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CYCAD (CYCADALES) CHROMOSOME NUMBERS ARE NOT CORRELATED WITH GENOME SIZE

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Premise of research. Change in base number of chromosomes per nucleus is usually believed to result from whole-genome duplication or from duplication or elimination of a single chromosome (aneuploidy). However, chromosome numbers can also change via mechanisms with no gain or loss of nuclear DNA, such as fusion or fission of chromosomes.

Methodology. We previously determined amount of DNA per nucleus (2C values) using flow cytometry of leaf tissue. We tested whether Cycadales (cycad) chromosome numbers are correlated with these 2C values using ordinary least squares (OLS) and phylogenetic generalized least squares (PGLS) regression.

Pivotal results. Regardless of branch length estimation technique and evolutionary model, OLS and PGLS yielded no statistically significant relationship between chromosome number and genome size in our analysis of 22 species, encompassing all 11 extant cycad genera.

Conclusions. Within cycad genera, chromosomal fissions or fusions are much more likely than large additions or deletions of nuclear DNA.

Keywords: aneuploidy, chromosomal fission and fusion, Robertsonian fission and fusion, genome size, pseudopolyploidy, whole-genome duplication.

Online enhancements: appendix figures, supplementary data.

Introduction

Large, radical evolutionary changes are often caused by duplication of the entire genome and/or large-scale deletions from the genome (Soltis and Soltis 1993). Smaller evolutionary changes usually arise from less draconian chromosomal changes, such as rearrangements of existing chromosomes, including fissions and fusions (Gorelick and Olson 2013). The evolution of cycads has seemed conservative over their roughly 250–300-million-year history (Zhifeng and Thomas 1989; Axsmith et al. 2003; Taylor et al. 2009; Gorelick and Olson 2011), with much less morphological diversity than exists in Mesozoic conifers and gnetophytes (Taylor et al. 2009), let alone in modern angiosperms or monilophytes. Modern conifers range from massive trees to short shrubs, have both evergreen and deciduous species, and even possess species that are parasitic (*Parasitaxus usta*). In the Mesozoic conifer family Cheirolepidaceae, "growth forms included small herbs, thick tangled mats, small stem succulents, large pachycauls, mangrove forests, and large woody trees" (Gorelick 2001, p. 413 [citations omitted]). The three extant genera of Gnetales are as morphologically divergent as any three angiosperm genera (Pearson 1929), with Mesozoic gnetophytes being even more diverse (Taylor et al. 2009). By contrast, the most remarkable morphological innovations over cycad evolution are the absence of ovulate cones in *Cycas* and concentric vascular cambia in *Cycas* (very unusual outside of lianas; there are no cycad lianas recorded in the fossil record). We therefore tested whether chromosome evolution has been equally conservative in cycads by examining whether cycad chromosomes have evolved mostly via rearrangements of existing genetic material. To do this, we compared nuclear DNA content with chromosome number, predicting no significant relationship between these variables if cycad chromosome evolution has largely been due to fissions or fusions, with no substantial gain or loss of DNA.

There are two different proximate mechanisms for duplicating the number of chromosomes, namely, whole-genome duplication and dividing all (nontelocentric) chromosomes in two at the centromere, which is sometimes known as karyotypic fissioning or pseudopolyploidy (Vandel 1937; Tobias 1953; Todd 1970; Kolnicki 2000; Gorelick and Olson 2013). Fissioning 2x metacentric chromosomes at their centromeres results in 4x telocentric chromosomes. Whole-genome duplication of 2x telocentric chromosomes also results in 4x telo-

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centric chromosomes. Looking for a preponderance of telocentric (vs. metacentric) chromosomes cannot help in determining the mechanism by which chromosome numbers doubled because telocentric chromosomes can become metacentric by growing new centromeres from repetitive elements (Kidwell and Lisch 2001). While it may be impossible to operationally distinguish whole-genome duplication from pseudopolyploidy without measuring C values or 2C values, these two evolutionary mechanisms for increasing the number of chromosomes have very different evolutionary consequences, which we thoroughly described in Gorelick and Olson (2013).

Evolution of cycad chromosomes is still poorly understood. Some authors have claimed that there is no whole-genome duplication in cycads (Levin and Wilson 1976; Crepet and Niklas 2009; Gorelick and Olson 2011). Early notions were that chromosomal fusion was fairly common in cycads (Marchant 1968; Norstog 1980), but more recent work indicates that chromosomal fission is much more common and important in cycads, at least in the Zamiaceae (Moretti and Sabato 1984; Caputo et al. 1996; Olson and Gorelick 2011). There is no good evidence of large-scale duplication of individual genetic loci in cycads, except that cycad 2C values are relatively large for vascular plants (Gregory 2004; Murray et al. 2012).

We do not expect whole-genome duplication in the Cycadales because chromosome numbers in extant Cycadales vary by less than a factor of 2, being between 2n=16 and 2n=28 (table 1). We therefore expect that cycad 2C values will be uncorrelated or weakly correlated with their chromosome numbers and hypothesize that aneuploidy or karyotypic fission (pseudopolyploidy) is the most likely explanation for the observed variation in chromosome number. Karyotypic fissioning will not affect telocentric chromosomes and hence can result in a large increase in number of chromosomes but less than a doubling of chromosome number. Sporophytic chromosome numbers vary between genera in the Cycadales, but intrageneric variation occurs only in *Zamia*.

The ancestral sporophytic number in *Zamia* appears to be 2n=16 (Gorelick and Olson 2011; Olson and Gorelick 2011). However, it is difficult to estimate the ancestral number among all extant Cycadales for three reasons. First, *Cycas* has 2n=22 (Selvaraj 1980; Tian et al. 2002). Second, the base of the Cycadales phylogenetic tree contains both extant families: Cycadaceae, which includes only *Cycas*, and Zamiaceae, which includes all other extant genera (Bogler and Francisco-Ortega 2004; Nagalingum et al. 2011). Both clades contribute to the estimation of the ancestral state. *Cycas* is not necessarily ancestral. Third, many extinct cycads are missing from the base of most phylogenetic trees (Hermsen et al. 2006). We will report below our ancestral character state estimation for number of chromosomes, albeit with caution.

Inferring ancestral chromosome numbers in cycads would be simpler if sister taxa were known. Cycadales is possibly sister to the Medullosales (Crane 1985), which were strictly Paleozoic. It is possible but highly unlikely that fossilized chromosomes will be found in the Medullosales, as was recently reported with Jurassic Osmundaceae (Bomfleur et al. 2014). Cycads are probably more closely related to ginkgos than to other extant seed plants (Crane 1985; Bowe et al. 2000; Finet et al. 2010; Ruhfel et al. 2014). *Ginkgo biloba* has 2n=24 (Terasaka 1993), sometimes claimed to be 2n=22+XY (Chen et al. 1993). This provides extremely tenuous evidence that *Cycas*, with 2n=22, has an ancestral number of chromosomes in the Cycadales.

We compared the amount of DNA per nucleus (2C value) with chromosome numbers across a large number of Cycadales species. If increases in chromosome number are caused by pseudopolyploidy (fissioning or duplication of all centromeres without duplication of any other part of each chromosome), then there should be no net change in genome size (2C value), and thus there should be no correlation between chromosome number and 2C value. If increase in chromosome number is caused by whole-genome duplication, which we do not expect, then 2C value should double, and thus there should be a strong positive correlation between chromosome number and 2C value. Aneuploidy should lie somewhere in between, with a weak positive correlation. We therefore tested for statistically significant relationships (nonzero slope) between chromosome number and 2C value in Cycadales, both with and without phylogenetic correction.

Methods

Flow Cytometry and Chromosome Numbers

Genome sizes (2C values) were obtained via flow cytometry of nuclei from leaf tissue for 22 species. We previously reported these methods and data in Zonneveld (2011).

We repeated all analyses after also including 2C values of all four cycad species from the Gymnosperm DNA C-Values Database (Murray et al. 2010). This database includes only four species of cycads, one of which we had also sampled. The only additional cycad species included in the most recent Gymnosperm DNA C-Values Database (Murray et al. 2012) were contributed by Zonneveld (2011). We report results both with and without these four values from the C value database because of potential problems with those four values (see "Discussion").

We computed coefficients of variation of 2C values for each genus, using as input the mean 2C value for each species. We collected chromosome numbers, largely sporophytic, from the literature and the *Index to Plant Chromosome Numbers* (IPCN; Goldblatt and Johnson 2008). Gametophytic chromosome numbers were reported for only two species, *Cycas revoluta* Thunberg (n=11) and *Bowenia serrulata* (W. Bull) Chamberlain (n=9), whose values were exactly half that reported for sporophytes. We therefore conducted all analyses using sporophytic numbers.

Chromosome numbers and 2C values were never computed from the same individual plant. However, there is no known variability in chromosome number within the species for which we measured 2C values (see references in table 1).

DNA Isolation, Polymerase Chain Reaction (PCR), and Sequencing

DNA sequences, 2C values, and chromosome numbers were obtained from the same species (see app. A for voucher information and GenBank accessions), but different individ-

Table 1

2C Values and Ploidy Levels

Species	2C pg	2n	References for chromosome counts
Bowenia serrulata (W. Bull) Chamberlain	43.9	18	Marchant 1968; Terasaka 1982; Kokubugata et al. 2000, 2001
Bowenia spectabilis Hooker ex Hooker f.	41.4	18	Kokubugata et al. 2000, 2001
Ceratozamia mexicana Brongniart	63.3	16	Sax and Beal 1934; Marchant 1968; Moretti 1990; Kokubugata and Kondo 1998; Tagashira and Kondo 1999, 2001; Kokubugata et al. 2004
Ceratozamia norstogii D.W. Stevenson	63.2	16	Moretti 1990; Kokubugata et al. 2004
Chigua (*Zamia*) *restrepoi* D.W. Stevenson	42.5	18	Moretti et al. 1993
Cycas circinalis Linnaeus	29.5	22	Ohri and Khoshoo 1986
Cycas diannanensis Z.T. Guan & G.D. Tao	26.2	22	Tian et al. 2002
Cycas revoluta Thunberg	27.4	22	Selvaraj 1980; Terasaka 1982; Ohri and Khoshoo 1986; Hizume et al. 1992, 1998; Kokubugata and Kondo 1994
C. *revoluta* Thunberg	25.5	22	Selvaraj 1980; Terasaka 1982; Ohri and Khoshoo 1986; Hizume et al. 1992, 1998; Kokubugata and Kondo 1994
Dioon edule Lindley	50.3	18	Marchant 1968; Moretti 1990
Dioon merolae De Luca, Sabato & Vázquez-Torres	48.7	18	Moretti 1990
Encephalartos altensteinii Lehman	58.7	18	Marchant 1968
Encephalartos horridus (Jacquin) Lehman	57.9	18	Marchant 1968
Encephalartos lebombensis I. Verdoorn	59.1	18	Marchant 1968
Encephalartos lehmanii Lehman	59.5	18	Marchant 1968
Encephalartos manikensis (Gilliland) Gilliland	58.2	18	Marchant 1968
Encephalartos villosus Lemaire	42.2	18	Ohri and Khoshoo 1986
Lepidozamia hopei (W. Hill) Regel	60.3	18	Marchant 1968
Lepidozamia peroffskyana Regel	55.3	18	Marchant 1968
Macrozamia moorei F. Mueller	53.0	18	Moretti 1981–1982
Microcycas calocoma (Miquel) de Candolle	41.2	26	Moretti 1990; Kokubugata and Kondo 1998
Stangeria eriopus (Kunze) Baillon	39.0	16	Marchant 1968; Kokubugata et al. 2001, 2002, 2004
Zamia angustifolia Jacquin	24.1	16	Ohri and Khoshoo 1986
Zamia furfuracea Linnaeus f.	36.3	18	Norstog 1980; Moretti and Sabato 1984; Moretti 1990; Fuchs et al. 1995; Tagashira and Kondo 1999, 2001
Zamia integrifolia Linnaeus f.	38.2	16	Moretti 1990; Tagashira and Kondo 1999, 2001[a]
Zamia neurophyllidea D.W. Stevenson	37.7	18	Norstog 1980[b]

Note. 2C values from the Gymnosperm DNA C-Values Database (Murray et al. 2010) obtained via Feulgen microdensitometry are underlined. We analyzed data both with and without these samples. However, only results without these samples are reported herein. All 2C values not underlined are from Zonneveld (2011).

[a] Kondo and Tagasihira (1998) reported 2n=23 for *Z. integrifolia* but repudiated this count in their later article (Tagashira and Kondo 2001).

[b] As *Zamia skinneri* (see Olson and Gorelick 2011).

uals were used for each data type. A TissueLyser (Qiagen, Valencia, CA) was used to disrupt 5–10 mg of silica-dried leaf tissue with two 3-mm stainless steel ball bearings in a 1.6-mL tube (240 s at 30 Hz). DNA was extracted using the DNeasy Plant mini kit (Qiagen) following the manufacturer's instructions.

Samples were PCR amplified and bidirectionally sequenced for the large ribosomal subunit (26S; high copy nuclear), chlorophyll a/b binding protein (*CAB*; single-copy nuclear, chloroplast expressed), maturase K (*matK*; plastid), NADH dehydrogenase subunit 1, intron 2 (*nad1*; mitochondrion), *NEEDLY* (single-copy nuclear), and the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbcL*; plastid).

Individual amplifications took place in a 15-μL volume containing 1.5 μL buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% v/v Triton X-100, 5% w/v sucrose, 0.025% w/v cresol red), 0.2 mM dNTPs, 0.025 μg/μL BSA, 0.4 M betain (not used for *CAB* or *nad1*), 0.5 μM (26S and *nad1*) or 1 μM of each primer, 1 unit of *Taq* polymerase, and 0.5 μL (26S, *matK*, *nad1*, and *rbcL*) or 1.5 μL (*CAB* and *NEEDLY*) genomic DNA.

Amplification and internal sequencing primers were designed for 26S from publicly available *Ginkgo, Zamia*, and *Cycas* sequences (GenBank AY095475, DQ008666, and DQ008667; 5′-AAG-TAC-CGC-GAG-GGA-AAG-AT-3′ and 5′-CTA-AAC-CCA-GCT-CAC-GTT-CC-3′). Cycling conditions were 95°C 150 s; 35 cycles: 95°C 30 s, 57°C 30 s, 72°C 180 s; 600-s final extension. In addition to the amplification primers, internal sequencing primers were also used (5′-TCT-CAA-ACT-TTA-AAT-AGG-3′, 5′-TTT-AAC-AGC-CTG-CCC-ACC-C-3′, 5′-GCA-TGA-ATG-GAT-TAA-CGA-G-3′, 5′-CCC-GCG-CTT-GGT-TGA-ATT-TC-3′, 5′-GGA-ACC-TTT-CCC-CAC-TTC-AG-3′, and 5′-CGG-GCC-TCC-ACC-AGA-GTT-TC-3′).

Amplification and sequencing primers were designed for *CAB* from publicly available *Zamia* and *Cycas* sequences (GenBank CB090681, CB092784, DY034831, FD769126, and FD769420; 5′-GGG-AGG-AGT-TCA-TGG-AGA-AG-3′ and 5′-CCG-AAC-ATG-GAA-AAC-ATG-G-3′). Cycling conditions were 95°C 150 s; 35 cycles: 95°C 30 s, 58°C 30 s, 72°C 30 s; 600-s final extension.

The region containing *matK* and the *trnK* intron was amplified using primers designed by Tsumura et al. (1995; 5′-

AAC-CCG-GAA-CTA-GTC-GGA-TG-3′ and 5′-TCA-ATG-GTA-GAG-TAC-TCG-GC-3′). Cycling conditions were 95°C 150 s; 35 cycles: 95°C 30 s, 55°C 30 s, 72°C 120 s; 500-s final extension. In addition to the amplification primers, internal sequencing primers were also used (5′-ATC-TNT-YAG-ATG-GAT-CAT-T-3′, 5′-CTG-GAT-YCA-AGA-TGC-TCC-TT-3′, 5′-GTT-TTK-TGA-TAT-CTC-AGG-3′, 5′-TGG-ATC-GAA-TTY-CTT-GGT-AAT-G-3′, 5′-GTT-CGA-ACC-AAA-ATY-TCC-GG-3′, and 5′-GCG-ACC-TGA-TCG-CTC-TCC-TGA-CT-3′).

The second intron of *nad1* was amplified using primers designed by Demesure et al. (1995; 5′-GCA-TTA-CGA-TCT-GCA-GCT-CA-3′ and 5′-GGA-GCT-CGA-TTA-GT-TT-CT-GC-3′). Cycling conditions were 95°C 150 s; 35 cycles: 95°C 30 s, 57°C 30 s, 72°C 60 s; 600-s final extension. In addition to the amplification primers, a pair of internal sequencing primers was used for all species (5′-AGA-GTA-AAG-GGC-TGT-AGG-3′ and 5′-CTA-AAA-AGA-AGC-TGC-GTG-AGG-3′); for *Cycas* species, an additional pair of sequencing primers was required (5′-TAT-GGC-CGA-TCT-GTC-ACC-3′ and 5′-TAT-GAG-GAG-TAG-GAG-CAG-3′).

A portion of the *NEEDLY* coding region (with small embedded introns) was amplified using primers specifically designed to amplify *NEEDLY* (GenBank AF105111 and AF105108) to the exclusion of LEAFY (GenBank AF105107 and AF108228; 5′-AGA-TGG-GYT-TCA-CYG-CTA-ACA-CTC-3′ and 5′-AAR-ACS-CCY-TCR-ATG-TCC-CAA-TTG-3′). Cycling conditions were 95°C 150 s; 35 cycles: 95°C 30 s, 55°C 30 s, 72°C 90 s; 600-s final extension. For sequencing, a pair of internal sequencing primers was used in addition to the amplification primers (5′-CAC-AGC-AGT-GAC-AGC-GAC-G-3′ and 5′-GGC-RAG-TTC-CCC-GGG-CTC-3′).

Amplification primers for the coding region of *rbcL* were F1 (5′-ATG-TCA-CCA-CAA-ACA-GAA AC-3′) and 1379R (5′-TCA-CAA-GCA-GCA-GCT-AGT-TCA-GGA-CTC-3′). Cycling conditions were 95°C 150 s; 35 cycles: 95°C 30 s, 55°C 30 s, 72°C 60 s; 600-s final extension. For sequencing, a pair of internal sequencing primers was used in addition to the amplification primers (5′-GCG-TTG-GAG-AGA-TCG-TTT-CT-3′ and 5′-GAA-ACG-GTC-TCT-CCA-ACG-CAT-3′).

PCR products were treated with ExoSAP-IT (Affymetrix, Santa Clara, CA) and sequenced with BigDye 3.1 chemistry on an ABI 3730 sequencer (Life Technologies, Carlsbad, CA; High-Throughput Genomics Unit, University of Washington, Seattle).

Constructing Phylogenies

Sequences were assembled and edited in Sequencher 4.10 (Gene Codes, Ann Arbor, MI), automatically aligned with MUSCLE 3.8 (Edgar 2004), and manually adjusted with Jalview 2.7 (Waterhouse et al. 2009). Additional binary characters describing insertion/deletion (indel) span and distribution were coded using simple gap coding (Simmons and Ochoterena 2000) as implemented in 2matrix (Salinas and Little 2014).

A combined data matrix was analyzed with TNT 1.1 (Goloboff et al. 2008) and RAxML 8.0.17 (Stamatakis 2006). Sequence indels (indicated by dashes) were treated as missing data (the binary characters describing them were not). For the TNT analysis, branches were collapsed if supported ambiguously. One thousand random addition sequence replicates holding two trees per replicate were conducted. Each replicate was first exhaustively swapped with subtree pruning and regrafting followed by tree-bisection-reconnection (TBR) and then subjected to a 200-iteration ratchet (Nixon 1999) perturbing 10% of the informative characters using equal probability for up- and downweighting with other parameters set to the default. Trees were then swapped to completion using TBR. One thousand jackknife resamplings were used to assess support (Farris et al. 1996). Each resampling was searched using 10 random addition sequences holding 10 trees per search and swapped with TBR. The strict consensus of each resampling was used to calculate jackknife frequency (fig. 2). For the RAxML analysis, the general time-reversible substitution model was applied to nucleotide characters, and the binary model was applied to indel characters. Both models used γ correction for rate heterogeneity (the α parameter was automatically estimated). We conducted 10 random addition replicates, followed by branch swapping. We calculated 1,000 rapid bootstrap resamplings (Stamatakis et al. 2008) to assess support (fig. 2).

We have only a partial *matK* sequence for *Chigua restrepoi*. We have full sequence data for all other markers for all other species analyzed. All sequences generated for this study were deposited in GenBank (app. A).

Because of uncertainty in placement of *Bowenia* and *Stangeria* within the Cycadales (Zgurski et al. 2008), we constructed nine alternative phylogenetic trees (figs. B1–B9, available online), based solely on the following criteria (reviewed in Zgurski et al. 2008): (1) monophyletic genera, with the exception of *Zamia* containing *Chigua*; (2) monophyletic Cycadaceae; (3) monophyletic Zamiaceae; (4) monophyletic *Ceratozamia*/*Microcycas*/*Zamia*/*Chigua*; (5) monophyletic *Microcycas*/*Zamia*/*Chigua*; (6) monophyletic *Macrozamia*/*Lepidozamia*/*Encephalartos*; (7) monophyletic *Lepidozamia*/*Encephalartos*; (8) *Bowenia* floating within Zamiaceae; and (9) *Stangeria* floating within Zamiaceae.

Phylogenetic Comparative Methods

All phylogenetic comparative analyses were done using the GEIGER package in R (Harmon et al. 2008). All analyses were performed for the single most parsimonious tree (fig. 2) and nine alternative trees with different placements of *Bowenia* and *Stangeria* (figs. B1–B9).

We tested for a relationship between 2C value and chromosome number using phylogenetic generalized least squares (PGLS) regression. PGLS creates a covariance matrix based on the distance of taxa on the tree (Grafen 1989; Paradis 2006). PGLS does not compute ancestral values, as do phylogenetically independent contrasts, thus avoiding assumptions about ancestral states. PGLS also enables the variation of evolutionary model, unlike independent contrasts, which are modeled only as a Brownian motion process (Felsenstein 1985). The maximum likelihood method of PGLS also enables model selection using ad hoc statistics, thereby enabling selection of model in the absence of a priori evolutionary knowledge.

Using Akaike Information Criterion, we selected among branch length estimation techniques and evolutionary models. We used three evolutionary models: Brownian motion (stochasticity), Ornstein-Uhlenbeck (OU; stabilizing selection; Martins and Hansen 1997; Butler and King 2004), and Blomberg's model of varying evolutionary rates (Blomberg et al. 2003).

The Brownian motion model is

$$V\{X_i\} = \sigma^2 T_a .$$

The Ornstein-Uhlenbeck model is

$$V\{X_i\} = \frac{1 - d^{2(T_{ij} - T_j)}}{1 - d^2} \sigma^2 \gamma .$$

The Blomberg model is

$$V\{X_i\} = \frac{1 - g^{-(T_{ij} - T_j)}}{1 - g^{-1}} \sigma^2 \gamma .$$

In these equations X_i is the variance of the base-to-tip branch length for species i, $V\{X_i\}$ is the resulting covariance matrix, T is the distance between the root of the tree and the most recent common ancestor (therefore, T_{ij} is the distance between taxa i and j), and σ^2 is the variance due to Brownian motion. For the Blomberg model of varying evolutionary rates, g is the rate of evolution. For the OU model, d describes the degree of stabilizing selection, where $d = 1$ is Brownian motion and $d < 1$ indicates strong stabilizing selection (Blomberg et al. 2003).

We used two branch length estimation techniques, including setting all branch lengths to 1 (Pagel 1992) and Grafen's branch length scaling factor. Grafen's method sets all branch lengths as the number of branches minus 1 and then scales the branch lengths based on the degree of evolution above and below a focal branch (Grafen 1989).

To test for the effect of branch length assignment on type I error rate (P value), we used an iterative procedure. For each iteration we randomly assigned branch lengths to the cycad tree and computed the type I error rate. We repeated this procedure over 10,000 iterations for each of the nine trees and for all three evolutionary models listed above (for a total of 270,000 iterations).

Ancestral State Reconstruction

To visualize ancestral character states for chromosome numbers in Cycadales, we created character maps using the contMAP function in the phytools R package (Revell 2013). Stochastic character mapping involves evaluating the proportional probability of character states along the tree. Continuous trait mapping involves estimating ancestral states at each node in the tree using a maximum likelihood approach followed by interpolation of trait states along each branch (Revell 2013). In this case, however, we used two phylogenetic trees, the single most parsimonious tree and the maximum likelihood tree, so we show the results of two stochastic character maps.

Results

We compiled data on 2C values and numbers of chromosomes for 22 cycad species, which included at least one species in each of the 11 extant Cycadales genera (table 1; fig. 1). Table 1 also includes four samples (indicated by underlining) from the Gymnosperm DNA C-Values Database (Murray et al. 2010). We did not have plant material suitable for flow cytometry for three of the published species: *Cycas circinalis* L., *Encephalartos villosus* Lem., and *Zamia angustifolia* Jacq. The fourth species, *Cycas revoluta*, was in the Gymnosperm DNA C-Values Database (25.5 pg), and we performed flow cytometry on it (27.4 pg). These two independent estimates for *C. revoluta* are somewhat similar. However, the Gymnosperm DNA C-Values Database values for the other three species are very different from the measurements we obtained for the congeners (data not shown). 2C values are consistent within species (data not shown), and values within genera hardly differed (table 2).

Figure 1, which does not account for phylogeny, shows no significant correlation between numbers of chromosomes and 2C values; i.e., the regression between these two variables is not statistically different from 0 ($P = 0.06$, t-test $= -1.975$, df $= 1$, $R^2 = 0.17$).

Analysis of sequence data produced a single most parsimonious tree (length = 2639, consistency index = 0.71, retention index = 0.86; fig. 2). Jackknife support for generic monophyly is uniformly high, but a portion of the tree backbone is not well supported—reflecting instability in the placement of *Bowenia* and *Stangeria* commonly observed in Cycadales data sets (Zgurski et al. 2008). Maximum likelihood analysis resulted in the same tree topology (ln likelihood = −32,163.288159; fig. 2), with similarly depressed support values along a portion of the tree backbone. Preliminary phylogenetic analyses, using a subset of the sequence data reported herein, resulted in several different placements of *Bowenia* and *Stangeria*—indicating inherent instability of the data set or the underlying phylogeny.

Table 3 details tests of relationship between genome size and chromosome number, after accounting for phylogeny, using the single most parsimonious tree/maximum likelihood tree and using three methods of branch length estimation and three different evolutionary models. Regardless of branch length estimation technique or model used, genome size was unrelated to number of chromosomes. None of the models yielded a statistically significant regression at the 0.05 level.

Because of the unresolved placement of *Bowenia* and *Stangeria* in the Cycadales, we reran the phylogenetic comparative methods with nine alternative phylogenetic trees (figs. B1–B9). None of the analogues of table 3 based on these nine alternative trees—either with or without inclusion of values from the Gymnosperm DNA C-Values Database—yielded a statistically significant correlation at the 0.05 level; i.e., there were no statistically significant correlations out of 162 individual tests. Table S1, part a, and table S1, part b, available online, are analogues of table 3, part a, and table 3, part b, but with the nine alternative trees in lieu of the single most parsimonious tree/maximum likelihood tree.

We used an iterative procedure to test for the effects of branch length estimation and evolutionary model on type I

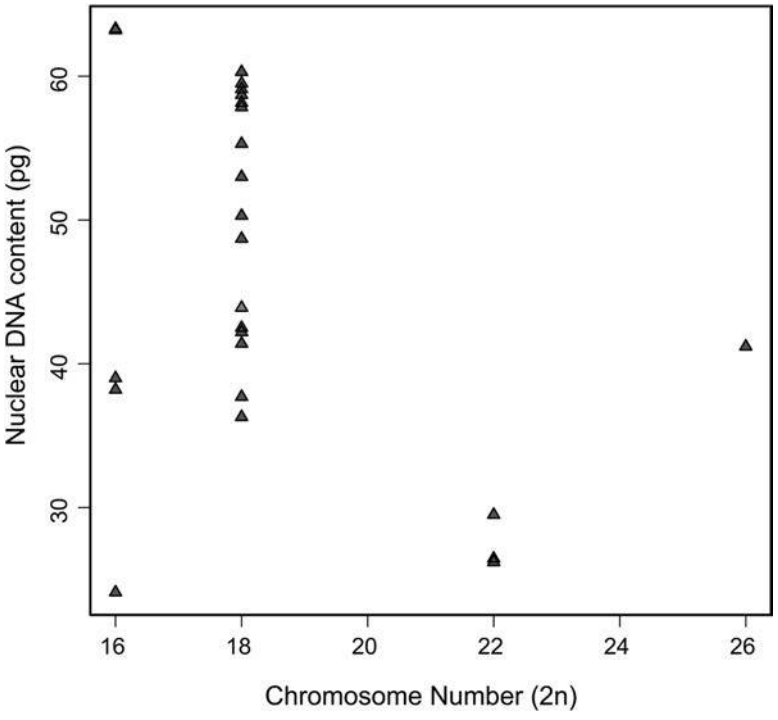

Fig. 1 2C value versus chromosome number in Cycadales ($P = 0.06$, t-test $= -1.975$, $R^2 = 0.17$). Without accounting for phylogeny, there is no correlation between cycad DNA content and chromosome number. Slope of the regression is statistically indistinguishable from 0.

error rate. For all three models and all phylogenetic trees (our single most parsimonious tree/maximum likelihood tree plus the nine alternative trees as suggested by Zgurski et al. [2008]), 90%–99% of the 10,000 iterations yielded a nonsignificant relationship between 2C value and chromosome number among the sampled cycad taxa (type I error rates of 0.90–0.99). Even with randomly assigned branch lengths, none of the models yielded a statistically significant correlation at the 0.05 level.

We reconstructed the ancestral chromosome number as 2n=22 for all Cycadales (fig. 2), with stasis or a subsequent reduction in chromosome numbers in the ancestor leading to all but one other cycad taxa used in this study. *Microcycas calocoma* is the only species to subsequently show an increase in chromosome number. However, had we included several species of *Zamia* in our analysis—with 2n ranging between 16 and 28 (Olson and Gorelick 2011), species for which we could not obtain specimens for flow cytometry to measure 2C values—the ancestral character reconstruction in figure 2 would probably have been far different.

Table 2

Coefficients of Variation of 2C Values with 22 Species

Genus	No. species studied	No. species in genus	CV
Bowenia	2	2	.029
Ceratozamia	2	27	.001
Chigua	1	0	…
Cycas	2 (3)	107	.022 (.055)
Dioon	2	14	.016
Encephalartos	5 (6)	65	.023 (.661)
Lepidozamia	2	2	.043
Macrozamia	1	41	…
Microcycas	1	1	…
Stangeria	1	1	…
Zamia	3 (4)	71	.037 (.170)

Note. Values in parentheses are with 25 species after inclusion of data from the Gymnosperm DNA C-Values Database. The coefficients of variation (CV) were computed using the mean value of genome content for each species in a genus, with the mean computed using one value for each specimen accessioned. The total number of species per genus is from Osborne et al. (2012), who subsumed *Chigua* in *Zamia*.

Discussion

To determine whether chromosome numbers are the result of DNA duplication, we estimated the relationship between chromosome numbers and nuclear DNA content. If a positive correlation exists, then large portions of chromosomes were likely duplicated, as with whole-genome duplication or polysomy. If no correlation exists, then chromosome numbers likely changed via chromosomal fission or fusion, as well as replication or deletion of individual genetic loci. DNA duplication can include transposable elements or duplicated satellite DNA, which can substantially alter 2C values without altering ploidy. For example, the congeneric palms *Pinanga coronata* Blume and *Pinanga subintegra* Ridl. each have 16

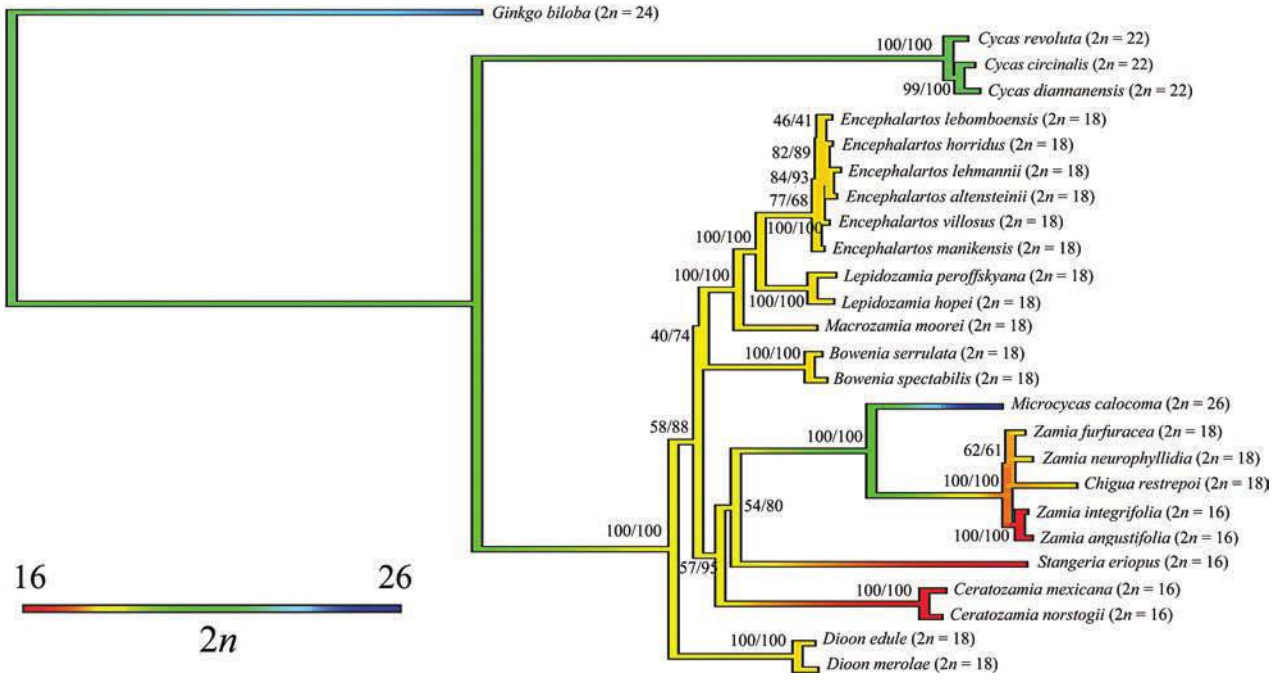

Fig. 2 Single most parsimonious tree and maximum likelihood tree of combined 26S, *CAB*, *matK*, *nad1*, *NEEDLY*, and *rbcL* used for phylogenetic generalized least squares (with only a partial *matK* sequence for *Chigua restrepoi*) with ancestral character estimates of chromosome numbers. Branch lengths reflect the minimum number of parsimoniously reconstructed changes. Parsimony jackknife (left of slash) and likelihood bootstrap (right of slash) values are indicated at nodes. Tree length = 2639 steps; consistency index = 0.71 (excluding uninformative characters); retention index = 0.86; ln likelihood = −32,163.288159. Colors reflect chromosome counts of the branch tips, as well as ancestral character reconstruction of chromosome numbers.

pairs of chromosomes (Sarkar 1970) but a huge difference in 4C values, 35.4 versus 55.6 pg (Röser et al. 1997). Likewise, all plants in *Helleborus* Gueldenst. (Ranunculaceae) have 16 pairs of chromosomes, yet 2C values vary from 19.0 to 35.7 pg (Zonneveld 2001). Similar ranges in 2C content without differences in chromosome numbers are found in kangaroo rats *Dipodomys* Gray (John and Miklos 1979), due to differences in satellite DNA, and a two-fold difference in 1C values in rice *Oryza* L., due to transposable elements (Chen and Wu 1982; Piegu et al. 2006). For our study, such duplicated satellite DNA or transposons are indistinguishable from other forms of duplicated DNA.

To see that, ceteris paribus, duplication of genetic loci, chromosomal fission, and chromosomal fusion does not result in a relationship between chromosome numbers and 2C values, consider each of these phenomena separately. Proliferation of transposable elements within a genome will increase C value without altering chromosome number. In fact, duplication of genetic elements should eventually result in more stable (i.e., less fissionable) chromosomes because duplicated loci are usually highly heterochromatic, albeit out of necessity to silence the transposable elements (Kejnovsky et al. 2012; Slotkin et al. 2012). By contrast, without proliferation of transposable elements, repeated chromosomal fissions will increase the number of chromosomes without altering 2C values. In either case, correlation between chromosome numbers and 2C values is attenuated.

We examined chromosome numbers that varied between 2n=16 and 2n=26, reflecting virtually the entire known range in the Cycadales (which extends to 2n=28). In our data set, Cycadales 2C values varied from 26.2 to 63.3 pg. There does not appear to be any relationship between chromosome numbers and 2C values, either with or without accounting for phylogeny (table 3 and fig. 1, respectively; also see table S1, pt. a, pt. b), consistent with the prevailing notion that there has been no recent whole-genome duplication in Cycadales or duplication of any significant portion of the genome (Gorelick and Olson 2011). The nonsignificant (statistically no different from 0) relationship between 2C values and numbers of chromosomes indicates that neither whole-genome duplication nor polysomy has occurred. Cycadales chromosome numbers have instead largely varied by rearrangement via chromosomal fission/fusion or replication/deletion of individual genetic loci.

The low jackknife and bootstrap support values along the middle backbone of our single most parsimonious tree and the maximum likelihood tree (fig. 2) reflect instability in the final data set. Sampling plastid genomes has not resolved placement of *Bowenia* and *Stangeria* (Zgurski et al. 2008); hence, we sampled nuclear and mitochondrial markers in addition to plastid markers but still cannot report a robust placement of *Bowenia* or *Stangeria*. Our phylogenetic trees are in agreement with Zgurski et al. (2008), with the exception of *Stangeria*. However, our regression results (PGLS) were insensi-

Table 3

Correlations between 2C Values and Chromosome Numbers including and excluding Four Values from Gymnosperm C-Value Database

Branch length and model	AIC	±SD	ΔAIC	*t*	*P*
a. Including database values:					
Grafen's method:					
Brownian motion	138.71	…	.00	−.60	.56
Blomberg's ACDC	140.54	…	1.83	−.48	.64
Ornstein-Uhlenbeck	143.05	…	4.34	−.75	.46
All branch lengths set to 1:					
Brownian motion	142.61	…	.00	−.53	.60
Blomberg's ACDC	142.78	…	.17	−.20	.84
Ornstein-Uhlenbeck	160.37	…	17.76	−2.84	.01
Random assignment:					
Brownian motion	185.46	4.69	.00	…	1.00
Blomberg's ACDC	185.97	4.00	.51	…	1.00
Ornstein-Uhlenbeck	189.74	2.00	4.28	…	1.00
b. Excluding database values:					
Grafen's method:					
Brownian motion	138.71	…	.00	−.60	.56
Blomberg's ACDC	140.10	…	1.39	−.88	.39
Ornstein-Uhlenbeck	143.05	…	4.34	−.75	.46
All branch lengths set to 1:					
Brownian motion	142.61	…	.00	−53.00	.60
Blomberg's ACDC	142.78	…	.17	−.20	.84
Ornstein-Uhlenbeck	160.37	…	17.16	−2.84	.01
Random assignment:					
Brownian motion	142.13	3.10	.00	…	1.00
Blomberg's ACDC	145.63	2.40	3.50	…	1.00
Ornstein-Uhlenbeck	154.33	1.69	12.20	…	1.00

Note. The best-fit model is underlined. None of the correlations is statistically significant. ACDC, accelerated/decelerated; AIC, Akaike Information Criterion; SD, standard deviation.

tive to the fluid/uncertain placement of *Bowenia* and *Stangeria* within the Cycadales. Regardless of phylogeny used, PGLS always yielded regression coefficients that were not statistically different from 0, i.e., no significant correlation.

2C values are consistent within species, and values within genera hardly differ, which may reflect low species sampling or that within each extant genus, the oldest extant species diverged from a common ancestor not more than 12 million years ago (Nagalingum et al. 2011). Clearly, the 2.5-fold range of 2C values in species we examined shows that large portions of the genome have been lost, gained, or duplicated since the last common ancestor of all extant Cycadales. *Ceratozamia* provides a possible example of duplication, with 2C values of 63 pg, whereas all other members of the Zamioideae (*Zamia, Ceratozamia, Microcycas, Bowenia*) have 2C values around 40 pg. This suggests a possible whole-genome duplication from 40 to 80 pg, with a subsequent loss of one or more chromosomes to end up with a 2C value of 63 pg, plus massive chromosome fusion or diploidization, resulting in extant *Ceratozamia* with 2n=16 chromosomes. It is also possible that ancestors of *Ceratozamia* simply underwent massive duplication of parts of their genome.

Unfortunately, we only have a few 2C values from *Zamia*, the genus of Cycadales with the largest variation in numbers of chromosomes, from 2n=16 to 2n=28, including all intermediate values except 2n=20 (Olson and Gorelick 2011). Our data set contains only 2n=16 and 2n=18 for *Zamia*, although we have included the closely related *Microcycas calocoma* (Miq.) DC with 2n=26. Our results would be more robust if 2C values could be obtained for *Zamia* species with high and variable numbers of chromosomes, such as *Zamia paucijuga* Wieland (2n=19, 23–28), *Zamia pracina* W. Bull. (2n=17, 22–28), and *Zamia roezlii* Regel (2n=22, 24–26). This will require obtaining 2C values and chromosome numbers from the same specimen, which has not yet been done for cycads. Nonetheless, *Zamia* 2C values are relatively variable (B. J. M. Zonneveld, unpublished data), contrary to the relatively constant 1C values alluded to by Cafasso et al. (2009), who conducted flow cytometry of *Z. roezlii* and six specimens of *Z. paucijuga*. Previously, we examined chromosomal morphology in *Zamia* (Olson and Gorelick 2011), which provided evidence for extensive chromosomal fission in this genus, consistent with our more general results herein for all Cycadales.

Coverage of Cycadales in the IPCN (Goldblatt and Johnson 2008) is uneven. IPCN coverage is good for several genera, such as *Zamia* (34 species); *Bowenia* (2 species); and *Chigua*, *Microcycas*, and *Stangeria* (1 species each). However, IPCN coverage is not as thorough in *Dioon* (1 species) and *Encephalartos* (2 species). Coverage is intermediate for *Ceratozamia* (9 species), *Cycas* (19 species), and *Macrozamia* (8 species). See table 2 for the currently accepted number of spe-

cies per cycad genus (Osborne et al. 2012). Correlation between chromosome number and 2C values could change when data are collected for more species.

Currently, 1C and 2C values exist only for four species of cycads in the Gymnosperm DNA C-Values Database (Murray et al. 2012), plus the values reported in Zonneveld (2011). While inclusion of these additional four data points did not affect our primary result of no correlation between chromosome numbers and 2C values (table 3, part a vs. part b), some of the values in the Gymnosperm DNA C-Values Database seem inconsistent with the 2C values that we measured. Our flow cytometry measurements were constant within each species and within each genus (table 1). However, data from the Gymnosperm DNA C-Values Database, especially those of *Encephalartos villosus* and *Zamia angustifolia*, were very different from the 2C values we measured for those genera, greatly increasing coefficients of variation for those genera (table 2). The four values in the Gymnosperm DNA C-Values Database were all obtained using Feulgen microdensitometry, which is known to require much practice (Bennett and Leitch 1997, 2003; Palomino et al. 1999; Greilhuber 2005; Zonneveld 2011). Phenolic compounds, which are present at high concentrations in cycads (Wallace 1972), can impede DNA quantification using the Feulgen reaction (Greilhuber 1998) and are perhaps responsible for the discrepancy between the results obtained by Feulgen microdensitometry and flow cytometry (Doležel et al. 2007). Therefore, despite flow cytometry also having pitfalls (Greilhuber et al. 2007), albeit fewer pitfalls (Doležel et al. 2007), we place greater weight in table 3, part b, than in table 3, part a.

Our results indicate that recent cycad genome evolution has probably been primarily due to fusion/fission of chromosomes or replication/deletion of individual genetic loci, as indicated by how 2C values are constant within each genus but different between genera. This is also consistent with an ancestral chromosome number of 2n=22 in the Cycadales (fig. 2), especially given that 2n=22 is the midpoint between the minimum (2n=16) and maximum (2n=28) number of chromosomes found in extant cycads.

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Appendix A

Voucher Information and GenBank Accessions for Analyzed DNA Sequences

Ginkgoaceae: *Ginkgo biloba* L., *Little and Brenner 915* [NY], 26S: KF221113, *matK*: JQ512414, *nad1*: KF221159, *NEEDLY*: KF221197, *rbcL*: KF221173.

Cycadaceae: *Cycas circinalis* L., *Little and Stevenson 1099* [FGT, NY], 26S: KF221102, *CAB*: KF221123, *matK*: KF221149, *nad1*: KF221163, *NEEDLY*: KF221201, *rbcL*: KF221177. *Cycas diannanensis* Z.T. Guan & G.D. Tao, *Little and Stevenson 960* [FGT, NY], 26S: KF221103, *CAB*: KF221125, *matK*: KF221150, *nad1*: KF221164, *NEEDLY*: KF221202, *rbcL*: KF221178. *Cycas revoluta* Thunb., *Little and Stevenson 987* [FGT, NY], 26S: KF221104, *CAB*: KF221124, *matK*: KF221151, *nad1*: KF221165, *NEEDLY*: KF221203, *rbcL*: KF221179.

Zamiaceae: *Bowenia serrulata* (W. Bull) Chamb., *Little and Stevenson 1004* [FGT, NY], 26S: KF221098, *CAB*: KF221133, *matK*: GQ203825, *nad1*: GQ203934, *NEEDLY*: GQ203971. *Bowenia spectabilis* Hook. ex Hook.f., *Little and Stevenson 1005* [FGT, NY], 26S: KF221099, *CAB*: KF221134, *matK*: GQ203826, *nad1*: KF221160, *NEEDLY*: KF221198, *rbcL*: KF221174. *Ceratozamia mexicana* Brongn., *A. VB. 191* [XAL], 26S: KF221100, *CAB*: KF221126, *matK*: KF221146, *nad1*: KF221161, *rbcL*: KF221175. *Ceratozamia mexicana* Brongn., *Little and Stevenson 1009* [FGT, NY], *NEEDLY*: KF221199. *Ceratozamia norstogii* D.W. Stev., *Little and Stevenson 1014* [FGT, NY], 26S: KF221101, *CAB*: KF221127, *matK*: KF221147, *nad1*: KF221162, *NEEDLY*: KF221200, *rbcL*: KF221176. *Chigua restrepoi* D.W. Stev., *Stevenson 693* [NY], *matK*: KF221148. *Dioon edule* Lindl., *Little and Stevenson 1092* [FGT, NY], 26S: KF221105, *CAB*: KF221135, *matK*: GQ203838, *nad1*: GQ203951, *NEEDLY*: GQ203968, *rbcL*: KF221180. *Dioon merolae* De Luca, Sabato & Vázq.-Torres, *Little and Stevenson 1122* [FGT, NY], 26S: KF221106, *CAB*: KF221136, *matK*: GQ203840, *nad1*: GQ203949, *NEEDLY*: GQ203969, *rbcL*: KF221181. *Encephalartos altensteinii* Lehm., *Little and Stevenson 1019* [FGT, NY], 26S: KF221107, *CAB*: KF221142, *matK*: KF221152, *nad1*: KF221166, *NEEDLY*: KF221204, *rbcL*: KF221182. *Encephalartos horridus* (Jacq.) Lehm., *Little and Stevenson 1028* [FGT, NY], 26S: KF221108, *CAB*: KF221141, *matK*: KF221153, *nad1*: KF221167, *NEEDLY*: KF221205, *rbcL*: KF221183. *Encephalartos lebomboensis* I. Verdoorn, *Little and Stevenson 1030* [FGT, NY], 26S: KF221109, *CAB*: KF221140, *matK*: KF221154, *nad1*: KF221168, *NEEDLY*: KF221206, *rbcL*: KF221184. *Encephalartos lehmannii* Lehm., *Little and Stevenson 1031* [FGT, NY], 26S: KF221110, *CAB*: KF221143, *matK*: KF221155, *nad1*: KF221169, *NEEDLY*: KF221207, *rbcL*: KF221185. *Encephalartos manikensis* (Gilliland) Gilliland, *Little and Stevenson 1033* [FGT, NY], 26S: KF221111, *CAB*: KF221144, *matK*: GQ203847, *nad1*: GQ203946, *NEEDLY*: GQ203979, *rbcL*: KF221186. *Encephalartos villosus* Lem., *Little and Stevenson 1047* [FGT, NY], 26S: KF221112, *CAB*: KF221145, *matK*: GQ203849, *nad1*: GQ203940, *rbcL*: KF221187. *Lepidozamia hopei* (W. Hill) Regel, *Little and Stevenson 1049* [FGT, NY], 26S: KF221114, *CAB*: KF221138, *matK*: KF221156, *nad1*: KF221170, *NEEDLY*: KF221208, *rbcL*: KF221188. *Lepidozamia peroffskyana* Regel, *Little and Stevenson 1050* [FGT, NY], 26S: KF221115, *CAB*: KF221139, *matK*: GQ203851, *nad1*: GQ203938, *NEEDLY*: GQ203974, *rbcL*: KF221189.

Macrozamia moorei F. Muell., *Little and Stevenson 1059* [FGT, NY], 26S: KF221116, *CAB*: KF221137, *matK*: GQ203853, *nad1*: GQ203942, *NEEDLY*: GQ203973, *rbcL*: KF221190. *Microcycas calocoma* (Miq.) A. DC., *Little and Stevenson 1063* [FGT, NY], 26S: KF221117, *CAB*: KF221128, *matK*: GQ203854, *nad1*: GQ203920, *NEEDLY*: GQ203954, *rbcL*: KF221191. *Stangeria eriopus* (Kunze) Baill., *Little and Stevenson 1006* [FGT, NY], 26S: KF221118, *matK*: GQ203855, *nad1*: GQ203919, *NEEDLY*: GQ203970, *rbcL*: KF221192. *Zamia angustifolia* Jacq., *Little and Stevenson 1066* [FGT, NY], 26S: KF221119, *CAB*: KF221130, *matK*: GQ203857, *nad1*: GQ203927, *NEEDLY*: GQ203957, *rbcL*: KF221193. *Zamia furfuracea* L.f., *Little and Stevenson CC288* [FGT, NY], 26S: KF221120, *CAB*: KF221131, *matK*: GQ203858, *nad1*: GQ203924, *NEEDLY*: GQ203960, *rbcL*: KF221194. *Zamia integrifolia* L.f., *Turnbull FA62291A* [NY], 26S: KF221121, *CAB*: KF221129, *matK*: KF221157, *nad1*: KF221171, *NEEDLY*: KF221209, *rbcL*: KF221195. *Zamia neurophyllidia* D.W. Stev., *Stevenson 1255* [NY], 26S: KF221122, *CAB*: KF221132, *matK*: KF221158, *nad1*: KF221172, *NEEDLY*: KF221210, *rbcL*: KF221196.

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