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

**Graciela Esther González* and Lidia Poggio**

Instituto de Ecología, Genética y Evolución (IEGEB, Consejo Nacional de Investigaciones Científicas y Técnicas - CONICET) - Laboratorio de Citogenética y Evolución (LaCyE), Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina.

**Running title:** Genomic affinities and intergenomic restructuring in *Zea*.

**Author for Correspondence:** Graciela Esther González, Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. Int. Güiraldes Nº 2620, Pabellón II, Lab. 110, 4° Piso (1428), Ciudad Autónoma de Buenos Aires, Argentina. Tel.: +54-11-4576-3300 ext. 218. e-mail address: gegonzalez@ege.fcen.uba.ar
Abstract

The present work compares the molecular affinities revealed by GISH with the analysis of meiotic pairing in intra e interspecific hybrids between Zea species, 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 ssp. mays) and both Zea mays ssp. parviglumis and Zea mays ssp. mexicana. On the other hand, when Zea mays ssp. 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 that enabled to recognize the genomic source of the chromosomes involved in the meiotic configurations of the Zea perennis x maize hybrids. These findings allow postulating that maize has a parental genome not shared with Zea 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 Zea perennis genome evolution.

Keywords: GISH, Intergenomic restructuring, Maize, Teosintes, Zea
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 all tetraploids with a basic chromosome number of five (x=5). On the other hand, Z. perennis (2n=40) is, in all probability, an amphiploid (Naranjo et al. 1990, 1994; Poggio et al. 1990, 1999a, 2000a, 2000b, 2005; González et al. 2004, 2006, 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, 2000b, 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 Zea species were proposed, being AxAxBxBx for 2n=20 species, and ApApApBp1Bp1Bp2Bp2 for Z. perennis (2n=40) (Naranjo et al. 1990, 1994; Poggio et al. 1990, 2000b, 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; Schanable et al. 2011).
The genomic in situ hybridization (GISH) is a valuable tool for discriminating between closely related genomes in plants, because 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, 2000a, 2000b, 2005; González et al. 2004, 2006).

In this work, the results of comparative GISH experiments, using the wild related Zea species (teosintes) genomic DNA 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 ssp. mays were legated by Vavilov Lab., Facultad de Agronomía de la Universidad de Buenos Aires (FA-UBA): 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 come from México and Guatemala: Zea mays ssp. parviglumis (Valle del Río Balsas, Guerrero, México, Leg. by CIMMYT), Zea mays ssp. mexicana (Chalco-Amecameca, Mesa Central, México, Leg. by Dr. A. T. Kato Yamakake from Colegio de Postgraduados, Montecillo, México), Zea mays ssp. 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, San Gabriel, Jalisco, México, Leg. by Dra. C. Prywed), All materials were cultivated and are maintained in the greenhouse of FA-UBA.

**Cytological preparations**

Metaphase chromosome preparations from *Zea mays* ssp. *mays* were obtained from root tips. Seeds were placed in Petri dishes on wet filter paper. Root tips were pre-treated in 0.02 M 8-hydroxyquinoline (Merck) for 3 hours at room temperature and fixed in 3:1 ethyl alcohol – acetic acid for 24 – 48 hours. Fixed root tips were washed in 0.01 M citric acid – sodium citrate, pH 4.6 buffer to remove fixative, and then were transferred to an enzyme solution containing 2% of 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 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), while for biotin-labelled probes, the slides were treated with Streptavidine-CY3 conjugated (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 \( \mu g/ml \) 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 Lab.). 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 an image analyzer Leica IM 1000.

Results

In GISH experiments total genomic DNA of \( Z. \) \textit{m. ssp. mexicana} (Fig. 1 A and B) and \( Z. \) \textit{m. ssp. parviglumis} (Fig. 1 C and D) 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 being weak or absent in the pericentromeric region and in the region proximal to 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 (Fig. 1 A and B).

When genomic DNA of \( Z. \) \textit{m. ssp. huehuetenanguensis} was probed on maize chromosomes, many chromosome regions were unlabelled. Three chromosomal pairs displayed one chromosome arm highly labelled and the other one 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 (Fig. 1 E-F).

Genomic DNA from \( Z. \) \textit{luxurians} showed uniform hybridization signals over all maize chromosomes, being higher on the knob regions (Fig. 1 G and H). In contrast,
probing with genomic DNA from *Z. diploperennis* hybridization signals were observed over all maize chromosomes, although they were weaker than the observed in the species already described. Besides, ca. 10 maize chromosomes showed very weak hybridization signal. The knob regions presented the same level of hybridization than that observed along the chromosome arms (Fig. 1 I and J).

Probing several accessions of maize with genomic DNA from *Z. perennis*, high level of hybridization in 4 chromosome pairs and absence of hybridization on other 4 chromosome pairs were revealed. The rest of the chromosomes (2 pairs) presented hybridization signals in a large portion of one of the chromosome arms. The knob regions lacked hybridization signals (Fig. 1 K and L).

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

**Discussion**

For a long time maize has been considered to be a diploid species, however noteworthy data indicate that it is a cryptic or diploidized polyploid. Several studies evaluated the genome affinity based on the estimation of the chromosome meiotic association of species and hybrids of genus *Zea*. These studies provided cytogenetic evidence confirming the cryptic polyploid nature of the genus and established that all *Zea* species have a basic chromosome number of five (x=5), 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, 1999a, 1999b, 2000a, 2000b, 2005; González *et al*. 2004, 2006, 2011). These hypotheses were further confirmed by molecular studies.
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 the two subspecies of the same taxonomical Section Zea (Doebley 1990), Z. m. ssp. parviglumis and Z. m. ssp. mexicana, have regular meiosis, with ten bivalents and high pollen viability (revisited in Poggio et al. 2005). When maize chromosomes were hybridized with labelled genomic DNA from Z. m. ssp. parviglumis and Z. m. ssp. 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. 1999a; González et al. 2004, 2011, 2013). However, the pericentromeric regions and the NOR region presented faint hybridization signals with Z. m. parviglumis and Z. m. 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. m. ssp. parviglumis and Z. m. ssp. mexicana are in agreement with the meiotic pairing of their hybrids. All these results indicate that the tree subspecies are very close, with little divergences among its genomes.

When maize chromosomes were hybridized with labelled genomic DNA from Z. m. ssp. 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 Z. m. ssp. 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 the first generation of newly synthesized Brassica allopolyploids (Ma and Gustafson 2005), among others (Soltis et al. 2014a, 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 F1 artificial hybrids Z. luxurians x maize 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. m. ssp. parviglumis and Z. m. ssp. 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, because *ca.* 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 F1 hybrids *Z. diploperennis* x maize, 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 autoalloctoploid species (2n=40) from the Section Luxuriantes, with a genomic formulae ApAp Ap′Ap′ Bp1Bp1 Bp2Bp2 (Doebley 1990; Naranjo *et al.* 1990). The meiotic analysis of the hybrids *Z. perennis* x maize (2n=30), whose genomic formulae is ApAp′Am Bp1Bp2 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 to recognize 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 4 chromosome pairs and absence of hybridization on other 4 chromosome pairs, while the remaining 2 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 *Z. perennis* x maize 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 was reported on several genus (revisited in Soltis *et al.* 2014a, 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.* 1999a, 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 *Zea* species 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, 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 *Zea* species and the origin of domesticated maize.

**Acknowledgements**

Authors gratefully acknowledge grants from the Agencia Nacional de Promoción Científica y Técnica, University of Buenos Aires and the National Council of Scientific Research of Argentina (CONICET). The authors specially acknowledge the valuable suggestions from the Associate Editor and the two anonymous reviewers, which have improved the manuscript.

**References**


**Fig. 1:** GISH on mitotic metaphase chromosomes of *Zea mays* ssp. *mays* probed with:

Unable to Convert Image

The dimensions of this image (in pixels) are too large to be converted. For this image to convert, the total number of pixels (height \times width) must be less than 40,000,000 (40 megapixels).