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Organization of a dispersed repeated DNA element in the *Zamia* genome

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Abstract

Occurrence and genomic organization of dispersed elements containing ZpS1 satellite repeats have been investigated in a wide representation of species of the old plant genus *Zamia* (*Zamiaceae*, *Cycadales*). In *Z. paucijuga*, the ZpS1 repeat is organized as long satellite DNA arrays and as short arrays inserted into AT-rich dispersed elements. A comparative study by Southern analysis shows that these unusual dispersed elements containing the ZpS1 repeat are present with different organizations in all investigated *Zamia* species. In some species these elements are present with a low copy number, while in other species secondary amplification events, involving specific sequence clusters, appear to have generated characteristic dispersed elements in a high copy number. Among *Zamia* species, several groups share similar restriction patterns, as the *Zamia loddigesii* complex and the Caribbean species suggesting a general correlation between organization and genomic representation of the dispersed repeated sequence and the pattern of phyletic relationships in the genus. However, the finding of different patterns also among closely related species suggests a complex history of amplifications and losses of these dispersed repetitive elements that cannot be always easily traced through the phylogenetic reconstruction of this ancient plant group.

Additional key words: cycads, dispersed sequences, genome evolution, repetitive DNA.

Introduction

Repetitive DNA sequences represent a large proportion of higher plant genomes and are implicated as a major contributor to variation in DNA content among organisms of similar complexity (Harding *et al.* 1992, Charlesworth *et al.* 1994, Kubis *et al.* 1998, Sharma and Raina 2005). DNA content varies largely among plant species and, comparing to angiosperm, gymnosperms typically have larger genomes: their modal C-value (15.8 pg) is over 20 times greater than in angiosperms (Leitch and Bennett 2002). Large C-values (≥ 14.0 pg) is even typical in cycads (mean 1C = 14.7 pg), regarded as the basal group of gymnosperms (Ohri and Khoshoo 1986). Investigations of genome organization in these old plant groups are thus important to our understanding of the evolution of plant genome. At the same time, the study of the physical organization of repetitive DNA sequences and of the degree of conservation and/or divergence of these sequences among related species may represent a way to gain knowledge of the evolution of and within a species group (Bedbrook *et al.* 1980, Bachmann and Sperlich 1993, Capriglione *et al.* 1998). In fact, repetitive DNA not only represents a dynamic and fast evolving component of the eukaryotic genome, but may also show conservation within taxonomic groups, thus providing evidence for phylo-genetic relationships (King *et al.* 1995, Pich *et al.* 1996, Alix *et al.* 1998, Kubis *et al.* 1998, Macas *et al.* 2006).

Cycads are an archaic and isolated group of seed

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Abbreviations: DAPI - 4,6-diamidino-2-phenylindole; DIG - digoxigenin; PCR - polymerase chain reaction.
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plants consisting of three families (Johnson 1959, Norstog and Nicholls 1997, Stevenson 1990), 11 genera and over 150 species (Stevenson 1992). The genus *Zamia* (*Zamiaceae*) has the widest ecological and morphological diversity among all cycad genera, ranging from arborescent species to species with small, subterranean stems, to epiphytic taxa (Stevenson *et al.* 1995, Norstog and Nicholls 1997). Fossil and phylogenetic evidences indicate a derivate position for this genus among the neotropical cycads (Norstog and Nicholls 1997). *Zamia* is also unique among cycads in showing interspecific and intraspecific karyotype variation (Marchant 1968, Norstog 1980, Moretti 1990a,b). Occurrence of Robertsonian changes, such as centric fission of metacentric chromosome or centric fusion of telocentric chromosomes, has been suggested to explain the causes of the high chromosome variability reported in *Zamia* (Norstog 1980, Moretti and Sabato 1984, Schutzman *et al.* 1988, Moretti 1990b, Moretti *et al.* 1991).

Repetitive elements may show different organization in the genome, typically ranging from a more or less even dispersion over genome (dispersed elements) to localization in few large arrays (satellite DNA) (Kubis *et al.* 1998).

A family of repeated sequences with an intermediate and unusual distribution pattern has been recently characterized in the genome of *Zamia paucijuga* (Cafasso *et al.* 2003). In this species, a 320 bp-long repeat sequence named ZpS1 (GeneBank Accession number: AJ315634), is organized both as typical satellite DNA arrays located in subtelomeric position of most chromosomes and in small dispersed repeated elements of 1 - 6 repeats which are flanked by AT-rich lateral arms (GeneBank Accession numbers: AJ315635, AJ416334). While the ZpS1 satellite DNA arrays are recognizable after digestion with the restriction endonucleases *Apa*I and *Eco*RV, the dispersed repeated elements are recognizable after digestion with the restriction endonuclease *Eco*RI which digests the AT-rich flanking elements, but not the ZpS1 satellite arrays (Fig. 1). These unusual dispersed repeated elements have been likely originated by a rearrangement of pre-existing satellite DNA repeats that have become inserted into an AT-rich sequence by site-specific recombination during the long life history of this ancient plant group (Cafasso *et al.* 2003).

Preliminary analysis indicated that these dispersed repeated elements are present in other *Zamia* species (Cafasso *et al.* 2003). To investigate the occurrence, representation and genomic organization of these dispersed elements within the genus *Zamia*, Southern blot and PCR experiments has been carried out on genomic DNAs of several *Zamia* species representative of all major clades recognized in the genus (Caputo *et al.* 2004).

Specifically, we asked how widespread (and consequently how old) these dispersed repeated elements are among members of genus *Zamia* and which rearrangements occurred in their organization in the different phyletic lineages of this ancient plant group.

Materials and methods

Synthesis of digoxigenin-labelled DNA probes: Digoxigenin (DIG)-labeled probes for hybridization experiments were obtained by PCR amplification by adding 11-dUTP in the polymerase chain reaction (PCR DIG probe synthesis kit, *Boehringer*, Mannheim, Germany). Probes of ZpS1 repeat were amplified from clones obtained from genomic DNA (Cafasso *et al.* 2003) using the universal M13 forward and reverse primers. Two other probes were obtained from genomic DNA with the primer pairs Z1-Z5 and Z4-Z8 (Cafasso *et al.* 2003). The first primer pair selectively amplifies the AT-rich element flanking the ZpS1 dispersed repeats, while the probe obtained with the second primer pair is homologous to both ZpS1 repeat and AT-rich element (Fig. 1; Cafasso *et al.* 2003). Unincorporated nucleotides were removed from probes by *Microcon 100* column filtration (*Amicon*, Millipore, Bedford, USA).

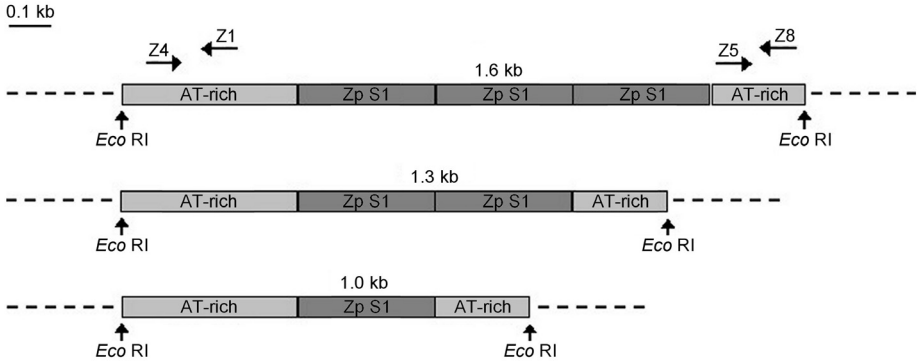

Fig. 1. Diagram illustrating the structure of the dispersed repeated elements containing one (1.0 kb long), two (1.3 kb long) or three (1.6 kb long) ZpS1 repeats, respectively. Restriction sites for *Eco*RI enzyme and position and orientation of the Z1-Z5 and Z4-Z8 primer pairs are indicated by *arrows*. *Dark blocks* correspond to the ZpS1 satellite repeat and *bright blocks* correspond to the AT-rich flanking elements.

Southern blot analysis: Genomic DNA from *Zamia* species was isolated from fresh or silica gel-dried leaf material as described by Doyle and Doyle (1987). After treatment with RNAse, the DNA amount was estimated spectrophotometrically. All plant material came from the botanical collections of the Naples Botanical Garden and the New York Botanical Garden, or has been field collected by the authors (Table 1).

The same exact amount of genomic DNA (500 ng) of different *Zamia* species (Table 1) was digested overnight at 37 °C with an excess of *Eco*RI or *Apa*I restriction endonucleases [3 - 5 U μg^{-1}(DNA)] to avoid partial digestion, electrophoresed in a 1.3 % agarose gel, and transferred and fixed to a nylon membrane according to standard procedures (Sambrook *et al.* 1989). Filters were hybridized at 65 °C with the DIG-labeled probe amplified with primer pair Z4-Z8 (Fig. 1), and washed at same temperature in 0.1 % SDS, 0.1× SSC (stringent conditions). The DIG detection kit was used to detect hybrid bands, following the manufacturer's instructions.

Flow cytometry analyses: Estimation of relative genome content was performed for all species for which fresh leaf material was available *(i.e.* all *Zamia* species, but *Z. manicata*, present in the collection of Naples Botanical Garden, see Table 1). Flow cytometry is based on the measure of fluorescence emission by light-excited, stained interphase nuclei (Uberall *et al.* 2004, Dolezel

Table 1. List of examined *Zamia* species with authors, geographic origins of samples, places of cultivation (NBG - Naples Botanical Garden; NYBG - New York Botanical Garden; FC - field collected) and accession numbers.

Zamia species	Geographic origins	Cultivation	Accession No.
Z. neurophyllidia Stevenson	Panama	NYBG	Z1
Z.acuminata Oerested ex Dyer	Panama	NYBG	Z2
Z. tuerckeimii Smith	Guatemala	NYBG	Z3
Z. ipetiensis Stevenson	Panama	NYBG	Z4
Z. cremnophila Vovides, Schutzman, Dehgan	Tabasco (Mex)	FC	Z5
Z. portoricensis Urban	Puerto Rico	NBG	Z6
Z. pumila Linnaeus	Dominican Republic	NBG	Z7
Z. purpurea Vovides, Rees, Vazquez Torres	Oaxaca (Mex)	FC	Z8
Z. muricata Willdenow	Colombia	NYBG	Z9
Z. wallisii Braun	Colombia	NBG	Z10
Z. skinneri Warszewicz ex Dietrich	Panama	FC	Z11
Z. inermis Vovides, Rees, Vazquez Torres	Veracruz (Mex)	NBG	Z12
Z. fisheri Miquel	San Luis Potosì (Mex)	NBG	Z13
Z. vazquezii Stevenson, Sabato, Moretti	San Luis Potosì (Mex)	NBG	Z14
Z. soconuscensis Schutzman, Vovides, Dehgan	Chiapas (Mex)	NBG	Z15
Z. poeppigiana Martius, Eichler	Ecuador	FC	Z16
Z. amplifolia Hort ex Masters	Colombia	NBG	Z17
Z. standley Schutzman	Honduras	FC	Z18
Z. roezlii Regel	Colombia	FC	Z19
Z. lecointei Ducke	Brasile	NYBG	Z20
Z. manicata Linden ex Regel	Colombia	NBG	Z21
Z. chigua Seemann	Colombia	FC	Z22
Z. sylvatica Chamberlain	Oaxaca (Mex)	NBG	Z23
Z. splendens Schutzman	Chiapas (Mex)	NBG	Z24
Z. integrifolia Linnaeus fil. in Aiton	Cuba	NBG	Z25
Z. picta Dyer	Guatemala	FC	Z26
Z. spartea De Candolle	Oaxaca (Mex)	NBG	Z27
Z. boliviana (Brogniart) A. De Candolle	Bolivia	FC	Z28
Z. loddigesii Miquel	Veracruz (Mex)	FC	Z29
Z. furfuracea Linnaeus fil.	Veracruz (Mex)	NBG	Z30
Z. paucijuga Wieland	Oaxaca (Mex)	FC	Z31
Z. paucijuga Wieland	Oaxaca (Mex)	FC	Z32
Z. paucijuga Wieland	Michoacan (Mex)	NBG	Z33
Z. paucijuga Wieland	Nayarit (Mex)	NBG	Z34
Z. paucijuga Wieland	Nayarit (Mex)	NBG	Z35
Z. paucijuga Wieland	Jalisco (Mex)	NBG	Z36
Z. polymorpha Stevenson, Moretti, Vàzquez Torres	Campeche (Mex)	FC	Z37
Z. polymorpha Stevenson, Moretti, Vàzquez Torres	Campeche (Mex)	FC	Z38
Z. polymorpha Stevenson, Moretti, Vàzquez Torres	Quintana Roo (Mex)	NBG	Z39
Z. polymorpha Stevenson, Moretti, Vàzquez Torres	Belice	FC	Z40
Z. polymorpha Stevenson, Moretti, Vàzquez Torres	Belice	NBG	Z41
Z. polymorpha Stevenson, Moretti, Vàzquez Torres	Belice	NBG	Z42

and Bartos 2005, Kovářová *et al.* 2007, Yang *et al*. 2008). The latter were released by chopping up fresh leaf fragments in Petri dishes using a razor blade, and they were stained with 4,6-diamidino-2-phenylindole (DAPI), which has the property of binding specifically to the chromosomes. The DNA-DAPI complexes of each species were then successively excited by the laser beam, which in turn produced specific wavelengths emission patterns depending on the DNA contents of the interphase nuclei. This emitted light was picked up by detectors, and produced DNA histograms that allowed comparing the patterns obtained among samples and inferring their relative genome content. As controls for the calibration of the flow cytometer (see also the procedure detailed by Aron *et al.* 2003) we used an accession of *Zamia angustifolia*. The genome content (1C = 12.05) of this species was previously determined by Ohri and Khoshoo (1986).

Quantitative dot blot analysis: The amount of the repeated DNA sequences in the *Zamia* species, for which sufficient DNA was available, was estimated by quantitative dot blot analysis using varying amounts of genomic DNA. A linearized plasmid DNA containing an insert of dispersed repeated sequence was used as a standard. After tissue extraction, the genomic DNA of the species used for the dot blot analysis was treated with RNAse, precipitated twice with one volume of 88 % (v/v) isopropyl alcohol + 0.2 M potassium acetate and resuspended in TE buffer. The DNA amount was estimated spectrophotometrically and then diluted at concentrations multiple of one another. Carrier sonicated salmon sperm DNA was added to each sample up to a final amount of 0.5 µg DNA per sample to avoid errors due to differences in the hybridization kinetics. Dot blot was hybridized with a probe of the ZpS1 satellite DNA. The digoxigenin-labelling and hybridization detection procedures were the same as those used for Southern blot analysis.

PCR reaction: Dispersed sequences were amplified by PCR using 10 ng of genomic DNA as template and 2.5 pmol of the primers in a final volume of 0.05 cm^3. The primer pair Z4 and Z8 (Cafasso *et al.* 2003) was chosen to amplify a portion of the flanking AT-rich dispersed elements and the internal ZpS1 satellite arrays (Fig. 1). All PCR reactions were carried out in a thermal cycle (*Applied Biosystem 9700*, Foster City, USA) for 30 cycles with the following conditions: 30 s denaturation at 94 °C, 1 min annealing at 55 °C, 2 min extension at 72 °C; extension in the last cycle was for 7 min. Amplification products were separated by electrophoresis on 1.5 % agarose gel with TBE buffer and visualized by ethidium bromide staining. A 100 bp ladder was used as a DNA molecular size standard (*Pharmacia*, Buckingham-shire, UK). Specificity of amplification products was then controlled by Southern blot hybridization with a probe of ZpS1 repeat as described above.

Results

Southern blot analyses of DNA digested with the restriction endonuclease *Apa*I, which cuts the ZpS1 satellite repeat, showed that this repeated sequence displays the typical ladder pattern of satellite DNA in all species of *Zamia* examined, but was absent in the closely related genera *Ceratozamia* and *Microcycas* (data not shown).

Upon digestion with *Eco*RI (this enzyme does not cut the ZpS1 satellite repeat, but only the AT-rich element flanking dispersed ZpS1 repeats), the hybridization patterns of different *Zamia* species, revealed a variable occurrence and organization of the dispersed repeated elements.

Samples of all *Zamia* species examined by using flow cytometry yielded a virtually identical profile to the reference standard (*Z. angustifolia*). This result suggests that it is fairly likely that all samples/species tested have very similar genome content (*i.e.*, around 1C = 12.05). Quantitative dot blot analysis (Fig. 2), even if carried out only for a limited representation of taxa, from which a larger DNA amount was available, indicates that the amount of the tandem ZpS1 repeats varies from 3.0 %, as in row A, to 0.3 %, as in row F, of total DNA among species.

Southern blot experiments using a probe that recognizes both the ZpS1 repeats and the AT-rich flanking sequences (*i.e*. the probe from Z4-Z8 primer pair amplification) show that *Zamia* species belonging to different groups can be clearly distinguished according to

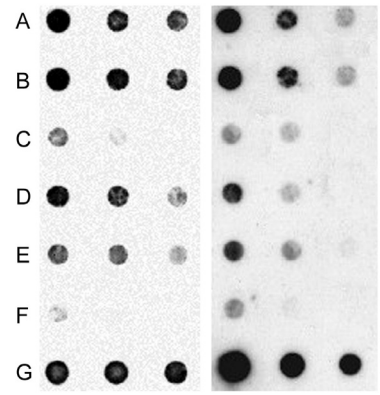

Fig. 2. Quantitative dot blot hybridization. *Row* A: *Z. paucijuga* (Z31); B: *Z. furfuracea* (Z30); C: *Z. wallisii* (Z10); D: *Z. inermis* (Z12); E: *Z. fisheri* (Z13); F: *Z. amplifolia* (Z17); G: (*from left to right*) 100, 50, 25 ng of linearized DNA clone containing the dispersed repeated sequence. For each *Zamia* species the genomic DNA is in the same amounts as those in row G. Hybridization was carried out with a digoxygenin-labelled probe of the repeated sequence ZpS1 (*panel on the left*) and of the AT-rich dispersed sequence amplified with Z1-Z5 primer pair (*panel on the right*).

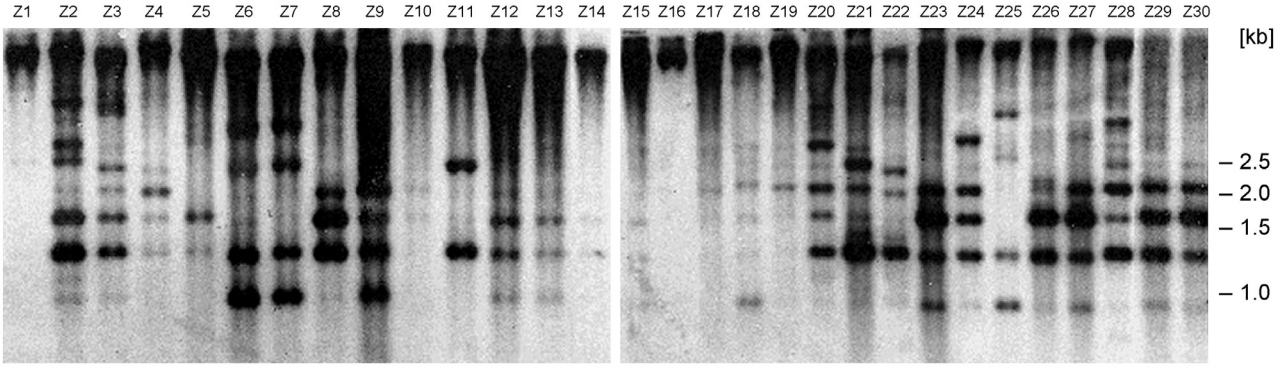

Fig. 3. Southern blots of genomic DNA of *Zamia* species. DNAs have been digested with *Eco*RI and hybridized with the dispersed repeated sequence amplified with Z4-Z8 primer pair. See Table 1 for the list of species.

their *Eco*RI digestion patterns (Figs. 3, 4). The observed differences in the overall signal intensity among the DNAs of the different species confirms that the copy number of ZpS1 repeats organized as satellite DNA and as *Eco*RI dispersed repeated elements varies broadly among taxa. In these blots, hybridization bands of high molecular mass associated to DNA poorly digested by *Eco*RI, correspond to satellite DNA, while the discrete bands in the range 1 - 3 kb correspond to the dispersed element containing one (approx. 1 kb band) or more ZpS1 repeats.

In some species containing ZpS1 organized as satellite DNA, the dispersed repeated elements are hardly detectable, as in *Z. vazquezii* (Z14), *Z. neurophyllidia* (Z1) and *Z. poeppigiana* (Z16) or present in a very low copy number as in *Z. amplifolia* (Z17), *Z. cremnophila* (Z5), *Z. roezli* (Z19), *Z. soconuscensis* (Z15), *Z. standley* (Z18), *Z. wallisii* (Z10) which are characterized by a strong hybridization signal at high molecular mass (satellite DNA) and by none or only few faint clusters of dispersed elements (bands about 980, 1300, 1620, and 1940 bp long). To exclude that, in these species, the strong hybridization signal at high molecular mass was not the result of varying effectiveness of DNA digestion with the *Eco*RI restriction enzyme, we performed additional digestions as described in Cafasso *et al.* 2003. Briefly, we extensively digested the DNAs with *Eco*RI and then we recovered from gel the fraction of high molecular mass DNA that was not digested by *Eco*RI and redigested it with *Eco*RV that, as expected, generated the typical satellite DNA ladder of multimers of the 320 bp repetitive unit (data not shown). However, PCR amplifications (Fig. 5) showed that dispersed elements containing ZpS1 repeats are present also in these species, suggesting that mutations leading to a complete or partial loss of the *Eco*RI site in the AT-rich element have occurred.

Secondary amplification events, involving only some clusters of dispersed repeated sequences, likely generated the different *Eco*RI digestion patterns and the high number of copies of dispersed repeated elements observed in all the other species. Among these, a group characterized by the abundant presence of clusters with very similar pattern is represented by the Mexican species of the *Z. loddigesii* group (Vovides and Olivares 1996, Stevenson *et al.* 1998). These are *Z. furfuracea* (Z30), *Z. loddigesii* (Z29*), Z. picta* (Z26), *Z. paucijuga* (Z31, Z32, Z33, Z34, Z35, Z36), *Z. polymorpha* (Z37, Z38, Z39, Z40, Z41, Z42), *Z. purpurea* (Z8), *Z. spartea* (Z27), *Z. splendens* (Z24), and *Z. sylvatica* (Z23). This group of species is clearly recognizable by the presence of four discrete clusters of repeats (980, 1300, 1620, 1940 bp), with the 1300 bp and the 1620 bp clusters, that contain two and three ZpS1 repeat copies respectively, being the most represented (Figs. 3 and 4).

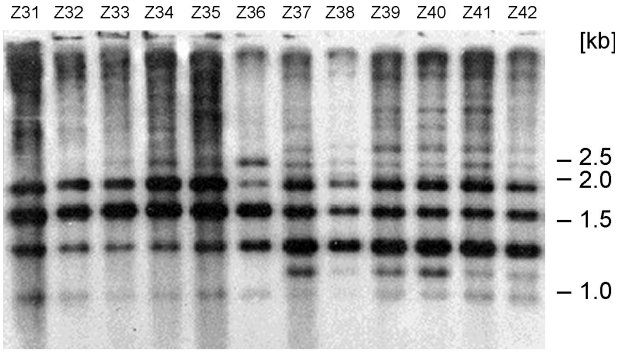

Fig. 4. Southern blots of genomic DNA of *Z. paucijuga* and *Z. polymorpha.* Accessions of *Z. paucijuga* and *Z. polymorpha* from different geographic origins have been digested with *Eco*RI and hybridized with the dispersed repeated sequence amplified with Z4-Z8 primer pair. See Table 1 for the list of species.

Together with the above described pattern, *Z. picta* (Z26) displays also an additional band at 2200 bp, *Z. splendens* (Z24) a faint band at 2900 bp, and *Z. polymorpha* (Z37, Z38, Z39, Z40, Z41, Z42) a faint band at 1200 bp.

When possible (*e.g.*, with *Z. paucijuga* and *Z. polymorpha*, for which several individuals from different populations were available), the hybridization patterns were examined from different and unrelated

geographic accessions. This resulted in a highly uniform pattern among accessions (Fig. 4). The *Z. loddigesii* (Z29) hybridization pattern, even if quantitatively less represented, is present also in the other Mexican isolated species *Z. fisheri* (Z18), *Z. soconuscensis* (Z15) and *Z. inermis* (Z12).

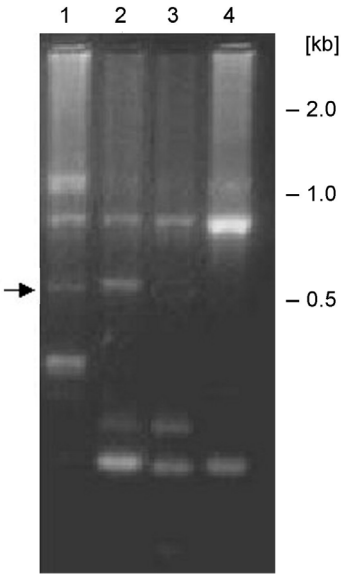

Fig. 5. PCR amplification with Z4-Z8 primer pair of the dispersed sequence containing the repeated sequences of a sub set of *Zamia* species whose southern blots showed none or few hybridization bands with the dispersed repeated sequence (see the text for explanation). *Line* 1: *Zamia vazquezii, line* 2: *Z. poeppigiana, line* 3: *Z. neurophyllidia, line* 4: *Z. roezli*. An *arrow* points the dispersed elements without the inserted satellite array.

Another clearly distinguishable group is represented by the Caribbean species *Z. integrifolia* (Z25), *Z. portoricensis* (Z6) and *Z. pumila* (Z7). These taxa are characterized by four strongly representative clusters (980, 1300, 2500, and 3700 bp), which provide an exclusive pattern for this group.

The Panamanian species *Z. skinneri* (Z11), is characterized by two large clusters of 1300 and 2500 bp. Similarly, *Z. tuerckheimii* (Z3) and the closely related *Z. acuminata* (Z2), both from the Guatemala-Panama region, are characterized by a similar basic pattern with 1300 and 1620 bp clusters being the most represented and with additional, fainter, higher mass *Eco*RI clusters (2500, 3200, and 4600 bp). *Z. ipetiensis* (Z4) from Panama is characterized by a pattern with the 1940 bp long repeat as the most represented cluster but with no additional higher mass *Eco*RI clusters. *Z. manicata* (Z21) and *Z. chigua* (Z22), from the border region between Panama and Colombia, display a similar hybridization pattern (1300, 1450, 1620, 1940, 2400, and 2900 bp) with the 1300 bp long cluster being the most represented. On the contrary, the Colombian *Z. muricata* (Z9) has a unique pattern with five strong bands (980, 1300, 1620, 1940, and 2260 bp) and additional higher mass *Eco*RI clusters.

Finally, two geographically related and morphologically similar species, *Z. lecointei* (Z20) and *Z. boliviana* (Z28), which range from Ecuador to the western Amazon Basin, display a similar pattern with the 1300 and 1940 bp clusters being the most represented plus additional, higher mass *Eco*RI clusters (2260 and 2580 bp long) (Fig. 3).

Discussion

Both repetitive elements, the satellite repeats and the dispersed elements show a different representation within the genome of *Zamia* species examined by Southern blots analyses. Due to the fact that all species display very similar genome content at flow cytometry, the different estimated amounts of satellite repeat and of the dispersed elements among species, are likely due to their diverse representation in copy number in their genomes as consequence of different events of sequence amplifications. Cycads are very rare and slow-growing plants, but for *Z. paucijuga*, for which several accessions where present in the collection of Naples Botanical Garden, *in situ* hybridization of satellite repeat have showed that chromosomes hybridization sites were detected in the subtelomeric regions at one ends of most of the chromosomes confirming the satellite nature of this repeated sequence (Cafasso *et al.* 2003). Sorely, the need of several active growing root tip meristems used to generate chromosome preparations (Moretti 1990), does not allow to perform a similar analysis on other rare species less represented in living collections as botanical gardens. Thus, with this limitation, we cannot asseverate if, species showing a different content of large tandem arrays also display different chromosome localization for these repeated elements.

The presence of ZpS1 hybridization signal in the *Apa*I digestions in all investigated species of *Zamia*, and its absence in the closely related genera, *Ceratozamia* and *Microcycas*, suggests that this repeated sequence was already present, as satellite DNA, in the last common ancestor of the genus *Zamia.* The finding of a common satellite repeat family in all investigated species of *Zamia*, whose stem lineage dates back at least to the early Cenozoic (Caputo *et al.* 2004) is an indication of an old generation event for the origin and amplification of this repetitive sequence.

At the same time, the ubiquity in the genus of dispersed elements containing satellite repeats strongly suggests that also this peculiar genomic rearrangement (*i.e.* the insertion of ZpS1 satellite repeats in the AT-rich dispersed sequence) occurred relatively early in the evolutionary history of *Zamia.*

This hypothesis is supported by the presence of these genomic rearrangements in all investigated species as showed by combined results of hybridization patterns and PCR amplifications. PCR amplifications (Fig. 5) showed, in fact, that dispersed AT-rich elements containing the repeated elements are present also in those species where hybridization patterns show only a strong hybridization signal at high molecular weight and none/little (*Z. vazquezii*, *Z. neurophyllidia, Z. poeppigiana)* or few hybridization bands (*Z. amplifolia*, *Z. roezli*, *Z. soconuscensis*, *Z. standley*, *Z. wallisii*) of the dispersed repeated elements. This is likely due to a complete or partial loss of the *Eco*RI restriction site in the AT-rich element. The stochastic nature of such type of event can also explain why, species belonging to different phyletic *Zamia* lineages can show a similar hybridization pattern. For instance, *Z. neurophyllidia* (Z1), that does not display hybridization bands for the dispersed repeated elements, is a derivate rather than a basal taxon of the Central American species (Caputo *et al.* 2004). Similarly, *Z. roezli* (Z10), that displays only few dispersed repeated elements, is a derivate species in the South American clade. Clearly, in these taxa, the absence of hybridization pattern (more likely due to a loose of *Eco*RI restriction sites) is a secondary derivate condition rather than a primitive condition.

The presence in all *Eco*RI Southern blots of strong ZpS1 repeat hybridization signals in undigested DNA indicates that only a fraction of the repeated arrays is present in the dispersed clusters in all *Zamia* species. Accordingly, all investigated *Zamia* species, when digested with endonucleases (*e.g*. *Apa*I) that cut within the ZpS1 repeat, produced the ladder pattern typical of satellite DNA with the absence of undigested DNA (Cafasso *et al.* 2003).

The hybridization profiles show that different groups of species may be distinguished according to their *Eco*RI digestion patterns as consequence of different genome rearrangements of the dispersed repeated elements (Fig. 3) and indicate that this family of sequences is actively evolving within the genus. Successive amplification events occurring in different lineages over evolutionary time are typical of interspersed or transposable elements and secondary rearrangements of dispersed elements have been often documented in various plant groups (Arnason *et al.* 1984, Hagemann *et al.* 1993, Schmidt and Kudla 1996, Hanson *et al.* 1998, Frediani *et al.* 1999).

The apparent lack of a strictly sequence-dependent role of tandem repeat sequences (Charlesworth *et al.* 1994) may result in their rapid divergence between related species. As a consequence of the nature of evolution of repetitive sequences, acquisition and loss of restriction sites and amplification of specific repeat clusters may happen during the evolution of phyletic lineages. Thus, similar patterns are common in closely related species and may be different between lineages (Kamm *et al.* 1995, King *et al.* 1995, Cardone *et al.* 1997, Hall *et al.* 2005), suggesting potential pattern of relationships among taxa. Several species that represent closely related taxa, accordingly to available molecular and morphological phylogenetic reconstructions, effectively show similar organization and representation of dispersed repeated elements. This is the cases of the *Z. loddigesii* group, of the Caribbean species (*Z. integrifolia, Z. portoricensis* and *Z. pumila*) and of the two closely related South-American species *Z. lecointei* and *Z. boliviana.* Similarity in the hybridization pattern among closely related species may indicate the presence of phylogenetic signal in the genomic organization of the dispersed repeated elements. However attempts to fit the occurrence and organization of these repetitive elements on the molecular phylogenetic trees available for the genus (Caputo *et al.* 2004) do not allow the reconstruction of a unique pattern for the molecular evolution of these dispersed repeated elements in *Zamia*. In fact, while some species groups are characterized by similar hybridization patterns, strong variations in occurrence and genomic organization of the dispersed repeated element were also observed between closely related species as, for instance, the Panamanian *Z. skinneri* and *Z. neurophyllidia*.

The simplest model for the origin of these dispersed repeated elements involves a site-specific recombination between the AT-rich dispersed sequence and circular ZpS1 satellite repeats occurring at a 45 bp-long common sequence (Cafasso *et al.* 2003). This recombination event likely took place early in the *Zamia* cladogenesis. In fact, the occurrence of these elements in different (all tested) *Zamia* lineages indicates that their evolution predates the radiation of the genus on both side of Panama isthmus. Several events of secondary amplification and losses of these dispersed repeated elements may have occurred in the long history of this ancient plant group. These secondary changes have likely hidden the evolution pattern of these dispersed repeated elements. However, a multiple and independent origin from the ZpS1 satellite DNA and the dispersed AT-rich element is also a possible scenario. In fact, due to the sequence homology and tandem organization of these two repetitive elements (Cafasso *et al.* 2003), we cannot rule out that both insertion and excision events may have occurred several times in the history of species cladogenesis. A characterization of the complete sequence of the dispersed elements will probably allow to determinate if their different genome representations in *Zamia* species are the consequence of several changes that occurred along a unique evolution pathway or the result of several independent events from a common genomic source. This characterization may further help to elucidate the mechanisms of genome evolution in this ancient plant group. At this regard it should be noted that in *Zamia*, contrarily to the other cycad genera, chromosome numbers vary greatly from species to species and, in some cases, even within species and populations (Moretti 1990b, Moretti *et al.* 1991). Typically, the repeat DNA organization and its chromosome distribution are related to genome evolution (Charlesworth *et al.* 1994, Fuchs

et al. 1995, Lin *et al.* 2005) and the scattered presence in the *Zamia* genome of these dispersed repeated elements that contain satellite repeat arrays could be involved in the unusual karyotype variations observed in this plant group. In this regard, if centric fission is accepted as the main cause of chromosomal changes in *Zamia* (Moretti and Sabato 1984), such a type of occurrence of repeated sequences scattered along the chromosomes, producing many areas of sequence homology in different chromosomes, could influence correct chromosome pairing and promote chromosome rearrangements.

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