *Zea* (teosintes) as probes on maize chromosomes, are discussed and compared with the genomic affinities revealed by meiotic pairing of intra- and interspecific hybrids. These data will provide new clues about the evolutionary relationships among maize and its allied species, revealing cryptic genomic divergences between these closely related taxa.

Materials and methods

Plant materials

The plants of *Zea mays* subsp. *mays* were provided by the Vavilov Laboratory, Facultad de Agronomía de la Universidad de Buenos Aires: Race Amarillo Chico (accession VAV 6451) from Santa Victoria, Salta, Argentina; Race Amarillo Grande (VAV 6669) from Manuel Belgrano, Jujuy, Argentina; and Race Blanco y Ocho Rayas (VAV 6483) from Trancas, Tucumán, Argentina. The teosintes were from México and Guatemala: *Zea mays* subsp. *parviglumis* (Valle del Río Balsas, Guerrero, México, Leg. by CIMMYT); *Zea mays* subsp. m*exicana* (Chalco-Amecameca, Mesa Central, México, Leg. by Dr. A.T. Kato Yamakake from Colegio de Postgraduados, Montecillo, México); *Zea mays* subsp. *huehuetenanguensis* (Huehuetenango, Guatemala, Leg. by Dra. C. Prywed from Colegio de Postgraduados, Montecillo, México); *Zea luxurians* (Guatemala, Leg. by Dra. C. Prywed); *Zea diploperennis* (San Miguel, Ciudad Guzmán, Jalisco, México, Leg. by Dra. C. Prywed); *Zea perennis* (Piedra Ancha, Jalisco, México, Leg. by Dra. C. Prywed). All materials were cultivated and maintained in the greenhouse of the Facultad de Agronomía de la Universidad de Buenos Aires.

Cytological preparations

Metaphase chromosome preparations from *Z. mays* subsp. *mays* were obtained from root tips. Seeds were placed in Petri dishes on wet filter paper. Root tips were pre-treated in 0.02 mol/L 8-hydroxyquinoline (Merck) for 3 h at room temperature and fixed in 3:1 ethylic alcohol – acetic acid for 24–48 h. Fixed root tips were washed in 0.01 mol/L citric acid – sodium citrate, pH 4.6 buffer to remove fixative, and then transferred to an enzyme solution containing 2% cellulase Onozuka R10 (Merck) and 20% liquid pectinase (SIGMA). After washing with the above buffer solution, root tips were squashed onto slides in a drop of 45% acetic acid. Preparations showing well-spread metaphase cells were selected by phase-contrast light microscopy. After removal of coverslips by freezing, the slides were air-dried to be used for in situ hybridization procedures.

DNA probes

Total genomic DNA was isolated from adult leaves from the teosintes with Wizard Genomic DNA Purification kit (Promega), following manufacturer's procedures.

DNA was labelled with Dig High Prime kit (Boehringer Mannheim) and Biotin Nick Translation kit (Boehringer Mannheim), following manufacturer's procedures.

Genomic in situ hybridization (GISH)

GISH technique was carried out according to González et al. (2004). Slide preparations were incubated in 100 μg/mL of RNase in 2xSSC (saline-sodium citrate) for 1 h at 37 C° in a humidified chamber and washed three times in 2xSSC for 5 min each at room temperature. The slides were post-fixed in freshly prepared 4% (*w*/*v*) paraformaldehyde in distilled water for 10 min and then were washed in 2xSSC for 15 min at room temperature. The preparations were dehydrated in a graded ethanol series and air dried. The hybridization mixture consisted of 50% (*w*/*v*) deionised formamide, 10% (*w*/*v*) dextran sulphate, 0.1% (*w*/*v*) SDS, and 0.3 mg/mL of salmon sperm in 2xSSC, then adding 100 ng of the labelled probe to 30 μL of hybridization mixture for each slide. The hybridization mixture was denatured for 15 min at 75 °C, loaded onto the slides and covered with a plastic coverslip. The slides were placed on a thermocycler at 75 °C for 7 min, 45 °C for 10 min, and 38 °C for 10 min. Then the slides were incubated overnight at 37 °C. Following hybridization, coverslips were carefully floated off by placing the slides in 2xSSC at 42 °C for 3 min each and then given an astringent wash in 20% formamide in 0.1xSSC at 42 °C for 10 min. After that, the slides were washed in 0.1xSSC at 42 °C for 5 min, 2xSSC at 42 °C for 5 min, then the slides were transferred to detection buffer (4xSSC, 0.2% (*v*/*v*) Tween 20) at 42 °C for 5 min and 1 h at room temperature in the same buffer.

To detect digoxigenin-labelled probes, slides were treated with sheep antidigoxigenin-FITC (Boehringer Mannheim), whereas for biotin-labelled probes, the slides were treated with streptavidine-Cy3 conjugate (SIGMA). The slides were incubated in a 1:40 solution of the corresponding antibody in detection buffer containing 2.5% bovine serum albumine (SIGMA), for 1 h at 37 °C and washed 3 times in detection buffer for 10 min each at room temperature. Slides were counterstained with 1 μg/mL of 4′6-diamino-2-phenylindole (DAPI) in 4xSSC/Tween buffer for 25 min at room temperature and then mounted in anti-fade solution (Vector Laboratories). Slides were examined with a Carl Zeiss Axiophot epifluorescence microscope with appropriate Carl Zeiss filters coupled with a Leica DC 250 digital camera and with the image analyzer Leica IM 1000.

Results

In GISH experiments, total genomic DNA of *Z. mays* subsp. *mexicana* (Figs. 1A and 1B) and *Z. mays* subsp. *parviglumis* (Figs. 1C and 1D) were labelled and used as probes on mitotic metaphases of the maize accessions studied. With both probes, strong signals of hybridization were observed along all maize chromosomes, but with weak or absent signals in the pericentromeric region and in the region proximal to the nucleolar organizer (NOR) located in the short arm of the chromosome pair 6. Moreover, strong fluorescent signals on the DAPI-positive bands (heterochromatic knob regions) and B chromosomes were detected (Figs. 1A and 1B).

When genomic DNA of *Z. mays* subsp. *huehuetenanguensis* was probed on maize chromosomes, many chromosome regions were unlabelled. Three chromosomal pairs displayed one chromosome arm highly labelled and the other chromosome arm was unlabelled or weakly hybridized. The chromosome pair with NOR showed very weak hybridization signals, except in the knob region. The rest of the chromosomes displayed a high level of hybridization, except in the pericentromeric regions. The knobs and B chromosomes also showed a high level of hybridization (Figs. 1E and 1F).

Genomic DNA from *Z. luxurians* showed uniform hybridization signals over all maize chromosomes, with higher signals on the knob regions (Figs. 1G and 1H). In contrast, probing with genomic DNA from *Z. diploperennis* hybridization, signals were observed over all maize chromosomes, although they were weaker than those observed with *Z. mays* subsp. *mexicana*, *Z. mays* subsp. *parviglumis*, and *Z. luxurians* probes. Besides, ~10 maize chromosomes showed very weak hybridization signal. The knob regions presented the same level of hybridization than that observed along the chromosome arms (Figs. 1I and 1J).

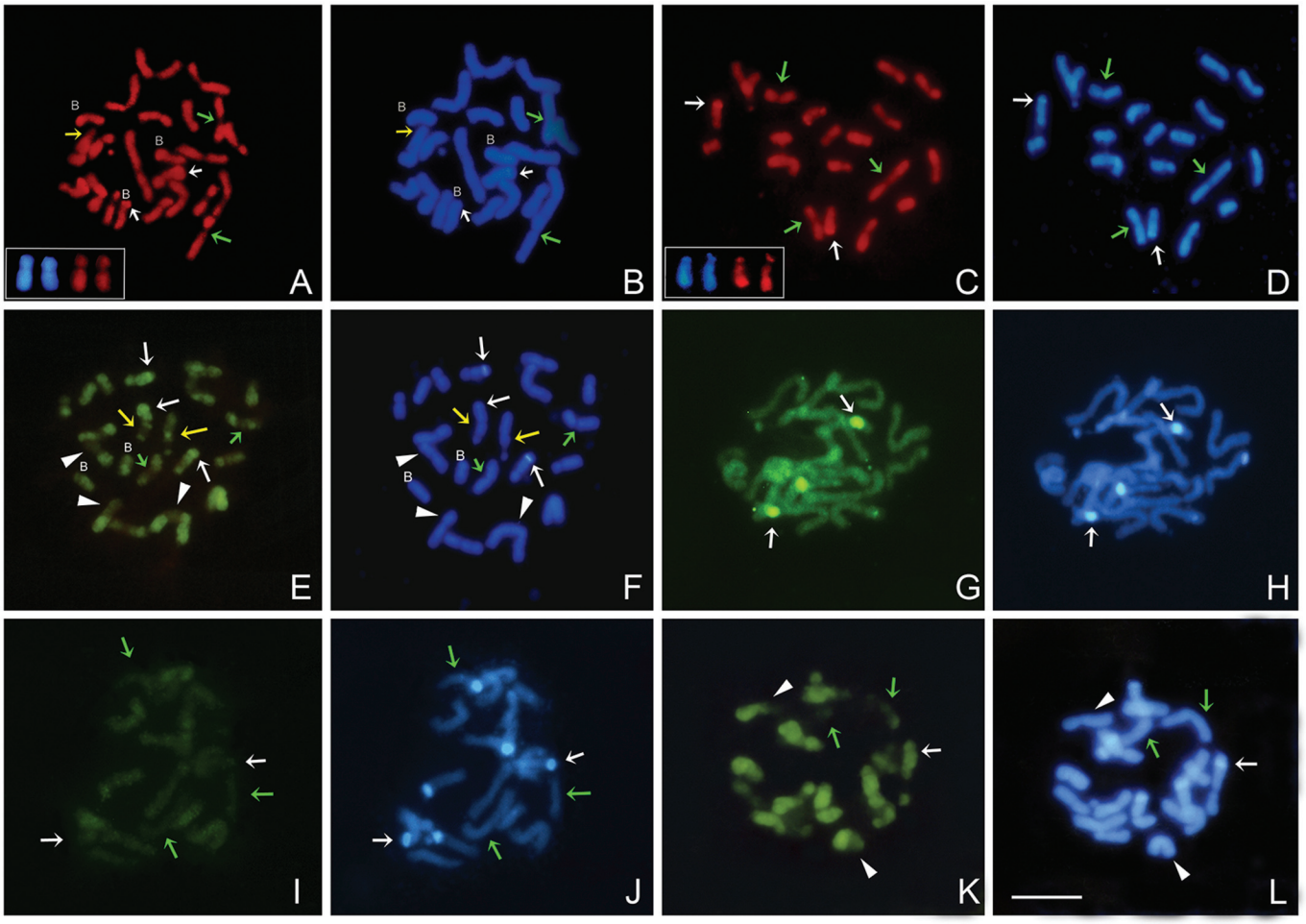
Probing several accessions of maize with genomic DNA from *Z. perennis*, high level of hybridization in four chromosome pairs and the absence of hybridization on another four chromosome pairs were revealed. The rest of the chromosomes (two pairs) presented hybridization signals in a large portion of one of the chromosome arms. The knob regions lacked hybridization signals (Figs. 1K and 1L).

The B chromosomes of maize showed high hybridization signal with all teosinte probes (Figs. 1A, 1B, 1E, and 1F).

Discussion

For a long time maize has been considered to be a diploid species; however, noteworthy data indicate that it is a cryptic or diploidyzed polyploid. Several studies evaluated the genome affinity based on the estimation of the chromosome meiotic association of species and hybrids of the genus *Zea*. These studies provided cytogenetic evidence confirming the cryptic polyploid nature of the genus and established that all species of the genus *Zea* have a basic chromosome number of five ($x = 5$), and that maize and its wild relatives ($2n = 20$) are tetraploids, while *Z. perennis* ($2n = 40$) is an allooctoploid (Naranjo et al. 1990, 1994; Poggio et al. 1990, 1999*a*, 1999*b*, 2000*a*, 2000*b*, 2005; González et al. 2004, 2006; González and Poggio 2011). These hypotheses were further confirmed by molecular studies (Moore et al. 1995; White and

Fig. 1. GISH on mitotic metaphase chromosomes of *Zea mays* subsp. *mays* probed with: (A) *Zea mays* subsp. *mexicana* DNA (detected with red-Cy3). Insert: detail of one chromosome pair (from another metaphase plate) lacking hybridization signal on the pericentromeric region; (C) *Zea mays* subsp. *parviglumis* DNA (detected with red-Cy3). Insert: detail of chromosome pair six (from another metaphase plate) without hybridization signal on the short arm; (E) *Zea mays* subsp. *huehuetenanguesis* DNA (detected with yellow-green FITC); (G) *Zea luxurians* DNA (detected with yellow-green FITC); (I) *Zea diploperennis* DNA (detected with yellow-green FITC); (K) *Zea perennis* DNA (detected with yellow-green FITC). (B), (D), (F), (H), (J) and (L) DAPI counterstaining. White arrows indicate several knob regions, yellow arrows indicate the short arm of chromosome six, arrowheads indicate chromosomes with hybridization signal on only one arm, and green arrows indicate a lack of hybridization signals on pericentromeric regions (A–F) or onto entire chromosomes (I–L). B, B chromosomes. Scale bar = 10 μm.



Doebley 1998; Soltis and Soltis 1999; Schnable et al. 2011; Swigonova et al. 2004; Wei et al. 2007).

In the present work, the genomic affinities revealed by GISH experiments, using teosinte probes on maize chromosomes, are analyzed and compared with those detected through meiotic analysis in previous works.

The hybrids between maize and two subspecies of the same taxonomical section *Zea* (Doebley 1990), *Z. mays* subsp. *parviglumis* and *Z. mays* subsp. *mexicana*, have regular meiosis, with 10 bivalents and high pollen viability (revisited in Poggio et al. 2005). When maize chromosomes were hybridized with labelled genomic DNA from *Z. mays* subsp. *parviglumis* and *Z. mays* subsp. *mexicana*, high hybridization signals throughout all chromosomes were observed. Similar results were reported by Takahashi et al. (1999). It is interesting to point out that, in the present work, bright hybridization signals were detected on the subtelomeric regions on some maize chromosomes, which correspond to the heterochromatic maize knobs (Poggio et al. 1999*a*; González et al. 2004, 2011, 2013). However, the pericentromeric regions and the NOR region presented faint hybridization signals with *Z. mays parviglumis* and *Z. mays mexicana* probes. This could be due to higher divergence in these regions, which consist mostly in repetitive sequences (Heslop-Harrison and Schwarzacher 2011). The high genomic affinities, revealed by GISH, between maize and *Z. mays* subsp. *parviglumis* and *Z. mays* subsp. *mexicana* are in agreement with the meiotic pairing of their hybrids. All these results indicate that the three subspecies are very close, with little divergence among their genomes.

When maize chromosomes were hybridized with labelled genomic DNA from *Z. mays* subsp. *huehuetenanguensis* (section

Zea), it was observed that, in addition to the absence of hybridization signals in the centromeric and NOR regions, entire chromosome arms or part of them were unlabelled. Taking into account the genomic formulae proposed for these taxa, AABB (Naranjo et al. 1990), this result let to postulate that one of the maize parental genomes (A or B) presents an important divergence with the *Z. mays* subsp. *huehuetenanguensis* genome. Moreover, the pattern of hybridization observed could be explained by the occurrence of intergenomic recombination between A and B maize parental genomes. This phenomenon is frequent in polyploids, indicating that parental genomes may have undergone some rearrangements following hybridization and whole genome duplication. Intergenomic restructuration was also reported in *Avena* (Hayasaki et al. 2000), *Poa jemtlandica* (Brysting et al. 2000), and in the first generation of newly synthesized *Brassica* allopolyploids (Ma and Gustafson 2006), among others (Soltis et al. 2014*a*, 2014*b*).

The meiotic analysis of the hybrids between maize and two teosinte species from section *Luxuriantes* (Doebley 1990), *Z. luxurians* and *Z. diploperennis*, showed 10 bivalents with regular meiotic pairing. However, both hybrids differ in pollen fertility and seed viability (Naranjo et al. 1990; González 2004; González and Poggio 2011). González and Poggio (2011) analyzed the meiotic behavior of F_1 artificial hybrids of the *Z. luxurians* × maize cross to determine the genomic relationships between both parental species. These hybrids presented 10 heteromorphic bivalents and several meiotic abnormalities that explained their high pollen sterility, which determine the postzygotic isolation between parental species. When labelled DNA from *Z. luxurians* was probed on maize chromosomes, homogeneous hybridization signals were observed through all chromosomes. However, the hybridization signals were weaker than those observed with *Z. mays* subsp. *parviglumis* and *Z. mays* subsp. *mexicana* probes, except in the knob regions, where high hybridization signals were seen. This indicates a high homology between knob sequences of maize and *Z. luxurians*, which is greater than that detected in the rest of the chromosomes. Even lower genomic affinity was observed when total DNA of the perennial teosinte *Z. diploperennis* was probed on maize chromosomes. In this experiment, all maize chromosomes showed hybridization signals, although variation in the intensity of the signals among chromosomes was found, as ~10 chromosomes showed lower intensity of hybridization. According to the hypothesis proposed by Naranjo et al. (1990), these results would suggest that the allotetraploids maize and *Z. diploperennis* differ in one parental genome (A or B), or that these genomes experimented divergence and (or) fractionation bias during the evolutionary process. On the other hand, these divergences are not reflected in the meiotic behavior of the F_1 hybrids of the *Z. diploperennis* × maize cross, which presented regular meiotic chromosome pairing and high pollen viability (Naranjo et al. 1990; Poggio et al. 2005). In relation to the knobs, *Z. diploperennis* probe showed low hybridization signals on these heterochromatic regions of maize chromosomes. This result was previously observed by Albert et al. (2010) and could be explained by the existence of divergences in the sequence composition of knobs of maize and *Z. diploperennis*.

Zea perennis is a perennial autoaloctoploid species ($2n$ = 40) from the section *Luxuriantes*, with a genomic formulae ApAp Ap′Ap′ Bp_1Bp_1 Bp_2Bp_2 (Naranjo et al. 1990). The meiotic analysis of the hybrids of the *Z. perennis* × maize cross ($2n$ = 30), whose genomic formulae is ApAp′Am Bp_1Bp_2 Bm, revealed that 5III+5II+5I was the most frequent chromosome configuration at metaphase I (Poggio et al. 2005; González et al. 2006). GISH experiments enabled the recognition of the genomic source of each chromosome involved in the meiotic configurations of these hybrids. These results revealed that, in these hybrids, five maize chromosomes have paring affinities with *Z. perennis* chromosomes, jointly constituting the trivalents (III), while the other five maize chromosomes remain as univalents (González et al. 2006). In the present work, an interesting result was obtained when *Z. perennis* genomic DNA was probed onto the maize chromosomes. It was observed a high level of hybridization on four chromosome pairs and the absence of hybridization on another four chromosome pairs, while the remaining two chromosomes pairs presented hybridization signal in only one of their chromosomal arms. The entire chromosomes and the chromosome arms with hybridization signals could represent one of the parental genomes of maize with high sequence homology with *Z. perennis* genomes. They would correspond to the "pan handle" of the trivalents of the hybrids of the *Z. perennis* × maize cross described by González et al. (2006). Besides, the chromosomes and chromosomal arms without hybridization signals could belong to the parental maize genome with low sequence homology with *Z. perennis* genomes and are, in all probability, the chromosomes that remain as univalents in the hybrids (González et al. 2006). Then, the analysis of the meiotic behaviour of the hybrids and the molecular affinities detected by GISH support the hypothesis that maize has a parental genome not shared with *Z. perennis*. Moreover, the two chromosome pairs showing hybridization signals in one arm reveal the existence of intergenomic restructuring between the maize parental genomes A and B. These intergenomic rearrangements would have occurred during the evolution and (or) domestication of maize. Genomic restructuring following allopolyploidization has been reported in several genera (revisited in Soltis et al. 2014*a*, 2014*b*).

Another interesting result that emerges when *Z. perennis* genomic DNA is used as a probe on maize chromosomes is the absence of hybridization signals on all the maize heterochromatic knobs. Also, the lack of hybridization signal on knobs was observed when genomic DNA from

Z. perennis was hybridized onto the other teosintes (Poggio et al. 1999*a*, 2005). These results would indicate that the knob sequences were lost during the *Z. perennis* genome evolution. In relation to this, it is interesting to point out that the *Z. perennis* DNA content per basic genome is smaller than in other species of *Zea* with $2n = 20$ (Tito et al. 1991). It has been frequently documented that the major trend in vascular plants is a decrease in the genome size per haploid genome when a polyploidization event occurs (Leitch and Bennett 2004; Leitch and Leitch 2013). This genome downsizing, which could be involved in the genetic and cytogenetic diploidization of polyploids, consists in non-random deleting of coding and non-coding sequences, changes in retroelements, chromosome reorganization, and loss of chromosomes or entire genomes (Ma and Gustafson 2006; Jones and Langdom 2013; Leitch and Leitch 2013; Poggio et al. 2014).

The present work provides new clues for understanding the genome organization and diversification of the species of *Zea* and the origin of domesticated maize.

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