

Mapping 5S ribosomal DNA on somatic chromosomes of four species of *Ceratozamia* and *Stangeria eriopus* (Cycadales)

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Somatic chromosomes of four species of *Ceratozamia*, *C. hildae*, *C. kuesteriana*, *C. mexicana* and *C. norstogii*, and *Stangeria eriopus*, were observed and compared by the fluorescence *in situ* hybridization method using 5S ribosomal (rDNA) probes. The four *Ceratozamia* species and *S. eriopus* showed the same chromosome number of $2n = 16$, and had similar karyotypes, comprising 12 metacentric (m), two submetacentric (sm) chromosomes and two telocentric (t) chromosomes. The four *Ceratozamia* species exhibited a proximal 5S rDNA site in the interstitial region of two m chromosomes. *Stangeria eriopus* exhibited a distal 5S rDNA site in the interstitial region of two m chromosomes, which probably indicates that the two genera differ in chromosome structure by at least one paracentric inversion. © 2004 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2004, 145, 499–504.

ADDITIONAL KEYWORDS: cycads – cytotaxonomy – fluorescence *in situ* hybridization.

INTRODUCTION

The genus *Ceratozamia* (family Zamiaceae; Stevenson, 1992) is endemic to Mega-Mexico 2, an extension of Mexico's border, based on physiographic, climatic and biotic factors, into Central America including northern Nicaragua (Rzedowski, 1993). There are approximately 20 species known in Mexico, with three distributed in the adjacent countries of Belize, Guatemala and Honduras. In contrast, the genus *Stangeria* is a monotypic genus in the family Stangeriaceae (Stevenson, 1992) in which the sole species, *S. eriopus*, is distributed along the east coast of South Africa (Goode, 1989). In most previous references, *Ceratozamia* and *Stangeria* have had little taxonomic or cytological connection with each other in the living Cycadales (e.g. Stevenson, 1992; De Luca *et al.*, 1995). However, Kokubugata *et al.* (2001) and Kokubugata, Hill & Kondo (2002a) reported that they shared some cytological characters.

Recently, the molecular–cytological techniques of the fluorescence *in situ* hybridization (FISH) method have been applied to cytotaxonomic studies in some cycad taxa. It has been shown to be a powerful method for analysing inter- and infrageneric cytotaxonomic relationships, e.g. in *Cycas* (Hizume, Ishida & Kondo, 1992; Hizume, 1995; Hizume *et al.*, 1998; Kokubugata, Hill & Kondo, 2002b); in *Bowenia* and *Stangeria* (Kokubugata *et al.*, 2000; Kokubugata, Hill & Kondo, 2002a); in *Ceratozamia* (Kokubugata & Kondo, 1998; Tagashira & Kondo, 2001), and in *Zamia* (Kondo & Tagashira, 1998). In *Ceratozamia*, taxonomic studies using morphological comparisons, karyotype analyses and molecular phylogeny have been advancing over recent years (Vovides, 1983, 1985; Vovides *et al.*, 1993; Vázquez-Torres & Vovides, 1998; Pérez-Farrera, Vovides & Iglesias, 2001a, b; Vovides, Pérez-Farrera & Iglesias, 2001; González & Vovides, 2002; Avendaño, Vovides & Castollo-Campos, 2003). However, molecular–cytological studies by the FISH method have not been applied to *Ceratozamia* previously, with the exception of *C. mexicana* (Kokubugata & Kondo, 1998; Tagashira & Kondo, 2001).

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Table 1. Accession numbers and chromosomal characters of four *Ceratozamia* species and *Stangeria eriopus*

Species	Accession no.*	2n	Karyotype†
<i>C. hildae</i>	TBG136413	16	12m + 2sm + 2t
<i>C. kuesteriana</i>	TBG136416	16	12m + 2sm + 2t
<i>C. mexicana</i>	TBG136414	16	12m + 2sm + 2t
<i>C. norstogii</i>	TBG136423	16	12m + 2sm + 2t
<i>S. eriopus</i>	TBG141347	16	12m + 2sm + 2t

*TBG = Tsukuba Botanical Garden, National Science Museum, Tokyo.

†Karyotype: m = median-centromeric chromosome; sm = submedian-centromeric chromosome; t = terminal-centromeric chromosome.

The specific aims of the present study are to compare distribution patterns of the 5S rDNA sites on somatic chromosomes of four *Ceratozamia* species and *Stangeria eriopus*, which are known to share some cytological similarities.

MATERIAL AND METHODS

PLANT MATERIAL AND CHROMOSOME PREPARATION

Material of four *Ceratozamia* species, *C. hildae* G. P. Landry et M. C. Wilson, *C. kuesteriana* Regel, *C. mexicana* Brongn. and *C. norstogii* D. W. Stevenson, and *Stangeria eriopus* (Kunze) Nash were obtained from plants grown at the Tsukuba Botanical Garden (TBG), National Science Museum, Tokyo (Table 1).

Young leaves were harvested and pretreated in 2 mM 8-hydroxyquinoline at 4°C for 24 h, fixed in acetic ethanol (1 : 3) at 4°C for 24 h and then stored in 70% ethanol at 20°C. Stored leaflets were macerated in an enzyme solution with 2% cellulase 'Onozuka' RS (Yakult) and 1% pectoryase 'Y-23' (Seisin) in distilled water (w/v; pH 4.5) at 36°C for 20 min before being washed in distilled water and 45% acetic acid at room temperature for 5 min.

PROBES AND LABELLING OF PCR-AMPLIFIED 5S rDNA

A 5S ribosomal DNA (5S rDNA) sequence, equivalent to a 5S ribosomal RNA gene, was amplified as a probe from the total genomic DNA of *C. kuesteriana* (TBG Accession no. 136416) by the polymerase chain reaction (PCR) following the method of Hizume (1995). The PCR-amplified 5S rDNA was labelled with digoxigenin- (DIG-) dUTP by the nick translation method (Roche). The DIG-labelled probe was dissolved in 50% formamide and 10% dextran sulphate in 2× SSC (w/v). This hybridization mixture was denatured at 75°C for 10 min, then immediately chilled in cold

water for 10 min and stored at -20°C. The final DNA concentration of 5S rDNA was adjusted to 5 ng μL^{-1} as the hybridization mixture.

FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

The macerated leaflet was placed on a slide and squashed in 45% acetic acid with a coverslip. The slide was dried at 36°C for 30 min after removing the coverslip by the dry-ice method. It was treated with 0.1% RNase in 2× SSC at 36°C for 1 h, washed in 2× SSC for 10 min, treated in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min and washed in distilled water for 10 min. It was then dehydrated in an ethanol series (75%, 85% and 100%) for 3 min each before being dried at 36°C for 20 min. The dried slide received 10 $\mu\text{g mL}^{-1}$ of the hybridization mixture and was covered with a coverslip and sealed with rubber cement. The DNA on the slide was then denatured at 85°C for 10 min on a hot plate and immediately incubated at 37°C in a humid chamber overnight for DNA hybridization to occur. Following the hybridization, the coverslip was removed and the slide was washed in 4× SSC at 40°C for 20 min. The hybridized probes on chromosomal DNA were detected with 20 $\mu\text{g mL}^{-1}$ anti-DIG-fluorescein, Fab-fragmentavidin (Roche) in 1% bovine serum albumin in 4× SSC (w/v) at 37°C for 1 h. The slide was washed in 4× SSC at room temperature for 20 min before receiving 30 μL of an anti-fade solution of 10% PBS, 90% glycerol with 0.1 g mL^{-1} 1,4-diazabicyclo [2.2.2.] octan with 1 $\mu\text{g mL}^{-1}$ propidium iodide (PI) for counter-staining. The slide was then mounted with a coverslip for counter-staining and held at 4°C for at least 2 h. The hybridization signals were made visible by anti-DIG-fluorescein, Fab-fragmentavidin as yellow fluorescence, with PI revealing non-hybridized regions with orange or dark red fluorescence using the double band pass excitation filter (Zeiss filter set no. 23).

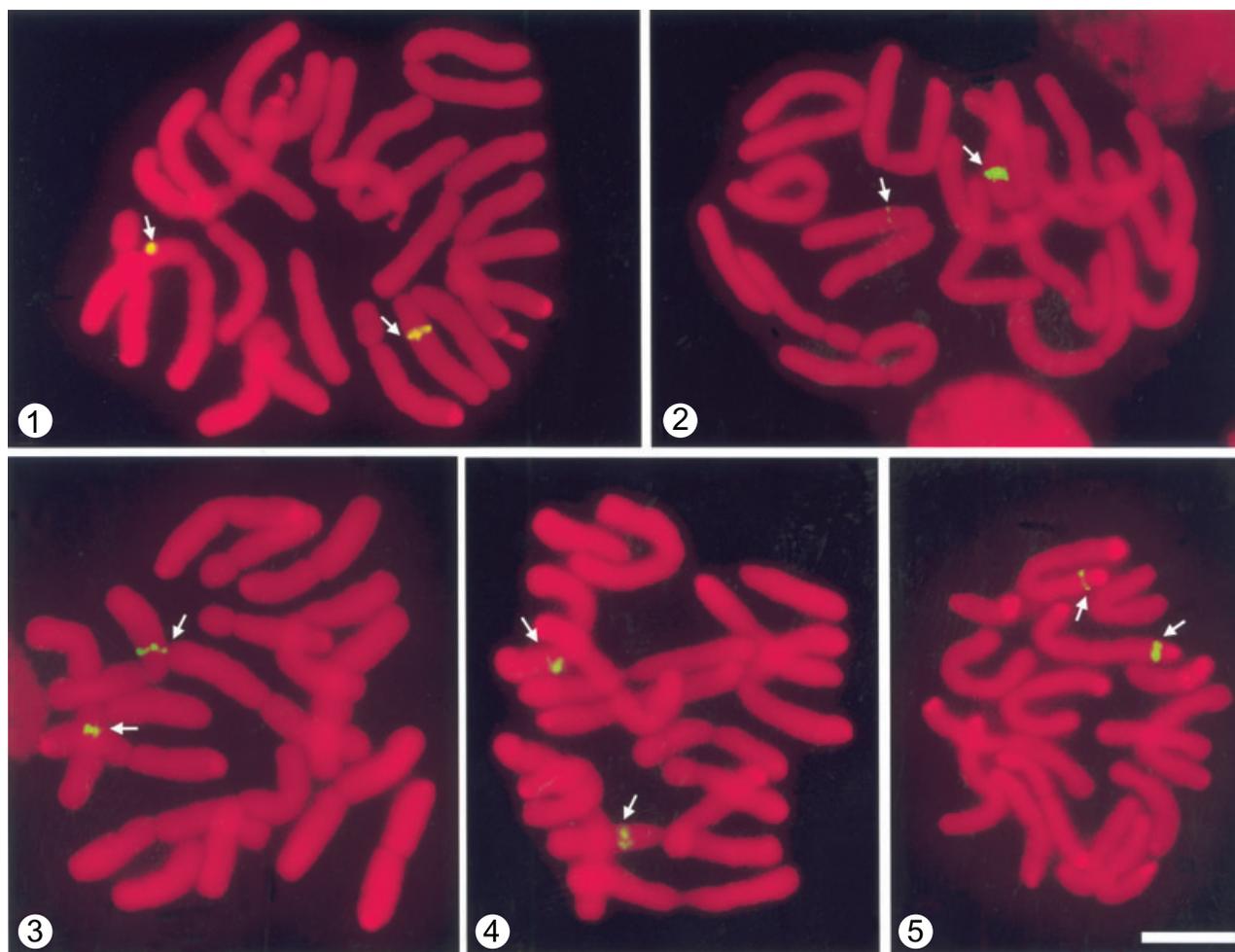
DESCRIPTION OF CHROMOSOMES

Chromosomes at mitotic metaphase were classified by arm ratio (R = long arm length/short arm length) following Levan, Fredga & Sandberg (1964). Median-centromeric (R = 1.0–1.7), submedian-centromeric (R = 1.8–3.0), subterminal-centromeric (R = 3.1–7.0) and terminal-centromeric chromosomes ($R \geq 7.1$) are abbreviated here as m, sm, st and t chromosomes, respectively.

RESULTS AND DISCUSSION

CHROMOSOME NUMBER AND KARYOTYPE

The four *Ceratozamia* species and *Stangeria eriopus* all had a chromosome number of $2n = 16$ (Figs 1–5).



Figures 1–5. Somatic chromosomes detected by FISH using 5S rDNA probes in four *Ceratozamia* species and *Stangeria eriopus*. Fig. 1. *C. hildae*. Fig. 2. *C. kuesteriana*. Fig. 3. *C. mexicana*. Fig. 4. *C. norstogii*. Fig. 5. *S. eriopus*. Arrows show 5S rDNA sites. Scale bar = 10 μ m.

The observations confirmed reports by Vovides (1983) for *C. hildae*; Vovides (1985) for *C. kuesteriana*; Sax & Beal (1934), Marchant (1968), Moretti (1990a, b), Kokubugata & Kondo (1998) and Tagashira & Kondo (2001) for *C. mexicana*; Moretti (1990a, b) and Pérez-Farrera *et al.* (2001a) for *C. norstogii* (Figs 1–4, respectively). The observations also confirmed reports by Sax & Beal (1968), Marchant (1993), Moretti (1990a, b), Kokubugata *et al.* (2001) and Kokubugata *et al.* (2002a) for *S. eriopus* (Fig. 5).

The karyotypes of the four *Ceratozamia* species and *Stangeria* all consisted of 12 m chromosomes (1–12), two sm chromosomes (13–14) and two t chromosomes (15–16; Table 1; Fig. 6). The karyotypes of four *Ceratozamia* species observed were consistent with Sax & Beal (1934), Marchant (1968), Vovides (1983, 1985), Moretti (1990b), Kokubugata & Kondo (1998), Vázquez-Torres & Vovides (1998) and Tagashira & Kondo (2001). That of *S. eriopus* was consistent with

Sax & Beal (1934) and Kokubugata *et al.* (2001, 2002a). In Figure 6, no attempt has been made to sort the m chromosomes into homologous pairs. They are ordered in series of decreasing length.

CHROMOSOMES OBSERVED BY FISH USING 5S rDNA

With the use of FISH, the four *Ceratozamia* species and *S. eriopus* all showed a 5S rDNA site on two metaphase chromosomes (Figs 1–5). Although the two m chromosomes with the 5S rDNA sites were not on exactly the same chromosomes among the five species, as determined from chromosome length, they were very close, in the range from the 2nd to the 4th (Fig. 6). Differential contraction rates of the exceedingly long chromosomes of cycads during pre-treatment make it difficult to pair homologues on size criteria alone (Vovides, 1983). Thus the two chromosomes with the 5S rDNA site are very proba-

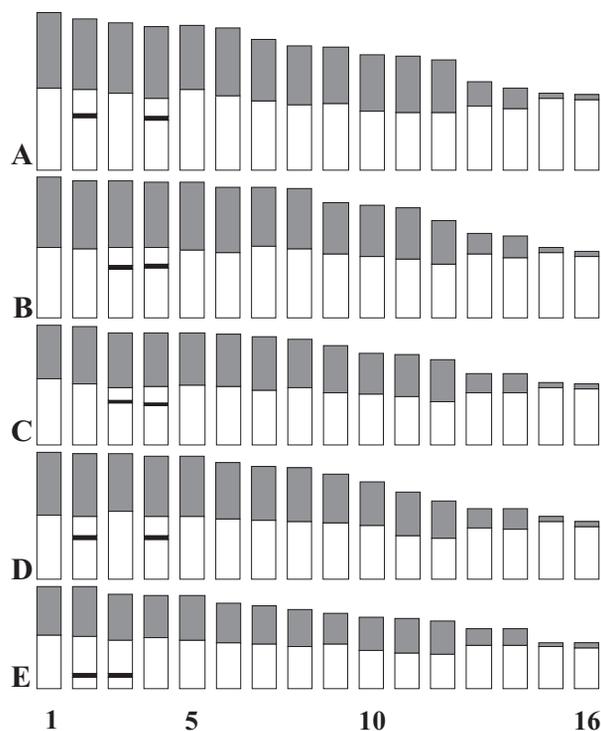


Figure 6. Ideograms detected by FISH using 5S rDNA probes in four *Ceratozamia* species and *Stangeria eriopus*. A, *C. hildae*. B, *C. kuesteriana*. C, *C. mexicana*. D, *C. norstogii*. E, *S. eriopus*. Shaded areas show long arms. Non-shaded areas show short arms. Solid areas show 5S rDNA sites.

bly homologous to each other among the five species.

The four *Ceratozamia* species exhibited a 5S rDNA site close to the centromere on the interstitial region of the long arm of the two m chromosomes (Fig. 6A–D). This is consistent with the findings of Tagashira & Kondo (2001) in *C. mexicana*. The plant of *C. kuesteriana* investigated showed a heteromorphic pair in the visual strength of the 5S rDNA sites (Fig. 2). Heteromorphic chromosomes indicated by FISH using 18S rDNA probes were previously reported in *Ceratozamia* (Kokubugata & Kondo, 1998) and in *Stangeria* (Kokubugata, Hill & Kondo, 2002a). There is a small possibility that the heteromorphic pair might be sex chromosomes. However, unfortunately, the sex of the plant of *C. kuesteriana* investigated was unknown. *Stangeria eriopus* exhibited its 5S rDNA site near the terminal on the interstitial region of the long arm of the two m chromosomes.

CYTOTAXONOMY

Earlier work on the group has found $2n = 16$ for *Ceratozamia* and *Stangeria* (e.g. Sax & Beal, 1934; Marchant, 1968; Moretti, 1990a), and Kokubugata

et al. (2001) reported that *Ceratozamia* and *Stangeria* had the most similar karyotypes in the Cycadales. Moreover, Kokubugata *et al.* (2002a) reported that *Ceratozamia* and *Stangeria* shared not only the same chromosome number and karyotype, but they also have similarly dispersed patterns of 18S–26S rDNA sites on their somatic chromosomes. This study shows a distinct difference in 5S rDNA site position between *Ceratozamia* and *Stangeria* for the first time. This difference suggests that the two genera possibly differ by a paracentric inversion in the region, indicating an increase in gene linkage (Stebbins, 1971), or dispersal of the 5S rRNA site. Evidence of dispersed 5S rRNA sequences has been observed in the fungi; for many of the species of *Pythium* the large-subunit rRNA gene and the 5S rRNA gene were found to have length heterogeneity (Belkhiri, Buchko & Klassen, 1992). In higher eukaryotes, the 5S rDNA sites are located independently of the 45S rDNA repeats containing 18S, 5.8S and 26S ribosomal RNA genes (Sone *et al.*, 1999).

By contrast, the four *Ceratozamia* species observed showed similarly distributed 5S rDNA sites, which might suggest gene linkage at genus level and that all *Ceratozamia* species might share a common pattern of 5S rDNA sites. FISH studies in the remaining members of *Ceratozamia* would clarify this idea.

Working with all *Ceratozamia* species known at present, González & Vovides (2002) showed *C. norstogii* to be phylogenetically far from the other three species by sequencing ITS in ribosomal DNA and *trnL-F* in the chloroplast DNA. However, the low level of variation detected among *Ceratozamia* spp. limited the conclusions on phylogenetic relationships because few clades were resolved fully, but the results enabled a phyto-geographical inference to be made. The molecular phylogeny of *Ceratozamia* revealed three main clades. The most basal clade, which includes *C. norstogii*, suggests south-east Mexico to be a probable ancestral geographical area for the genus *Ceratozamia*. Another implication is that speciation within the genus appears to be associated with the post-Pleistocene spread of floristic communities from proposed Pleistocene tropical refugia located in south-east Mexico north of the Trans Mexican Neovolcanic mountain range (González & Vovides, 2002). The three *Ceratozamia* species of the present study originate at the Trans Mexican Neovolcanic Mountain range (*C. mexicana*) or north of it (*C. hildae* and *C. kuesteriana*).

In view of the homogeneity of the sequence data in *Ceratozamia*, RAPD (random amplified polymorphic DNA) markers were used to detect variation among species of *Ceratozamia* and *Zamia* (Vovides *et al.*, 2003). RAPD is a polymorphism assay that is based on the amplification of random DNA segments using sets of primers of arbitrary nucleotide sequence. The polymorphism detected as DNA segments has allowed us to

examine variation in the whole genome. Thus far, five primers out of 20 have detected variation in *Ceratozamia* and six out of 23 in *Zamia*. By contrast, with DNA sequences the preliminary analyses of RAPD markers indicate high levels of variation within *Ceratozamia* spp. but moderate variation within *Zamia* spp.

Previously, Tagashira & Kondo (1998) have reported that *Zamia*, being classified in Zamiaceae with *Ceratozamia* (Stevenson, 1992), exhibited a 5S rDNA site close to the terminal on the interstitial region of two m chromosomes. In addition, Hizume (1995) and Kokubugata *et al.* (2002b) reported that *Cycas*, considered as the most primitive and most distinct genus in the Cycadales (Johnson, 1959; De Luca *et al.*, 1995; Norstog & Nicholls, 1997), commonly exhibited 5S rDNA sites close to the terminal on the interstitial region of two t chromosomes. The distribution pattern of 5S rDNA sites in the four *Ceratozamia* species in our study might be a character apomorphic from the other cycad members, probably a paracentric inversion that may have occurred during the divergence of the species in question with respect to *Stangeria*.

In this study, there are not enough data to explain the chromosomal evolution from *Stangeria* to *Ceratozamia* or vice versa, or whether the two genera are sister taxa in the Cycadales or not. However, based on our study, we are able to hypothesize that a paracentric inversion including the 5S rDNA site might have occurred on the interstitial region of the long arm of the m chromosome during the separation of the taxa at the genus level.

It is interesting to note that karyotype evolution has been very conservative in *Ceratozamia*, and apparently also in *Stangeria*. All species of *Ceratozamia* so far studied have the same karyotype, consisting of 12m + 2sm + 2t chromosomes. This contrasts with its sister genus *Zamia*, which is characterized by exceedingly variable somatic chromosome numbers ($2n = 16, 17, 18, 22, 23, 24, 25, 26, 27, 28$) and karyotypes which are indicative of Robertsonian changes (Vovides, 1983; Moretti & Sabato, 1984; Moretti, 1990a, b; Vovides & Olivares, 1996). More detailed discussion of *Zamia* chromosomal relationships have been given by Khoshoo (1969), Jones (1977), Norstog (1980, 1981) and Vovides *et al.* (2003).

From the evidence so far presented we can only speculate that karyotype evolution in *Ceratozamia* and *Stangeria* has been by way of chromosome inversions, whereas that of *Zamia* has been by way of chromosome translocations or Robertsonian changes.

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