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Evolutionary relationships in the genus *Zea*: analysis of repetitive sequences used as cytological FISH and GISH markers

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Abstract

The present study is a revision of our work on evolutionary cytogenetics of the genus *Zea*, including several new experiments which give a deeper insight into the nature of the DNA sequences involved in telomeric regions of *Zea luxurians*. These new experiments, based on the Southern blotting technique and *in situ* hybridization, have demonstrated the following: 1) *in situ* hybridization (FISH) demonstrated the presence of the 180-bp repeat maize-knob-repeat-sequence in DAPI-positive terminal heterochromatic blocks of *Z. luxurians* (ZL-THB region); 2) Southern blot analysis confirmed that the 180-bp repeat present in maize is also present in *Z. diploperennis*, *Z. luxurians* and *Tripsacum dactyloides*, but not in Z. *perennis*; 3) another sequence with targeted sites for endonucleases, but without recognition sites for the 180-bp repeat, may be interspersed with the 180-bp repeat in a tandem array sited in the ZL-THB region; 4) *in situ* hybridization (GISH) of probes and blocking-probes with chromosomes of Z. *luxurians* (using *Z. luxurians* as a probe and *Z. diploperennis* or *Z. perennis* as a blocking-probe) gave strong fluorescence in both cases. Since *Z. diploperennis* possesses the 180-bp repeat, fluorescence on *Z. luxurians* chromosomes was not expected. These results can be explained if the ZL-THB regions are composed not only of 180-bp repeats interspersed with other sequences, but also of other tandem arrays unique to *Z. luxurians,* which, according to our GISH results, are probably located at the subterminal position.

INTRODUCTION

Maize is one of the plants which has been most studied, but its origin, however, has not yet been solved. There is much data indicating that maize *(Zea mays* ssp. *mays)* with a chromosome complement of 2n = 20 is a cryptic polyploid with an ancestral basic number of five (x = 5). Previous cytological findings supporting this hypothesis are: a) the existence of chromosome pairing during the meiosis of haploids (McClintock, 1933; Ting, 1985), b) the secondary association of bivalents (Vijendra Das, 1970) and c) tridimensional chromosome distribution in somatic metaphases, where the chromosomes form four groups of 5 chromosomes each (Bennett, 1983, 1984).

The genus *Zea* (Tribe *Maydeae)* is composed of two sections: the Luxuriantes Section (Doebley & Iltis), which includes the perennials *Z. diploperennis* Iltis, Doebley & Guzman and *Z. perennis* (Hitch.) Reeves & Mangelsdorf and the annual Z. *luxurians* (Durieu & Ascherson) Bird, and the *Zea* Section with the annual *Z. mays* L., which has been divided into subspecies (Doebley and Iltis, 1980; Iltis and Doebley, 1980). All the above mentioned species have 2n = 20 except Z. *perennis*, which has 2n = 40.

The relative affinity between the genomes in the genus *Zea* can be evaluated by measuring the association of the chromosomes of hybrids at diakinesis-metaphase I, a method which is particularly useful in hybrids from polyploid species because pairing occurs in the same genetic background. Since 1987 our team have been analyzing meiotic configurations in *Zea* species and their intra- and interespecific hybrids. These studies, discussed below, have allowed us to record cytogenetic evidence confirming the cryptic polyploid nature of the genus.

Meiotic behavior of 2n = 30 hybrids

The most frequent meiotic configurations in 2n = 30 hybrids were 5 trivalents (III) + 5 bivalents (II) + 5 univalents (I) (Figure 1). Most of the trivalents were of the "frying pan" type, while bivalents were homomorphic even in those cases in which significant differences in chromosome size between parental species were observed (i.e., *Z. perennis* x *Z. luxurians*) (Molina and Naranjo, 1987; Naranjo *et al*., 1990; Poggio and Naranjo, 1995), and there is genetic evidence supporting the hypothesis that the univalents could have originated from the 2n = 20 species, when they occur in 2n = 30 hybrids (Longley, 1924). There is also a tendency for bivalents to be spatially grouped; we proposed that they could belong to a *Z. perennis* genome (Molina and Naranjo, 1987; Poggio and Naranjo, 1995; Poggio *et al*., 1998, 1999b).

Meiotic behavior of species and hybrids with 2n = 20 chromosomes

The secondary association of bivalents was observed at diplotene-diakinesis, with a maximum of five groups of two bivalents each (Molina and Naranjo, 1987; Naranjo *et al*., 1990; Poggio and Naranjo, 1995), which could be evidence of association between homologous chromosomes belonging to different genomes.

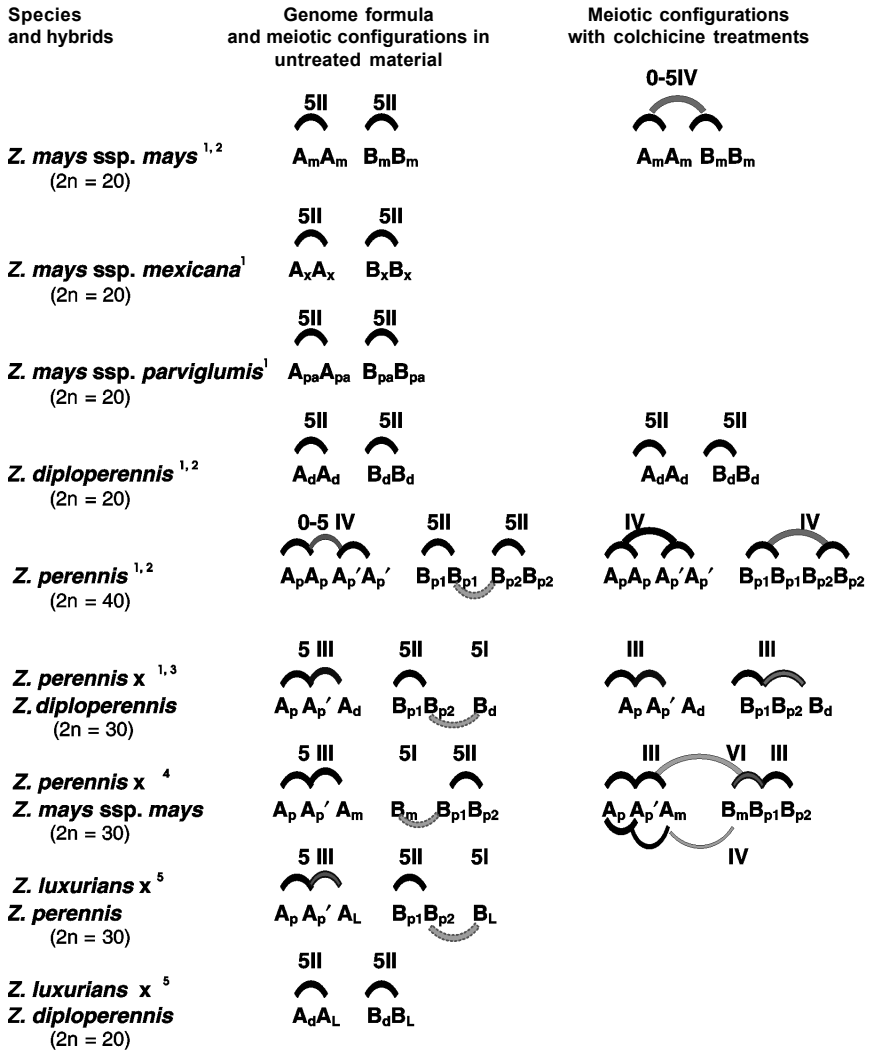

Figure 1 - Genome formulas and meiotic configurations for *Zea* species and hybrids with and without colchicine treatment. In untreated material: Black arcs of the top show the high-frequency meiotic association. Green arcs below indicate meiotic low frequency association. In treated material: Grey arcs indicate the new configurations that were observed and a proposed explanation for their formation. Data from: 1 = Molina and Naranjo, 1987; Naranjo *et al.*, 1990. 2 = Poggio *et al.*, 1990. 3 = Naranjo *et al*., 1994. 4 = Poggio, L. and Naranjo, C.A. (unpublished results). 5 = Poggio *et al.*, 1998; Poggio *et al.*, 1999b.

In a variable proportion of cells, the 10 bivalents were divided into two groups of five bivalents at diplotene-metaphase I which, together with other evidence, indicates that five is the basic chromosome number (x = 5) of the genus and suggests the separation of two relict genomes (designated as genomes A and B) (Naranjo *et al*., 1990; Poggio and Naranjo, 1995). In alloplasmic maize lines (composed of maize nuclei and *Z. mays* ssp. *mexicana* (teosinte) cytoplasm), the separation of bivalents into two groups was both noticeable and very frequent, suggesting that interactions between the nucleus and the cytoplasm influence the spatial separation of genomes (Poggio and Naranjo, 1995; Poggio *et al*., 1997).

Another important characteristic observed in alloplasmic lines and diploid hybrids was that in about 20-50% of pollen mother cells (PMC) at prophase I, MI and AI, the two groups of bivalents, are asynchronous, showing a slight displacement in the progress of meiosis, one group of 5 bivalents being more advanced with respect to the other (Poggio *et al*., 1997). Since this phenomenon is common in interspecific and intrageneric hybrids (Bennett, 1983; Jouve *et al*., 1977) it can be assumed that in *Zea* the two groups of 5 bivalents each correspond to different ancestral genomes (genomes A and B), a hypothesis also supported by the presence of two nucleoli in 10% of the cells in alloplasmic lines and hybrids, each nucleoli being associated with one of the groups of 5 bivalents (Poggio *et al.*, 1997).

Treatment of species and hybrids with dilute colchicine

Jackson and Murray (1983) demonstrated that dilute colchicine applied at the onset of meiosis promotes intergenomic pairing and reveals cryptic homologies in amphiploids. Poggio *et al*. (1990) and Naranjo *et al.* (1994) have studied the meiotic configuration of several *Zea* species and hybrids treated with colchicine. These workers have found that in *Z. mays* ssp. *mays* (2n = 20) 1 to 5 tetravalents are formed, demonstrating that cryptic homologies exist between the A and B genomes (described above), designated as Am and Bm for *Z. mays* ssp. *mays* (Figure 1). In *Z. diploperennis* (2n = 20) no quadrivalents were found, and no noticeable homologies between the Ad and Bd genomes were detected (Ad and Bd corresponding to *Z. diploperennis* genomes) (Figure 1). In *Z. perennis* (2n = 40) the number of quadrivalents increased to ten, revealing some cryptic homologies within the Ap genome and more within the Bp genome (Ap and Bp corresponding to *Z. perennis* genomes). The absence of octovalents demonstrated the lack of homology between Ap and Bp genomes (Figure 1). In *Z. diploperennis* x *Z. perennis* hybrids (2n = 30) the frequency of trivalents in cells not treated with colchicine was 25% while in colchicine-treated cells it was 90%, but hexavalents were not observed in this hybrid. These results show greater homologies between *Z. perennis* B genomes (Bp) and *Z. diploperennis* B genomes (Bd) than those detected in untreated material (Figure 1). In the hybrid *Z. mays* ssp. *mays x Z. perennis* (2n = 30) there was an increase in trivalent frequency, and quadrivalent and hexavalent formation was observed in 30% of the cells in agreement with the homologies detected between *Z. mays* ssp. *mays* genomes Am and Bm in colchicine-treated material (Figure 1). The formation of these hexavalents constitutes one of the most striking pieces of evidence supporting the hypothesis that maize is a cryptic tetraploid while *Z. perennis* is a cryptic octoploid (more details to be published later by Poggio and Naranjo).

Poggio *et al*. (1990) and Naranjo *et al*. (1994) showed that in species and hybrids of the genus *Zea*, colchicine treatment may disrupt a bivalent-promoting mechanism and reveal cryptic homologies, probably due to the presence of *Ph*-like gene (Pairing homologous) such as the *Ph1* gene which occur in *Triticum aestivum* (Feldman and Avivi, 1988).

It is interesting to note that molecular studies (Moore *et al.*, 1995) are in agreement with our cytogenetic findings and support the cryptic allotetraploid nature of maize.

Molecular cytogenetic analysis of species and hybrids

Genomic *in situ* hybridization (GISH) allows chromosomes from different parents or ancestors to be distinguished by means of differential hybridization of entire genomic probes (Bennett, 1995). Moreover, GISH has proved to be a very useful technique to demonstrate affinities among putative ancestors of *Z. mays* ssp. *mays* (Poggio *et al.*, 1998, 1999a,b).

By applying GISH to *Z. luxurians* we demonstrated that the DAPI positive bands located in all telomeric regions of this species did not hybridize either with *Z. perennis* or Z. *diploperennis* genomic probes, whereas the rest of the chomosome regions showed hybridization signals. *Z. luxurians* therefore has a repetitive sequence that can be used to identify its chromosomes by fluorescent staining techniques (Poggio *et al.*, 1999b).

We studied metaphase I in *Z. luxurians* x *Z. perennis* (2n = 30) hybrids, which (similar to the previous crosses) had a metaphase 1 chromosome complement of 5 trivalents + 5 bivalents + 5 univalents as the most frequent configuration (Figure 1). When fluorescent techniques were employed, the distinctive telomeres of Z. *luxurians* were found on the univalents but not on the bivalents (Poggio *et al*., 1999b), findings which show that the formation of bivalents and univalents is not a random event. In *Z. luxurians* x *Z. perennis* hybrids, bivalents result from pairing between chromosomes from the two genomes of Z. *perennis* with the exclusion of the corresponding chromosomes from the *Z. luxurians* genome (Figure 1). In this hybrid, bivalents from *Z. perennis* tend to be spatially separated and are very often observed forming an independent group of 5 bivalents. The GISH experiments confirm that genomes belonging to different species tend to occupy different domains in the nucleus, supporting the hypothesis that there is a tendency to genome separation.

Repetitive sequences can be used as species makers to differentiate chromosomes from different species. To gain further insight into the nature of these sequences, we present below the results of further hybridization experiments applied to different *Zea* species which we hope will contribute to resolving many of the questions relating to molecular affinities between our proposed genomes for the genus *Zea*.

MATERIAL AND METHODS

Plant material

The source of material for cytogenetical analysis was: *Zea perennis*: Mexico, Jalisco, Ciudad Guzmán, Leg. Dra. Prywer, cultivated at the "Instituto Fitotécnico Santa Catalina (IFSC)" since 1962; *Z. diploperennis*: Mexico, Jalisco, Sierra de Mananthan Occidental, 2 km from Las Joyas, Leg. Rafael Guzman and M.A. Guzman (No. 1120, Nov. 1980); *Z. luxurians*: Guadalajara, Mexico Cult. No. 2228 (IFSC) and *Z. mays* ssp. *mays* line (Santa Catalina 2), maintained in the greenhouse of the IFSC.

Materials analyzed by the Southern blotting method were: *Zea mays* ssp. *mays* (SC2 line and accession 13043, IFSC) and the following accessions provided by CIMMYT

(Centro International de Mejoramiento de Maíz y Trigo): Z. *luxurians* (9478), *Tripsacum dactyloides*, *Z. diploperennis* (933) and Z. *perennis* (8837).

Cytological analysis

For fluorescent *in situ* hybridization seeds were placed on Petri dishes on wet filter paper. Root tips were pretreated in 0.02 M 8-hydroxyquinoline and fixed in 3:1 (absolute alcohol:acetic acid). Fixed roots were washed in 0.01 M citric acid-sodium citrate buffer, pH 4.6, to remove fixative, transferred to an enzyme solution containing 2 ml of 2% cellulase (Onozuka R10) and 20% liquid pectinase (Sigma). The softened material was again washed in the above buffer solution. Finally, chromosomes were squashed onto slides in a drop of 45% acetic acid. Preparations showing well-spread cells were selected by phase contrast light microscopy. After removing the coverslips (by freezing the slides) the slides were air-dried.

Molecular analysis

DNA probes

The following probes were used for *in situ* hybridization: 1) a 180-bp repeat of the maize heterochromatic knob (Dennis and Peacock, 1984 - kindly provided by CSIRO Plant Industries), 2) the pTa 71 plasmid containing the 18S-5.8S-25S ribosomal sequences from *Triticum aestivum* (Gerlach and Bedbrook, 1979) and 3) genomic DNA isolated from adult leaves of the three species of the *Luxuriantes* Section (after Maniatis *et al*. (1982), with minor modifications). All probes were labeled by random priming with digoxigenin 11-dUTP (Boehringer, Mannheim, Germany) or by nick translation with biotin 14-dUTP (Bionick Labeling System, Gibco BRL).

Southern-blot hybridization

For this technique, 10 µg of DNA from each of the species listed in plant material was digested with *Hin*dIII, *Eco*RI, *Rsa*I and *Pst*I endonucleases. Fragments were electrophoretically separated on 0.8% agarose gels, blotted on nylon membranes and hybridized with digoxigenin labeled knob-specific probe (Maniatis *et al.*, 1982), with post-hybridization washes under high stringency conditions. Immunodetection of probes was carried out according to the manufacturer's instructions (Boehringer-Mannheim).

Fluorescent in situ hybridization

The technique, slightly modified, of Cuadrado and Jouve (1995) was used. Slide preparations were incubated in 100 µg/ml DNAse-free RNAse in 2x SSC for 1 h at 37°C in a humidified chamber and washed three times in 2x 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 2x 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, 0.1% (w/v) SDS and 0.3 mg/ml of salmon sperm DNA in 2x SSC, followed by adding 100 ng of labeled probe to 30 µl of hybridization mixture for each slide. In some cases, a blocking procedure (Bennett, 1995) was applied by adding unlabeled DNA from a different species and labeled total DNA probe from the same species in a proportion of 10:1, respectively. The hybridization mixture was denatured for 15 min at 75°C, loaded onto the slide preparation and covered with a plastic coverslip. The slides were placed on a thermocycler at 75°C for 7 min (denaturation), 10 min at 45°C and 10 min at 38°C. The slides were then incubated overnight at 37°C for hybridization.

Following hybridization, coverslips were carefully floated off by placing the slides in 2x SSC at 42°C for 3 min and then given a stringent wash in 20% formamide in 0.1x SSC at 42°C for 10 min. The slides were washed in 0.1x SSC at 42°C for 5 min; 2x SSC for 5 min at 42°C and transferred to detection buffer (4x SSC, 0.2% (v/v) Tween 20) for 5 min at 42°C and 1 h at room temperature.

To detect digoxigenin-labeled probes, slides were treated with sheep antidigoxigenin FITC (fluorescein isothyocyanate) (green fluorescence) while for biotin-labeled probes they were treated with streptavidine-Cy3 conjugate (red fluorescence) or avidin Texas red. Slides were then treated with 2.5% (w/v) BSA in detection buffer and incubated in a 1/40 solution of the corresponding antibody in detection buffer containing 2.5% BSA for 1 h at 37°C and washed 3 times in detection buffer for 10 min at room temperature.

Slides were counterstained with 1 µg/ml of 4'6-diamidino-2-phenylindole (DAPI) in McIlvaine's citrate buffer, pH = 7, for 10 min at room temperature and then mounted in anti-fade solution. Slides were examined with a Carl Zeiss Axiophot epifluorescence microscope with appropriate Carl Zeiss filters. Photographs were taken using Fuji color super G400 color print film.

RESULTS AND DISCUSSION

We performed several molecular hybridization experiments to obtain information about the DNA sequences which form the DAPI-positive telomeric regions of *Z. luxurians* chromosomes but which are not present in the other members of the *Zea* section. These experiments are described below.

In situ hybridization using the knob sequence as a probe for *Z. luxurians* chromosomes

Terminal heterochromatic blocks of *Z. luxurians* (ZL-THB) look like those of *Z. mays* ssp. *mays* (maize) when

observed using conventional C-banding techniques (Tito *et al.*, 1991) or by DAPI counterstaining (Figure 2A, C, and D). We therefore decided to assess the homology of the ZL-THB with respect to the 180-bp repeat (Dennis and Peacock, 1984) contained in maize heterochromatic knobs.

We performed an *in situ* hybridization experiment probing the knob sequence on mitotic chromosomes of *Z. luxurians* in which we observed that all DAPI-positive telomeric regions fluoresced (Figure 2B, D, F), indicating that the ZL-THB contain the 180-bp repeat.

Southern blot analysis using the knob sequence as a probe

Dennis and Peacock (1984) reported that the knob sequence is also the major component of heterochromatic knobs in the annual grass teosinte (*Z. mexicana*, Mexican teosinte, and *Z. luxurians*, Guatemala teosinte), as well as in *Z. diploperennis* and *Tripsacum.* We confirmed these findings by Southern blot analysis and also demonstrated that *Z. perennis* (or at least the accessions we used) lacks the knob sequence.

Genomic *in situ* hybridization of *Z. luxurians* chromosomes

As previously mentioned, GISH experiments had demonstrated that ZL-THB regions are not present on either *Z. perennis* or *Z. diploperennis* chromosomes (Poggio *et al.*, 1999b). The ZL-THB region should, therefore, be composed of more than one sequence, as the 180-bp repeat is not unique to Z. *luxurians* within its section, and in order to confirm this we performed other GISH experiments.

We carried out the hybridization procedure on mitotic chromosomes from *Z. luxurians* using labeled total genomic DNA from *Z. luxurians* as a probe, blocking with unlabeled DNA from *Z. perennis* (Figure 2H) or *Z. diploperennis* (Figure 2J). In both cases strong fluorescence was only present on telomeric regions having the same position as DAPI-positive bands (Poggio *et al.*, 1999b). Taking into account the observed homologies of the knob sequence to ZL-THB region this result was expected when using *Z. perennis* as a blocking agent. However, lack of fluorescence in the ZL-THB region should have been observed when GISH was performed using unlabeled DNA from *Z. diploperennis*, since this DNA should block the telomeric region because of its homology with the knob sequence. We have thus concluded that the ZL-THB region contains not only the 180-bp repeat, but also other tandem arrays unique *to Z. luxurians.* In fact, Figure 2J (blocked with *Z. diploperennis*) shows that fluorescent regions seem to be smaller and subterminal in most chromosomes compared to Figure 2E (blocked with *Z. perennis*), suggesting that the unique *Z. luxurians* sequence is probably located at the subterminal position.

Southern blot analysis using the knob sequence as a probe on *Z. luxurians* cleaved DNA

Previous work has shown that some endonucleases (including *Hin*dIII, *Eco*Rl, *Rsa*I and *Pst*I) cleave total genomic DNA from *Z. luxurians* in such a way that a ladder pattern can be visualized with ethidium bromide, but these endonucleases do not have recognition sites on the 180-bp repeat (Poggio *et al.*, 1999c).

However, when we performed Southern blot analysis using DNA from *Z. luxurians* digested with these endonucleases and hybridized with knob probe, we surprisingly observed that the probe hybridized producing a ladder pattern. The fact that the endonucleases employed do not have recognition sites on the 180-bp repeat suggests that in *Z. luxurians* another sequence with target sites for these enzymes could be interspersed with the knob repeat and be organized with them in a tandem array. These arrays of "knobs plus other sequences with target sites for endonucleases" are located on the ZL-THB regions as was demonstrated by using GISH (Figure 2F).

Z. mays ssp. *mays* and *Z. diploperennis* showed on Southern blot analysis a unique band of homology when hybridized with knob probe. Indeed, Phelps and Birchley (1997) stated that the 180-bp repeat seems to be the only sequence present in heterochromatic knobs of *Z. mays* ssp. *mays.* However, Ananiev *et al.* (1998) found a Tr-1 tandem repeat array up to 70 kb in length interspersed with stretches of 180-bp tandem arrays. A similar type of array could be present in *Z. luxurians.* Nevertheless, the interspersed array would be a different sequence because of the different patterns observed among the *Zea* species that were cleaved with the endonucleases and hybridized with the knob probe in our Southern blot analysis.

These observations indicate an important differentiation between the species of the genus *Zea.* A more detailed study on the organization of tandem arrays will perhaps provide a clue to determine the evolutionary relationships between these species, research that is currently in progress in our laboratories.

Genomic *in situ* hybridizations of *Z. mays* ssp. *mays* chromosomes

The heterochromatic knobs from *Zea mays* ssp. *mays* correspond to DAPI-positive bands on mitotic chromosomes of maize (Sumner, 1990). The distribution, number and size of knobs vary among different lines of maize. The GISH experiment using *Z. luxurians* as a probe for maize chromosomes showed stronger fluorescence in the knob regions than in the rest of the chromosome (Figure 2L). The same cell re-hybridized with *Z. diploperennis* as a probe produced strong fluorescence on the whole chromosome, including the knob regions (Figure 2M). Similar results were observed in other cells (Figure 2Q) suggesting that the affinity between *Z. diploperennis* and *Z. mays*

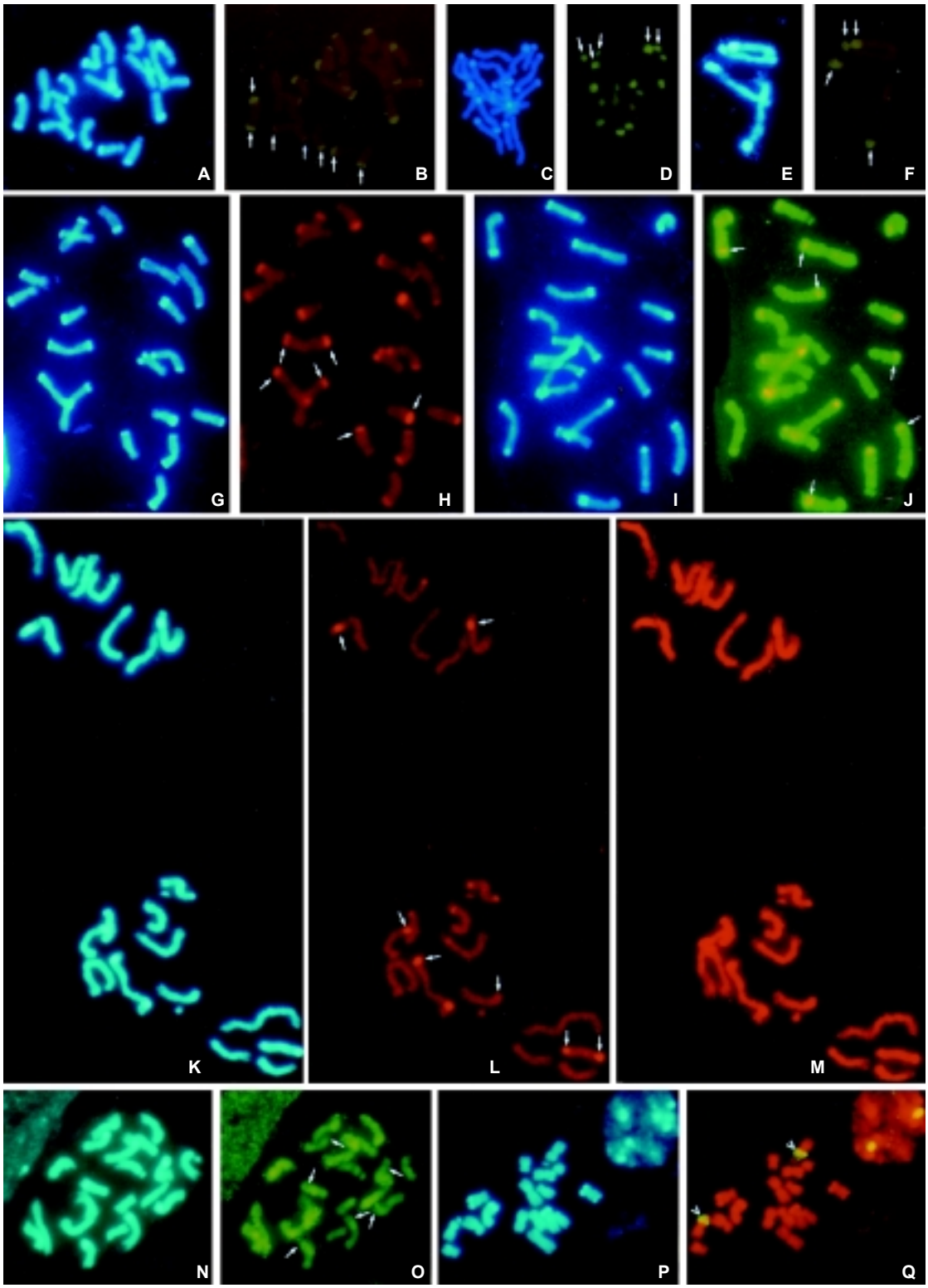

Figure 2 - A-J, Mitotic cells of *Z. luxurians*. **A**, Metaphase. **C**, Prophase. **A**, **C**, **E**, **G**, **I**, DAPI counterstaining. **B**, **D**, **F**, FISH using knob probe, detected with yellow green FITC (arrows indicate some of the telomeric regions with fluorescent regions coincident with DAPI + bands). **E**, Detail from four prophase chromosomes. **H**, GISH using DNA from *Z. luxurians* as a probe, detected with red Cy3, blocked with DNA from *Z. perennis* (arrows indicate some of the regions with strong fluorescence coincident with DAPI + bands). **J**, GISH using DNA from *Z. luxurians* as a probe detected with yellow green FITC, blocked with *Z. diploperennis* (arrows indicate some of the subtelomeric regions with fluorescence coincident with the DAPI + bands). **K-Q**, Mitotic cells from *Zea mays* ssp. *mays*. **K**, **N**, **P**, DAPI counterstaining. **L**, GISH using *Z. luxurians* DNA as a probe detected with avidin Texas red (arrows indicate some of the regions with strong fluorescence coincident with DAPI + bands). **M**, GISH using *Z. diploperennis* DNA as a probe detected with avidin Texas red. **O**, GISH using total DNA from *Z. perennis* as a probe detected with yellow green FITC (arrows show some of the regions of DAPI + bands exhibiting weaker fluorescence). **Q**, Double exposure photograph using total DNA from *Z. diploperennis* as a probe (red) and pTa 71 (yellow green, indicated with arrows).

ssp. *mays* is greater than that between *Z. luxurians* and *Z. mays* ssp. *mays*.

When total DNA of *Z. perennis* was probed on *Z. mays* ssp. *mays* chromosomes the results were in agreement with Southern blot analysis. As *Z. perennis* lacks the 180-bp repeat, weaker fluorescence was observed in the regions corresponding to heterochromatic knobs (Figure 2O). Interestingly, GISH analysis also revealed that the *Z. perennis* probe does not hybridize uniformly with *Z. mays* ssp. *mays* chromosomes.

This pattern of labeling could indicate that more than one species may have been involved in the origin of maize and that rearrangements between genomes could have occurred later, an idea which is in agreement with the proposed cryptic alloploid nature of maize.

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