Theor Appl Genet (1995) 90: 1094–1100

R. Aledo · R. Raz · A. Monfort · C. M. Vicient
P. Puigdomènech · J. A. Martínez-Izquierdo

Chromosome localization and characterization of a family of long interspersed repetitive DNA elements from the genus *Zea*

Received: 10 August 1994 / Accepted: 8 December 1994

Abstract This paper describes the characterization and chromosomal distribution of new long repetitive sequences present in all species of the genus *Zea*. These sequences constitute a family of moderately repetitive elements ranging approximately from 1350 to 1700 copies per haploid genome in modern maize (*Zea mays* ssp. *mays*) and teosinte (*Zea diploperennis*), respectively. The elements are long, probably larger than 9 kb, and they show a highly conserved internal organization among *Zea* subspecies and species. The elements are present in all maize chromosomes in an interspersed pattern of distribution, are absent from centromeric and pericentric heterochromatin, and with some clustering in the distal regions of chromosome arms.

Key words Genus *Zea* · Repeated DNA sequences · In situ hybridization · *Zea diploperennis* · Maize chromosome localization

Introduction

Dispersed repetitive sequences in mammalian genomes have been classified into two main groups according to their length (Singer 1982): short interspersed sequence elements (SINEs) or long interspersed sequence elements (LINEs). This classification can be generalized to non-mammalian eukaryotic genomes though it is true only in a broad sense because other dispersed repetitive elements, such as the viral superfamily and retrotransposons (Weiner et al. 1986; Moore et al. 1991), are not considered as either LINEs or SINEs. Dispersed repetitive sequences are usually moderately repeated in the genome and are thought to be transposable elements including transposons and retroposons (Schweizer et al. 1990). The latter are elements that spread via an RNA intermediate. If we consider the category of LINEs, they are usually a few kb long and in plants they vary from a few copies to more than 10 000 copies per haploid genome (reviewed by Grandbastien 1992). Most of these dispersed elements are randomly distributed in chromosomes, with some exceptions such as the HeT-A elements of *Drosophila* which are localized in telomeres and pericentric heterochromatin (Biessmann et al. 1992).

In a previous paper (Raz et al. 1991) we described the molecular characterization of a family of SINEs (ZEAR elements), with an average size of 253 bp, belonging to the genus *Zea*, and of moderately repeated sequences with about 2500 copies per haploid genome in both teosinte and maize. At that time it was not known whether the ZEAR elements were dispersed in the genome, though with some clustering, or if they were actually part of larger repetitive elements or LINEs (Raz et al. 1991). To determine the actual length of the repetitive elements, and to confirm their dispersion pattern, both Southern and chromosome in situ hybridization analysis are necessary.

Chromosome in situ hybridization has proven to be more difficult in plants than in animals. One of the main problems in plants is the presence of the rigid cell wall, which reduces the accessibility of the probe to the chromosomes (Ambros et al. 1986). This problem can be circumvented by using protoplasts as a source of chromosomes (Mouras et al. 1987).

The present study was planned in order to establish the type of repetitive elements previously cloned from teosinte (*Zea diploperennis*). We show that this family of moderately repeated elements is present in the other species of the genus *Zea*, including modern maize. Its localization in maize chromosomes is apparently random though with a preference for the distal parts of chromosome arms. The possible role of these long repetitive elements in the genome of *Zea* species and their usefulness as genetic markers for genome mapping or plant breeding, as well as in evolutionary studies, is discussed.

Communicated by I. Potrykus

R. Aledo · R. Raz · A. Monfort · C. M. Vicient · P. Puigdomènech
J. A. Martínez-Izquierdo (✉)
Departamento de Genética Molecular, CID-CSIC,
Jordi Girona Salgado 18-26, 08034 Barcelona, Spain

Materials and methods

Plant material, isolation of plant DNA and genomic clones

Teosinte, *Z. diploperennis* (2n=20), and the inbred line W64A of *Zea mays* ssp. *mays* (2n=20) were grown in our greenhouses. The original source of *Z. diploperennis* and the other plants used is described elsewhere (Raz et al. 1991). Plant DNA was prepared by the procedure of Dellaporta et al. (1983).

The isolation of genomic clones containing repetitive sequences from a lambda genomic library of *Z. diploperennis* is described by Raz et al. (1991).

Copy number estimation

Estimation of the copy number of the repetitive sequences by genomic reconstruction was done by slot-blot hybridizations with defined amounts of total genomic DNA from *Z. diploperennis* and maize w64A (250 ng and 50 ng) and dilutions (100 pg, 50 pg, 25 pg, 10 pg) of the different sequences used as probes. Slot-blotting was carried out with Zeta-probe (Bio Rad) under the conditions recommended by the supplier. Probes were labelled with α-^{32}P-dATP by random priming to a concentration of 2×10^6 cpm/ml. Hybridizations were done in phosphate buffer 0.25 M pH 7.2, 7% SDS, 1 mM EDTA at 65 °C, and afterwards the filters were washed in phosphate buffer 0.02 M pH 7.2, 1% SDS, 1 mM EDTA and exposed to X-ray films. The copy number was obtained by regression analysis of the values derived by tracing X-ray film bands using a densitometer. It was assumed that the haploid unreplicated genome contents of *Z. diploperennis* and W64A maize are 2.5×10^9 and 2.6×10^9 bp respectively (Laurie and Bennett 1985).

Southern hybridization

Southern blotting and hybridizations were performed essentially as described by Raz et al. (1991). ^{32}P-labelled probes were synthesized to a concentration of 10^8 cpm/µg DNA, using α-^{32}P-dATP as a radioactive precursor, either by nick translation (Raz et al. 1991) or by random priming (Sambrook et al. 1989).

Chromosome preparation

W64A maize chromosome metaphases and interphase nuclei were obtained from fixed protoplasts by a modification of the technique described by Ambros et al. (1986). Briefly, to accumulate cells in metaphase, 48-h-old root-tips were treated with 0.008 M hydroxyquinoline for 4 h at 17 °C or with a saturated solution of α-bromonaphthalene overnight at 4 °C. Root-tips were fixed with ethanol-acetic acid (3:1). To obtain a protoplast suspension, the root meristems were placed in 0.01 M citric acid/sodium citrate buffer (pH 5.2), and digested with a mixture of 2% cellulysin (Yakult) and 2% macerozyme (Yakult) for 2–3 h at 37 °C. The protoplast suspension, obtained after several steps of washing in citrate buffer and centrifugation, was subjected to cold fixation with ethanol-acetic acid (3:1). Protoplasts were dropped onto slides and these stored at –20 °C until use.

Synthesis of probes for in situ hybridization

Probes were labelled with digoxigenin-11-dUTP (Boehringer Mannheim) by nick-translation. Afterwards, probes were ligated and resuspended in hybridization mix (50% deionized formamide, 2×SSC, 10% dextran sulphate, 1% Denhardt's reagent, 0.1% SDS, 20 mM NaH_2PO_4, 20 mM Na_2HPO_4, pH 6.8, 100 µg/ml of sonicated salmon sperm DNA); the composition of SSC and Denhardt's reagent is described elsewhere (Sambrook et al. 1989). Probes were denatured for 10 min at 90°C prior to hybridization.

In situ hybridization

Slide preparations were incubated with 100 µg/ml of DNase-free RNase in 2×SSC. pH 7.0 for 1 h at 37 °C, rinsed twice in 2×SSC for 10 min at room temperature and dehydrated in an ethanol series (50%, 70%, 100%) for 10 min each and then air dried.

The slides were denatured in 70% deionized formamide, 2×SSC (pH 7.0) solution for 2 min at 70 °C and dehydrated in a cold ethanol series and air dried. To each slide 10 µl of the hybridization solution, containing the denatured probe, were added. Coverslips were applied over the hybridization solution. After overnight hybridization at 37 °C in a moist chamber, the slides were then washed at 42 °C twice in 50% deionized formamide, 2×SSC (pH 7.0) and twice in 2×SSC (pH 7.0) for 10 min each.

Signal detection

After post-hybridization washes, the slides were transferred to PBT buffer [0.15 M sodium chloride, 0.01 M phosphate buffer (pH 7.4), 0.1% (v/v) Tween-20, including 0.12% bovine serum albumin (BSA)] for 10 min. The detection of the digoxigenin incorporated into the hybridized probes was accomplished by means of sheep anti-digoxigenin FAB fragments conjugated to peroxidase for colorimetric methods or by fluorescence using FITC (fluorescein isothiocyanate)-conjugated sheep anti-digoxigenin (Boehringer Mannheim).

Peroxidase detection: the slides were incubated with the antibody conjugated to peroxidase (dilution 1:100) in buffer 1 [150 mM NaCl, 100 mM Tris-HCl (pH 7.5), 0.1% (v/v) Tween-20, including 1.2% BSA] for 45 min at 37 °C under a glass coverslip in a moist chamber, followed by a 10-min washing in 2×SSC (pH 7.0) and several rinses in PBT buffer. The colour was developed by incubation with 0.006% DAB (diaminobenzidine tetrahydrochloride, Sigma) in 0.0012% (v/v) hydrogen peroxide solution for 10 min in the dark. After washing in distilled water the DAB/hydrogen peroxide signal was amplified by silver precipitation as described by Burns et al. (1985). Then, the slides were washed in distilled water for 15 min and twice with 0.17 M acetic acid for 15 min and stained with 2% Giemsa in phosphate buffer for 4 min.

Fluorescence detection: the slides were incubated in PBT buffer with the anti-digoxigenin FITC antibody (dilution 1:8) for 1 h at room temperature, rinsed in PBT and counter-stained for 4 min with propidium iodide (final concentration 1 µg/ml). A drop of antifade solution was added and the slides were mounted with coverslips (Johnson and Araujo Nogueira 1981).

Results

Origin and structure of genomic clones containing repetitive elements

Screenings of a *Z. diploperennis* lambda genomic library resulted in the isolation of several genomic clones containing short repeated sequences. These sequences, flanked by *Hae*III sites and called ZEAR, were considered as belonging to a family of moderate short repetitive dispersed elements specific to the genus *Zea* (Raz et al. 1991). In order to determine whether the sequences flanking the ZEAR elements are also repetitive, i.e. if they constitute a part of larger elements, a map showing similar sequences between clones was constructed. For this purpose one of the longest clones was used as a source of probes by digestion with *Sac*I, resulting in four probes (Fig. 1) which were used to determine the location of similar sequences in the other four genomic clones. The results in terms of hybridizing bands are schematically presented in Fig. 1. Except for

probe SS1, which hybridized only with its own clone 7 and can not be considered as a part of the repetitive element, the other three hybridized with distinct digested bands. The fact that the organization of fragments in the various clones is not exactly the same can be explained by restriction-site polymorphism, as can be seen by the different enzyme targets flanking the fragments as shown in Fig. 1. In the case of clones 4 and 7 the total length of the three hybridizing bands is around 9 kb. Therefore, given the limitations of this approach, the size of the repetitive element is roughly 9 kb (Fig. 1). Although, this is large for a repetitive element, it is not exceptional, because even larger repetitive elements have been described in plants (Grandbastien 1992) and other eukaryotes. We tentatively call these repetitive DNA elements ZLRS (*Zea* long repetitive sequences).

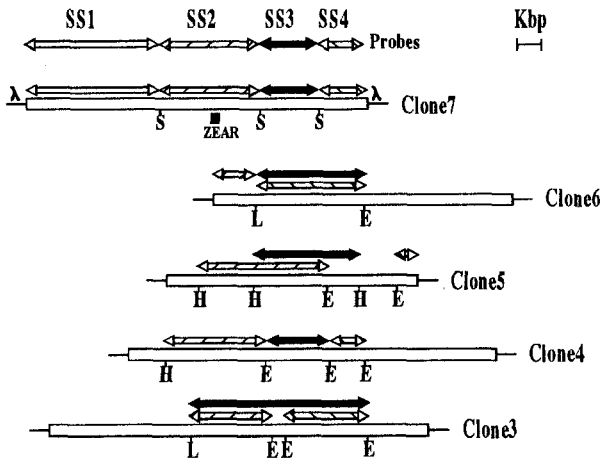

Fig. 1 Southern hybridization map of ZLRS-containing *Z. diploperennis* genomic clones. Maps of restriction sites (only relevant ones are represented) in five ZEAR-containing lambda phage teosinte genomic clones (*white open bars*) are shown. On the top of the figure are represented the four different *Sac*I fragments of clone ZLRS 7 (*double arrows*) that were used as probes for Southern hybridization of several digests of the five clones. Above every clone are represented, by the *same double arrows* as the probes depicted on the top, the minimum fragments showing significant hybridization which each probe. ZEAR-element location is indicated only below clone 7. *E*, *Eco*RI; *H*, *Hin*dIII; *L*, *Sal*I; *S*, *Sac*I

Abundance and species distribution of ZLRS

The copy number of ZLRS in the genomes of teosinte and W64A maize was assessed by using the complete insert of teosinte genomic clone 7, or different fragments of it (Table 1), as probes for slot-blot hybridization.

Except for probe SS1, which was not considered part of the ZLRS (see above and Fig. 1), and which gave a much lower number than the other three, these probes (SS2, SS3 and SS4) gave rise to comparable numbers, with a tendency to higher numbers with larger probes (Table 1) which can be explained by the higher stability of the hybridizing duplex. In summary, the copy number of ZLRS for the haploid genome of teosinte is around 1700. The number of copies obtained for ZLRS in maize (around 1350), although lower, is comparable to that in teosinte. This difference may be mostly explained by species-specific variation during hybridization, since the probes that come from teosinte should have a higher similarity with homologous teosinte, than with heterologous maize, DNA.

Considering a minimum size for ZLRS of 9 kb and a copy number of 1700, this family of ZLRS represents about 0.61% of the *Z. diploperennis* genome with a similar value for maize.

Due to the fact that ZLRS are present in two species of the genus *Zea*, we decided to investigate if these repetitive sequences are also present in the other *Zea* species and in close genera. To this end genomic DNA from the different species was digested with a frequent-cutting enzyme (*Hae*III) and analyzed by Southern hybridization. Five different probes were used individually: the full insert of clone 7 and the four fragments obtained after digestion with *Sac*I, which are shown in Fig. 1. SS1 hybridized with only one band after a much-longer film exposure than was needed to label bands with the other probes (data not shown), confirming what we previously knew, hamely that SS1 did not belong to the repetitive element. The other four probes gave the same pattern of hybridizing bands in all of the *Zea* species, with slight or no variation (Fig. 2). Most of the differences were due to band intensities, particularly in the case of *Z. perennis*, the only tetraploid species of the genus. Whereas probes SS2 and SS3 lit up discrete bands (Fig. 2b, c), SS4 and of course the full insert gave an additional smear hybridization (Fig. 2a, d). These results can be explained in general by a high conservation of the internal *Hae*III sites, and therefore of the different copies of ZLRS. In particular, the smear hybridization seen with the SS4 probe in the high-molecular-weight DNA zone (Fig. 2d) can be due either to the absence of *Hae*III sites in the SS4 region of ZLRS or because this region spans the end of ZLRS with the flanking *Hae*III sites being less conserved.

Table 1 Copy number of ZLRS based on slot-blot hybridization

Probes[a]		Copy number[b]	
Name	Size (bp)	*Z. diploperennis*	Maize W64A
Clone 7	14500	1750	1150
SS1	5700	100	145
SS2	4300	2300	1850
SS3	2500	1650	1250
SS4	2000	1100	1150

[a] The probes used are described in the legend of Fig. 1
[b] Copy numbers per haploid genome were obtained as described in Materials and methods. Final numbers are the average of at least two experiments

No hybridization bands can be observed in the case of *Tripsacum dactiloides*, the genus closest to *Zea*, or *Saccharum officinale*, which belongs to the same tribe as *Zea* (Mangelsdorf 1986).

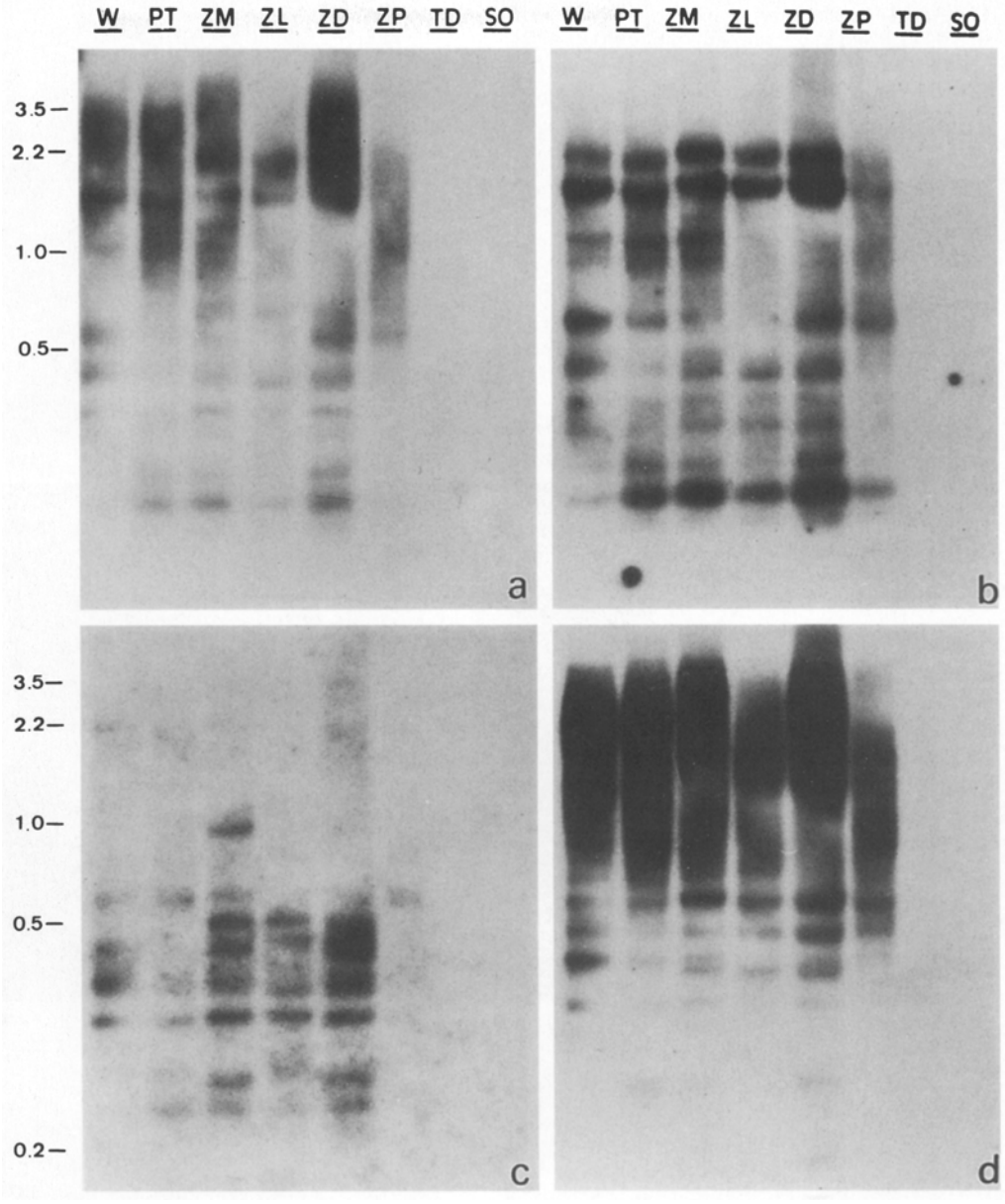

Fig. 2a–d The presence of ZLRS in *Zea* species revealed by Southern-hybridization analysis of genomic DNA. Ten micrograms of each DNA were digested with *Hae*III and loaded as follow: *Z. mays* ssp. *mays* W64A inbred line (*W*) and Palomero Toluqueño race (*PT*), *Z. mays* ssp. *mexicana* (*ZM*), *Z. luxurians* (*ZL*), *Z. diploperennis* (*ZD*), *Z. perennis* (*ZP*), *Tripsacum dactiloides* (*TD*), and *Saccharum officinale* (*SO*). The same filter was successively hybridized with the probes described in Table 1: **a** Clone 7 insert, **b** SS2, **c** SS3 and **d** SS4. Hybridized filters were exposed to Agfa Curix RP2 film at –70 °C with an intensifying screen for 7 h

Chromosome localization of ZLRS

The results of in situ hybridizations of teosinte genomic clone 7 to maize chromosomes are shown in Fig. 3. The technique was set up using a rDNA probe from wheat (Gerlach and Bedbrook 1979) as an in situ hybridization control probe. On one hand, this probe should provide, on one hand, an easy way to detect if the hybridization works, because ribosomal RNA genes are located on maize chromosome 6p in tandem arrays of several thousand copies per haploid genome (Phillips et al. 1979); on the other hand, the precise chromosomal localization of rRNA genes should also allow a discrimination between specific and background hybridization and between tandem and dispersed repeats. Figure 3c shows two maize interphase nuclei with two clear fluorescent sites per nucleus as result of hybridization with the rDNA probe. These sites are precisely defined at the NOR region of the 6p chromosomes in maize metaphase preparations (Fig. 3d). In contrast, the hybridization of ZLRS to maize chromosomes in interphase nuclei showed numerous fluorescent sites (Fig. 3b). A more clear picture appears when the hybridization of ZLRS was analyzed in metaphase chromosomes as presented in the karyotype of Fig. 3a. The hybridization sites, detected by the peroxidase reaction and silver amplification, are present in all the chromosomes in a dispersed manner, which is a typical distribution for interspersed repetitive elements. None of the hybridization signals are located in the centromeric regions, but some are present in the interstitial regions or in the distal zone of chromosome arms. Most of the repeated DNA characterized to-date has been found to reside in heterochromatic regions (Rayburn and Gill 1987). In maize the heterochromatin is distributed in the centromeric regions, the nucleolus organizer region,

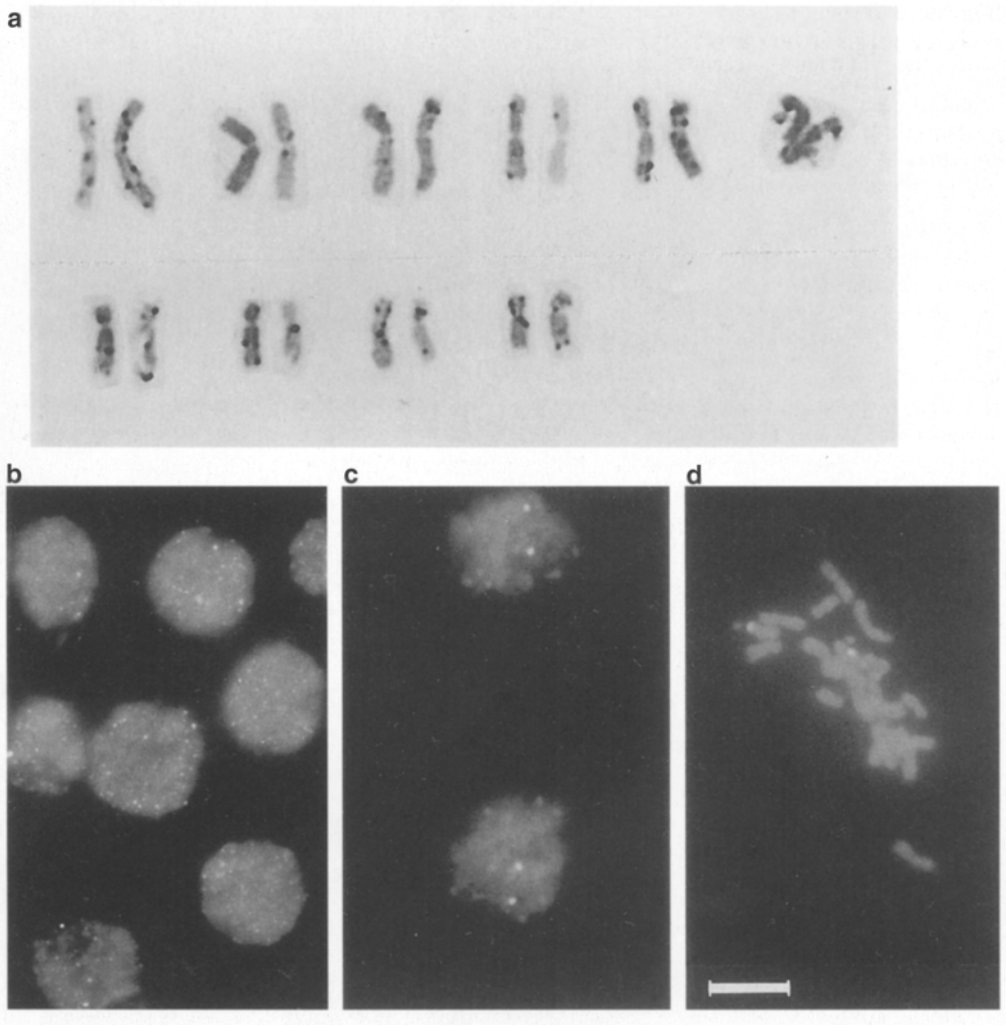

Fig. 3a–d In situ hybridization of ZLRS to maize chromosomes. Metaphase chromosomes **a** and **d** or interphase nuclei **b** and **c**, hybridized with teosinte genomic clone 7 in λCharon35 at 20 ng/μl in **a** and **b** or a wheat pTA71 rDNA probe (Gerlach and Bedbrook 1979) at 2 ng/μl in **c** and **d**. Probes were labelled as described in Materials and methods. **a** The ten pairs of chromosomes of one representative nucleus were arranged according to their size in descending order from left to lower right. The hybridized probe was detected by anti-DIG antibodies conjugated to peroxidase and silver amplified. **b**, **c** and **d** The hybridized probes were detected by anti-DIG antibodies conjugated to FITC. The bar in **d** represents 1 μ

telomeres and knobs, many of the latter being at the distal region of chromosome long arms (Pryor et al. 1980). Giemsa staining applied to plant chromosomes to identify heterochromatic C-bands also revealed knob heterochromatin in the case of maize, which is variable depending on the line (Hadlaczky and Kálmán 1975). Giemsa staining of W64A maize chromosomes showed most of the C-bands in the distal parts of chromosomes (data not shown). The numerous hybridization sites to ZLRS probes observed on the more distal regions of maize chromosomes (Fig. 3a) could therefore be associated with regions stained with Giemsa corresponding to knobs or telomeres.

Discussion

In this paper we describe the chromosomal localization of a previously undiscovered family of long repetitive elements specific to the genus *Zea*. These elements, named ZLRS, were found in independent clones of a genomic library of *Z. diploperennis* and were initially thought to constitute a moderately repetitive family of SINEs (Raz et al. 1991). Using consecutive restriction fragments of one ZLRS genomic clone as probes, we have through Southern hybridization experiments analyzing the other ZLRS-containing clones (Fig. 1) and slot-blot hybridizations (Table 1), demonstrated unambiguously that ZLRS are long repetitive elements, probably larger than 9 kb. This figure is not exceptionally high, because other repetitive elements from plants (Grandbastien et al. 1992; Manninen and Schulman 1993) and other eukaryotes (Biessmann et al. 1992; Springer et al. 1993) have similar or larger sizes.

Experiments on *Z. diploperennis* genomic reconstruction by slot-blot hybridization resulted in a copy number for ZLRS of 1700, consistent with a family of moderately repetitive elements. The extension of these experiments to the most important species of the genus *Zea*, modern maize, resulted in a similar, although lower, figure of 1350 copies. This difference can be explained by a lower degree of hybridization of the probe due to species divergence of ZLRS between teosinte and maize. A comparable conservation in the number of copies is also observed in the pattern of internal organization reflected by the genomic

Southerns shown in Fig. 2. The three different probes used revealed three different patterns of bands which in every case is essentially the same for all species of the genus *Zea*. The lowest intensity of bands observed in the case of *Z. perennis*, and previously noted also with ZEAR sequences (Raz et al. 1991), reflects the smallest number of copies in this species. This can be explained by the lowest heterochromatin and DNA content per basic genome for *Z. perennis* among *Zea* species (Tito et al. 1991). ZLRS are not present in the genera closest to *Zea*, *Tripsacum* and *Saccharum*, and they can therefore be considered specific to the genus *Zea*. The conservation of ZLRS internal organization patterns and the consistency in copy number point to either a recent amplification of these sequences, or alternatively to an old amplification coupled either with selection against variation or else with homogenization processes operating on the ZLRS. In the latter case the amplification would have occurred after the divergence of *Zea* species. The only sequence data available, corresponding to the teosinte ZEAR sequences mentioned before as part of ZLRS and analyzed elsewhere (Raz et al. 1991), showed a distinct degree of divergence inside each sequence and a global similarity among them of 70–86%, suggesting an old amplification coupled with selective constraint on some parts of the ZLRS, as for example the *Hae*III sites used in the Southern of Fig. 2.

The lack of G-bands in plant chromosomes, and which in mammal chromosomes is correlated with the presence of the LINE type of dispersed repetitive sequences (Schweizer et al. 1990), coupled with the fact that the heterochromatin stained in plants by Giemsa (C-bands) corresponds to highly repeated sequences, usually tandem arrays (Tito et al. 1991), leaves in situ hybridization as the only technique available for mapping dispersed repetitive sequences in plant chromosomes. The distribution of ZLRS hybridization signals over all the 20 chromosomes of maize reveals the dispersed nature of these sequences. However, the distribution of hybridization signals is not random, being absent from pericentric chromatin and more abundant in the distal zones of chromosomes (Fig. 3), possibly comprising part of the knobs or telomeres. This is in contrast to the irregular distribution of other long repetitive elements in plants (Sentry and Smyth 1985; Sonina et al. 1989). The fact that the number of hybridization spots (around 60) and the copy number of ZLRS were different can be explained, at least for the largest spots, by the contribution of several ZLRS hybridizing sequences located not very far away from each other to one spot in the photograph. A comparable bias to clustering has been reported for some long dispersed sequences, such as LINEs in humans (Chen and Manuelidis 1989) and retrotransposons in yeast (Bushman 1993), as well as for short moderately repetitive elements in rice (Wu et al. 1991).

We do not have direct evidence that ZLRS form part of the distal C-bands observed with Giemsa staining of W64A maize chromosomes which could correspond to heterochromatin in knobs and telomeres. Indeed the heterochromatin in knobs and telomeric regions is mainly constituted in cereal chromosomes by tandemly arrayed short highly repetitive sequences (Bedbrook et al. 1980; Jones and Flavell 1982; Dennis and Peacock 1984). In contrast, *Drosophila* telomeres are composed of very long repeated sequences (HeT-A) (Biessmann et al. 1992). Whether ZLRS, or at least those ZLRS present in the distal parts of chromosomes, are associated with euchromatin or with knob or telomere heterochromatin, and whether or not the ZLRS-rich chromosome zones, like human LINEs (Chen and Manuelidis 1989), are stained with Giemsa, are questions that remain to be answered.

Technical problems encountered in the accessibility of probes to plant chromosomes, for instance their higher chromatin condensation compared to animals and the surprisingly lower number of cloned dispersed repeats in maize, may have hampered the mapping of these repeats in maize chromosomes. To our knowledge, this is the first time that the in situ hybridization chromosome mapping of long interspersed repetitive elements in maize has been reported. The current programs on eukaryotic genome sequencing, and the need of suitable markers for genetic analysis by conventional RFLP or PCR-based analysis, make the finding of new genetic markers of increasing interest, especially dispersed repeated sequences like ZLRS because several loci can be scored simultaneously (Lee et al. 1990). ZLRS should also be valuable tools for taxonomic and evolutionary studies. Although it is still premature to propose a function for ZLRS, their number and their dispersion in the *Zea* genome suggest that these elements may be, or may have been, capable of movement and amplification. Such a mode of action is typical of transposable retroelements.

Acknowledgements We thank the Structure and Mutagenesis laboratory personnel, URA 620 CNRS, Institut Curie, Paris, for their technical advice in the chromosomal work. We are indebted to Dr. Stephen Jackson for carefully revising the manuscript. This work has been supported by a grant from DGPC (BIO 91/0129) to J.A.M.I. R.A. was the recipient of a postdoctoral reincorporation fellowship (MEC). R.R. was the recipient of a PFPI fellowship (MEC). A.M. was the recipient of a postdoctoral reincorporation fellowship (CIRIT). C.M.V. is the recipient of a FPI fellowship (CIRIT).

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