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Molecular Systematic Studies in Cycads: Evidence from *trn*L Intron and ITS2 rDNA Sequences

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I. Abstract

The results of a pilot DNA sequencing study of cycads conducted at the new molecular systematics laboratory at Fairchild Tropical Garden are presented and assessed with reference to previous phylogenetic analyses and classification schemes based on morphology and anatomy. Two DNA regions were sequenced and analyzed for variation, an intron in the *trn*L gene in the chloroplast genome (*trn*L intron) and the internal transcribed spacer region between the 5.8S and 26S ribosomal DNA subunits (ITS2). The *trn*L intron proved to be relatively conservative among cycad genera, while the ITS2 region contained higher levels of variation. Parsimony analysis of the sequences suggests a number of relationships, some of which were inferred by previous morphological studies, some of which are new. The sequences of *Cycas* are the most

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divergent among cycads, suggesting the longest isolation. *Dioon* is relatively isolated from the other genera and contains two major clades. *Stangeria* does not appear closely related to *Bowenia* but does seem to have a weak affinity with *Zamia* and *Microcycas*. *Lepidozamia* is more closely related to *Encephalartos* than to *Macrozamia*. Sequence variation among the species of *Ceratozamia* is low. *Microcycas* and *Zamia* are closely related.

II. Introduction

Fairchild Tropical Botanic Garden has long been an important center for the study of cycad biology. The extensive collections of cycads at Fairchild Tropical Garden and the associated Montgomery Botanical Center are among the best in the world and have been utilized in a wide variety of studies (Norstog, 1990; Norstog & Nicholls, 1997). Recently, a molecular systematics laboratory was jointly established between Florida International University and Fairchild Tropical Botanic Center for the purpose of promoting studies of cycads, palms, and other tropical plants. One of the first projects initiated in this new laboratory was a molecular systematics survey of cycads. Plans have been made for future, more extensive studies of molecular variation in the New World cycads. In this article we report the results of a pilot study of variation in *trn*L intron and ITS2 sequences in cycads.

Cycads are an ancient group of gymnosperms that were abundant and widely distributed during the Mesozoic but are today much less common and largely confined to isolated tropical regions (Norstog & Nicholls, 1997). The 11–12 genera of cycads currently recognized are thought to constitute a monophyletic group classified as a single order, the Cycadales, which is divided into three or four families (Johnson & Wilson, 1990; Stevenson, 1992). In these systems of classification, *Cycas* is distinguished from the other genera by its leaflike, loosely arranged, multiovulate megasporophylls, and it is placed in its own family, the Cycadaceae. *Stangeria* and *Bowenia* have distinctive leaves and are grouped together in the family Stangeriaceae (Stevenson, 1992) or placed in different families (Johnson & Wilson, 1990), but their precise affinities remain unsettled. The rest of the genera are grouped into Zamiaceae. Efforts to resolve the phylogeny of cycad genera more precisely using cladistic methods were made by Crane (1988), Stevenson (1990a), and Schutzman and Dehgan (1993). Those analyses have stimulated discussion about character states and evolutionary trends, but they differ in their results and conclusions. The formal classification of cycads proposed by Stevenson (1992), based on cladistic analysis of morphological and anatomical characters, serves as a useful point of reference and is shown in Fig. 1.

Molecular data have an advantage over morphological data in that they provide an almost unlimited number of discrete characters with which to compare taxa and also usually present fewer problems with homology assessment and character convergence (Hillis et al., 1996). One of the first molecular systematic studies of cycads was made by Caputo et al. (1991), who examined restriction fragment length polymorphisms (RFLPs) in the five genera of New World cycads and *Cycas,* which was used as an outgroup. Parsimony analyses of these data indicated that *Dioon* was the sister group to the other American genera, with *Ceratozamia* as the sister group to a clade containing *Microcycas, Zamia,* and *Chigua.* As indicated by bootstrapping support, these clades were strongly resolved, and the topology was congruent with the classification based on morphological characters. The RFLP analysis was extended to include the Old World genera (Caputo et al., 1993), but the results were at odds with classification based on morphology (sensu Stevenson, 1990a), although removal of *Macrozamia* from the analysis restored congruence. These studies demonstrated the potential utility of molecular markers for resolving generic relationships in the cycads.

In the last ten years DNA sequence data have become easier to obtain and consequently more widely used for reconstruction of plant phylogenies. In angiosperms the internal

Cycadales
- Suborder Cycadiineae
  - Family Cycadaceae
    - *Cycas*
- Suborder Zamiineae
  - Family Stangeriaceae
    - Subfamily Stangerioideae
      - *Stangeria*
    - Subfamily Bowenioideae
      - *Bowenia*
  - Family Zamiaceae
    - Subfamily Encephalartoideae
      - Tribe Diooeae
        - *Dioon*
      - Tribe Encephalarteae
        - Subtribe Encepahalartinae
          - *Encephalartos*
        - Subtribe Macrozamiinae
          - *Macrozamia*
          - *Lepidozamia*
    - Subfamily Zamioideae
      - Tribe Ceratozamieae
        - *Ceratozamia*
      - Tribe Zamieae
        - *Microcycas*
        - *Zamia*

Fig. 1. Classification of cycads, adapted from Stevenson (1992).

transcribed spacer (ITS) regions in nuclear ribosomal DNA have been especially important in phylogenetic studies comparing taxa at the generic level of divergence (Baldwin et al., 1995). These two spacers are located between the 18S and 5.8S ribosomal subunits (ITS1) and between the 5.8S and 26S ribosomal subunits (ITS2). Length variation in the ITS region of gymnosperms was studied by Liston et al. (1996), who reported relatively long ITS sequence lengths in cycads, similar to most other gymnosperms investigated. In order to confirm or deny our results from ITS sequencing and as part of an ongoing molecular survey of cycads, we wanted to include data from additional markers. Recently a number of chloroplast tRNA genes and spacers between them were investigated for their phylogenetic utility in angiosperms (Taberlet et al., 1991; Bayer & Starr, 1998). The intron in the *trn*L gene in the chloroplast genome is flanked by relatively conservative coding regions and is of moderate size, which makes it easy to amplify and sequence and therefore a good choice for us to try with cycads. In this article we present the results of a pilot molecular systematic study in cycads using ITS2 and *trn*L intron markers.

III. Methods

All specimens of cycads included in this study were obtained from the extensive living collections at Fairchild Tropical Botanic Garden and Montgomery Botanical Center in Coral

Table I

Cycad taxa included in this study, with Fairchild Tropical Garden accession numbers and voucher specimens deposited in the FTG herbarium

Taxon	Accession number	Voucher specimen
Bowenia serrulata (W. Bull) Chamberlain	FG 93786	Bogler 1202
Bowenia spectabilis Hook. ex J. D. Hook.	FG 9367C	Bogler 1203
Ceratozamia hildae Landry & M. Wilson	FG 7886	Bogler 1212
Ceratozamia kuesteriana Regel	FG 789355A	Bogler 1209
Ceratozamia miqueliana H. Wendl.	FG 76327C	Hubbuch et al. 106
Cycas rumphii Miq.	FG 66718B	Hubbuch et al. 215
Cycas wadei Merr.	FG 77967J	Hubbuch et al. 123
Dioon califanoi De Luca & Sabato	FG 93921A	Hubbuch and Walters 162
Dioon holmgrenii De Luca, Sabato & Vazquez-Torres	FG 88312A	Vovides 1138
Dioon mejiae Standley & L. O. Williams	FG 92224D	Bogler 1211
Dioon merolae De Luca, Sabato & Vazquez-Torres	FG 69420B	Tang s. n., 7 Nov 1983
Dioon spinulosum Dyer	FG 651320B	Hubbuch et al. 214
Encephalartos ferox G. Bertol	FG 614G	Stevenson 512
Encephalartos manikensis (Gilliland) Gilliland	FG 64761A	Fantz 4139
Lepidozamia hopei Regel	FG 69428G	Bogler 1242
Lepidozamia peroffskyana Regel	FG 59883A	Watson 1368
Macrozamia lucida L. Johnson	FG 60530A	Bogler 1207
Macrozamia moorei F. Muell.	FG 59302C	Hubbuch and Aronson 94
Microcycas calocoma (Miq.) A. DC	FG 77404W	Bogler 1201
Stangeria eriopus (Kunze) Baillon	FG 85130	Beck 1117
Zamia loddigesii Miq.	FG 93931A	Hubbuch and Walters 172
Zamia pseudoparasitica Yates	FG 76663	Kiem 61
Zamia pumila L.	FG 62291A	Eckenwalder 1416
Zamia spartea A. DC	FG 93926B	Watson 1920
Zamia standleyi Schutzman	FG 65990C	Hubbuch et al. 107

Gables, Florida (Table I). Most of these specimens were grown from seeds collected in the wild. Taxa were selected to represent the range of variation in the cycads and to test the utility of molecular markers at various levels of divergence. Fresh leaf material from two recently described genera, *Chigua* (Stevenson, 1990b), and *Epicycas* (de Laubenfels & Adema, 1998) was not available. All vouchers are deposited in the herbarium at Fairchild Tropical Botanic Garden (FTG). Leaf samples were cut into thin strips, ground to a powder in liquid nitrogen, and stored at –80°C. DNA was extracted from the leaf tissue using a standard 2X CTAB procedure (Doyle & Doyle, 1987). Prior to amplification by the polymerase chain reaction (PCR) the DNA samples were further purified by the Geneclean procedure (Bio. 101, Vista, CA), a process that entails binding DNA to tiny silica beads, washing them with an ethanol/salt solution, and eluting the purified DNA in water.

Target DNA sequences were amplified using a PTC–200 thermal cycler (MJ Research, Watertown, MA). The *trn*L intron was amplified with universal primers developed by Taberlet et al. (1991): primer "C" (59′–CGA AAT CGG TAG ACG CTA CG–3′) and primer "D" (5′–GGG GAT AGA GGG ACT TGA AC–3′). The ITS2 region was amplified using the primers of Liston et al. (1996): primer "ITS–3" (GCA TCG ATG AAG AAC GCA GC) and primer "26S–25R" (5′–TAT GCT TAA ACT CAG CGG GT–3′). The thermal regime consisted of an initial

denaturation at 96°C for 2 minutes, followed by 30 cycles of 96°C denaturation for 40 seconds, 55°C primer annealing for 90 seconds, and 72°C extension for 90 seconds. Following amplification, the PCR products were purified and concentrated by the Geneclean procedure and the amount of DNA quantified by fluorometry. Cycle sequencing was performed using reagents supplied in the Big Dye™ DNA Sequencing Kit (PE Applied Biosystems, Foster City, CA), sequencing in both forward and reverse directions using the primers mentioned above. Reactions were carried out in 10 μl volumes, with the Terminator Mix diluted 50:50 with 5X Amplitaq FS Buffer. Cycle sequencing conditions consisted of an initial denaturation at 96°C for 2 minutes, followed by 35 cycles of 96°C denaturation for 10 seconds, 55°C primer annealing for 5 seconds, and 60°C extension for 4 minutes. The cycle sequencing product was precipitated with EtOH/$NaNH_4OAc$ (6:1), the pellet dried under vacuum, resuspended in formamide/blue dextran loading dye (5:1), and loaded on an ABI 377 automated DNA sequencer. Sequences were edited and assembled using Autoassembler™ (ABI Prism) and aligned using Clustal X (Thompson et al., 1997).

The *trn*L intron sequences had very few indels, relatively low levels of base substitution, and their alignment was not ambiguous. The same cannot be said for the ITS sequences. Originally, we had planned to sequence both the ITS1 and ITS2 regions in all these taxa, but we ran into problems with ITS1. The high GC content and presence of homopolymeric regions (i.e., CCCCCCCCCCCC) in ITS1 made it very difficult to obtain sequences from *Zamia, Microcycas,* and some species of *Dioon.* The ITS1 sequences that we did obtain indicated that ITS1 sequences from different genera would be very difficult to align with confidence and would not serve as useful markers at that level of comparison. Although we had no problems obtaining ITS2 sequences and they are more conservative than ITS1, the alignment of ITS2 also proved challenging. We tried alignments using a number of gap penalties with ClustalX and picked the alignment that produced trees that were most congruent with results from the *trn*L sequence data. Clearly, more sequence studies are needed to confirm or refine the procedures, results, and conclusions reported in this pilot study.

Parsimony analysis was carried out with PAUP 4.0b2a (Swofford, 1999), using the branch and bound search routine. The robustness of the clades thus found was assessed by bootstrapping with PAUP, using 100 replications of simple heuristic searches. Data sets consisting of aligned sequences were analyzed separately and combined in one large data set. Pairwise distances between sequences and base composition were calculated with PAUP.

IV. Results

Both *trn*L intron and ITS2 sequences were obtained from 25 cycad taxa (Table I). The *trn*L intron in cycads is approximately 491 bp in length, with a short, 9 bp deletion in *Ceratozamia.* The mean base composition of the *trn*L intron was 0.266 (A), 0.230 (C), 0.185 (G), and 0.318 (T). The G/C content is 41.5%, which is low compared with ITS sequences. In terms of sequence variation, the *trn*L intron is relatively conserved in cycads, with simple pairwise distances ranging from 0.000 between the species of *Ceratozamia* to 0.087 between *Stangeria eriopus and Cycas rumphii.* Of the 491 bases in the *trn*L intron data matrix, 400 are invariant, 27 are parsimony-uninformative, and 64 parsimony-informative.

Branch and bound parsimony searching of the *trn*L intron sequence alignment produced 4 equally parsimonious trees 106 steps in length, with a consistency index of 0.896, and a retention index of 0.942. A strict consensus of these four trees is presented in Fig. 2. The taxa are resolved in this analysis, with three major clades. The tree was rooted with *Cycas,* based on its putatively primitive reproductive megasporophylls and high number of base substitutions.

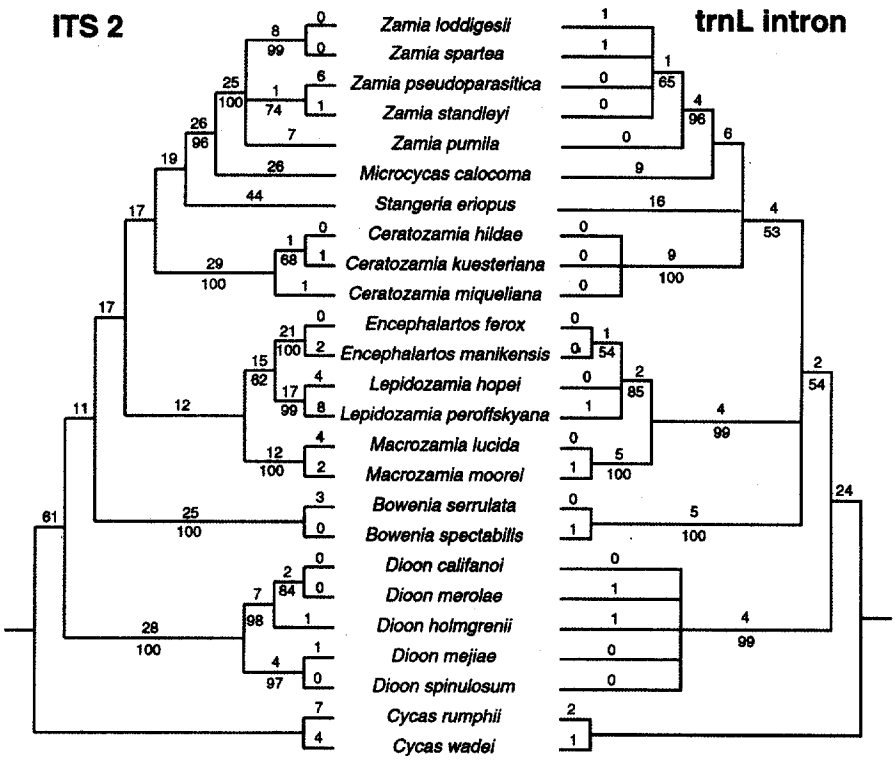

Fig. 2. *Left:* Strict consensus of 2 equally most parsimonious trees produced by analysis of ITS2 sequence data from cycads. Length = 478 steps, Consistency Index = 0.678, Retention Index = 0.828. *Right:* Strict consensus of 4 equally most parsimonious trees produced by analysis of *trn*L intron sequence data from cycads. Length = 106 steps, Consistency Index = 0.896, Retention Index = 0.942. The numbers above the lines are the base substitutions shared by a clade or distinguishing a terminal taxon. The numbers below the lines are the bootstrap support values.

Dioon occupies a somewhat isolated clade, which is the sister group to a large clade containing the remaining genera. In this analysis *Bowenia* and *Stangeria,* which have been united into the Stangeriaceae, show no close relationship at all. *Bowenia* is in a rather isolated position near the base of this clade, and *Stangeria* appears to be allied to *Ceratozamia* and *Zamia*. *Microcycas* forms a sister clade to Zamia. The Old World genera *Encephalartos, Lepidozamia,* and *Macrozamia* also constitute a clade, but the Australian genus *Lepidozamia* appears to be more closely related to the African genus *Encephalartos* than to the Australian genus *Macrozamia*. Within these genera there was almost no variation in *trn*L intron sequences.

The ITS2 sequences proved to be much more variable than the *trn*L intron. Most of the cycads had ITS2 sequences that were about 246–250 bp in length, but *Stangeria* had a slightly longer ITS2 sequence, 279. The mean base composition of ITS2 sequences was 0.112 (A), 0.329 (C), 0.342 (G), and 0.217 (T). The G/C content of the ITS2 was decidedly rich at 67.1%. Of the 306 characters in the ITS2 sequence data matrix, 100 were invariable, 22 were variable but parsimony-uninformative, and 184 characters were variable and parsimony informative.

This relatively high number of informative characters reflects the high level of variation found in these sequences. The pairwise distances between ITS2 sequences were also higher than in *trn*L intron sequences. Within genera, the pairwise distance values between species varied from 0.000 to 0.057 in *Dioon* and *Zamia* and from 0.004 to 0.012 in *Ceratozamia.* Between genera, the pairwise distances were much higher than with the *trn*L intron. The pairwise distance between ITS2 sequences of *Cycas* and all the other cycad genera was close to 0.400. The distance between *Zamia* and *Dioon* was about 0.347–0.360 and between *Zamia* and *Ceratozamia* was 0.312–0.353. The picture that emerges from the ITS2 data is one of relatively large distances between genera but rather small distances between species within a genus.

Parsimony analysis using the branch and bound search routine of PAUP resulted in two equally most parsimonious trees 478 steps in length, with a consistency index of 0.678 and a retention index of 0.828. A strict consensus of these two trees is presented in Fig. 2. The topology of this tree is remarkably similar to the *trn*L intron tree, but with better resolution of some of the clades. Once again, the tree was rooted with *Cycas* because of its primitive morphology and large number of base substitutions. *Dioon* is positioned in the same place between *Cycas* and the other genera. Within *Dioon* two major clades are firmly supported, suggesting that ITS sequences will be useful in resolving the species of this genus. Congruent with the *trn*L intron tree, the ITS2 data indicate *Bowenia* and *Stangeria* are not closely related. *Bowenia* is instead sister to a large clade containing the remaining genera, and *Stangeria* is again associated with the New World genera *Zamia, Microcycas,* and *Ceratozamia.* It must be pointed out, however, that the bootstrap support for the position of *Stangeria* here is not strongly supported by bootstrapping and that, with alternative alignments made with different gap penalties, the position of *Stangeria* switches to the base of the tree immediately after *Cycas.* The clade supporting *Macrozamia, Lepidozamia,* and *Encephalartos* is again present, with additional support for a close relationship between the African *Encephalartos* and Australian *Lepidozamia.* The ITS2 data place *Ceratozamia* in a clade along with *Stangeria, Zamia,* and *Microcycas.* The species of *Certaozamia* exhibit low levels of ITS2 sequence variation, suggesting that they are only recently diverged. There is strong support for a close relationship between *Microcycas* and *Zamia.* Within *Zamia,* the ITS2 data indicate a close relationship between *Z. loddigesii and Z. spartea* and indicate that these sequences may prove useful in resolving relationships of the other species.

Finally, the data were combined into a single matrix with 797 total characters, 248 of which were parsimony informative, and analyzed with the branch and bound option of PAUP. The result was a single most parsimonious tree that was 584 steps in length, with a consistency index of 0.717 and a retention index of 0.848. This tree is shown in Fig. 3. The finding of a single most parsimonious tree that is fully resolved suggests that resolution was slightly improved by combining the data. The C.I. and R.I. values are intermediate between those found in the separate analyses, as might be expected, but the bootstrap values were slightly higher on some of the clades in the tree from the combined data. The overall topology of the combined data tree closely resembles the strict consensus trees produced by the separate sequence data analyses, and what was said about those trees applies to this one as well.

V. Discussion

The results of this study provide some very interesting insights into the phylogeny of the cycads and information that may ultimately be useful in classification. The sequence data argue for or against some of the conclusions inferred in previous studies from morphological, anatomical, and cpDNA RFLP studies but also presents evidence for entirely new hypotheses of

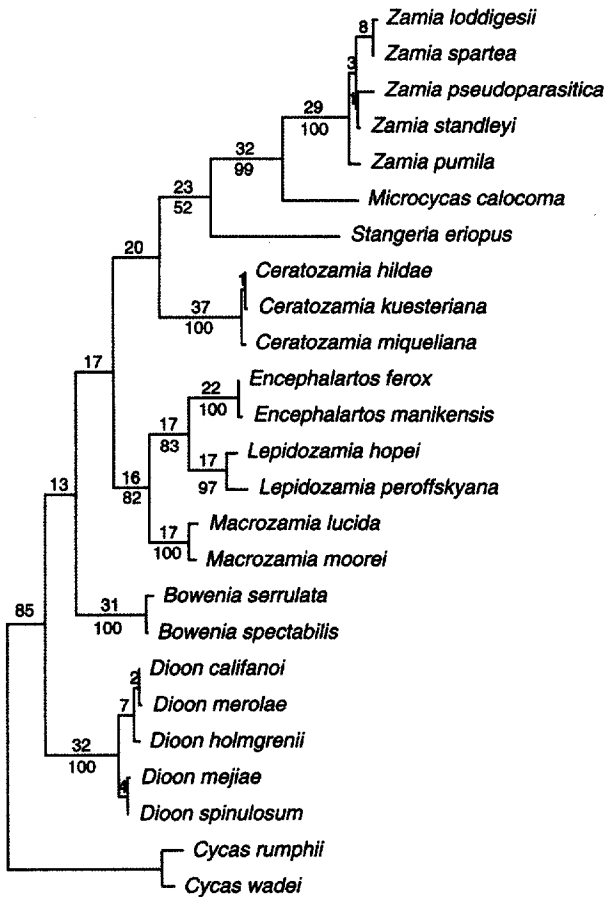

Fig. 3. Single most parsimonious tree found by parsimony analysis of combined *trn*L intron and ITS2 data from cycads. Length = 584 steps, Consistency Index = 0.717, Retention Index = 0.848. The numbers above the lines are the molecular characters shared by a clade. Branch lengths are proportional to the number of characters supporting the clade. The numbers below the lines are the bootstrap support values.

relationship. The topology that emerges from our data does not exactly match any previously published tree. The major conclusions based on the results of this *trn*L intron and ITS2 sequence study are discussed below, together with some general features of the genera and observations drawn from other sources.

A. *CYCAS* IS DISTANTLY RELATED TO THE OTHER GENERA OF CYCADS

The relatively distant position of *Cycas* with respect to the other genera of cycads is reinforced by the sequence data. *Cycas* contains approximately 30–50 species that are widely distributed from Japan, China, and Southeast Asia to Madagascar and, possibly, Africa (Jones, 1993; de Laubenfels & Adema, 1998). Morphologically, *Cycas* is separated from the other

cycad genera by the leaflets that have a distinct midrib, a single vascular strand, and are tightly coiled (circinnate) as they emerge. The seeds of *Cycas* are flattened (platyspermic); while in the other genera the seeds are more rounded. The female sporophylls are loosely arranged, leaflike affairs that do not form a compact cone as in other cycads. This arrangement has generally been considered a primitive feature retained from the seed-fern ancestor of all cycads. The male sporophylls do, however, form compact cones as in other cycads, so it has been suggested that the female sporophylls of *Cycas* may be more recently derived, with the loose arrangement related to pollination (Norstog & Nicholls, 1997). The sequence data do not tell us whether the female sporophylls are primitive or advanced, but the high level of sequence divergence in *Cycas* (85 unique characters in the combined analysis) reinforces the idea that this genus diverged from the others very early.

B. *DIOON* IS RELATIVELY ISOLATED AND CONTAINS TWO MAJOR CLADES

Dioon is a genus of about 10 species distributed mostly in Mexico, with one species in Honduras (Sabato & De Luca, 1985; Moretti et al., 1993). It appears that *Dioon* was formerly much more widespread, with fossils found in Eocene deposits in Alaska (Jones, 1993; Norstog & Nicholls, 1997). The plants have relatively large stems and stiff, occasionally spiny leaflets. The leaves of *Dioon* differ from those of other New World cycads in having firmly attached leaflets that are not shed separately (nonarticulate), and there are no prickles on the petiole. The cones are terminal and often massive. The female sporophylls of *Dioon* are loosely imbricated and end in an upturned shield that is considered leaflike by some, and therefore relatively primitive, intermediate between *Cycas* and the other genera. The sequence data firmly indicates just such an intermediate position of *Dioon,* although this does not necessarily indicate that the cones of *Dioon* are intermediate in an evolutionary sense.

An excellent study of the phylogeny of the species of *Dioon* was made by Moretti et al. (1993), including analysis of 187 informative cpDNA restriction fragments. Based on size, habitat, and cpDNA variation, *Dioon* can be divided into two major groups (Moretti et al., 1993; Norstog & Nicholls, 1997). The first group—the Spinulosum clade—has large trunks, leaves, and cones, occurs in mostly tropical forests, and includes *D. spinulosum, D. mejiae,* and *D. rzedowskii.* The second group—the Edule clade—has shorter trunks, smaller leaves, smaller cones, occurs in xeric habitats, and includes *D. edule, D. merolae, D. holmgrenii, D. purpusii, D. califanoi, D. caputoi,* and *D. tomasellii.* The ITS2 sequence variation also supports these two major clades and indicates a close relationship between *D. mejiae* and *D. spinulosum,* even though *D. mejiae* has a very disjunct distribution in Honduras and a unique seed and seedling morphology (Moretti et al., 1993; Sabato & De Luca, 1985). Broader sequence sampling in *Dioon* is needed to resolve the phylogeny of the species and to test the correlation of cladogenesis and vicariance events proposed by Moretti et al. (1993).

C. *BOWENIA* AND *STANGERIA* ARE NOT CLOSELY RELATED

Bowenia is unique among cycads in having bipinnately compound leaves. The two or three species occur in tropical areas of northeastern Australia. *Bowenia* has a large underground stem that is branched at the tip into many short branches, each of which may bear a cone or leaves. *Bowenia* has been considered to constitute a separate family, Boweniaceae (Stevenson, 1981; Johnson & Wilson, 1990), or combined with *Stangeria* in Stangeriaceae (Stevenson, 1992). *Stangeria* contains a single species, *S. eriopus,* restricted to southern Africa. *Stangeria* has an underground stem, fernlike leaves with prominent midribs on the leaflets,

and medium-sized cones with tightly overlapping sporophyll tips. It has been placed alone in a separate family, Stangeriaceae, (Johnson & Wilson, 1990), and combined with *Bowenia*. Two of the synapomorphies uniting *Bowenia* and *Stangeria* were the shared absence of cataphylls on vegetative shoots and the circular pattern of vascular bundles in the petioles (Stevenson, 1992). The *trn*L intron and ITS2 sequence data do not indicate a particularly close relationship between *Bowenia* and *Stangeria* and argue that they should not be placed together in a separate family. Our data support the position of Schutzman and Dehgan (1993), who argue that both genera do have cataphylls and do not share a similar petiolar anatomy. The sequence data indicate that *Bowenia* is rather isolated and not closely allied with any other cycad genus.

The position of *Stangeria* in the clade containing the New World cycads *Zamia* and *Microcycas* is one of the surprising results of this study, a result we certainly think needs additional evidence to verify. In general, the *trn*L intron sequence of *Stangeria* was similar to the sequence from the other cycads and aligned without difficulty. The ITS2 sequence of *Stangeria* appears to be more saturated with base changes and matches up with the other cycads in some regions, but it appeared not at all in others. To be certain there was no error, the ITS2 region was amplified and sequenced from 3 different individuals, each time with the same result. It is possible that this is a case of "long branch attraction," in which an overall very divergent sequence/lineage has a few regions/characters that are similar to a few region/characters in another lineage, and the whole branch jumps over to that lineage. In other words, the relationship displayed here between *Stangeria* and *Zamia/Microcycas* may be an artifact of the alignment or data analysis.

On the other hand, the sequence data may be indicating a real, though perhaps ancient, connection between *Stangeria, Zamia, Microcycas,* and *Ceratozamia,* obscured by time and long separation. The cycads have been around since the Mesozoic, when there were land connections between the continents of today; for example, between Africa and South America. It is also true that some cycad genera had a wider distribution in the past than at present. The population of *Stangeria* in South Africa may be the sole remnant of a formerly more widely dispersed genus. The relationship is supported by the finding of fossils similar to *Stangeria* in the New World, such as *Mesodescolea* in Argentina (Artabe & Stevenson, 1999) and *Eostangeria* in Wyoming (Kvacek & Manchester, 1999). Although we cannot attempt a thorough morphological and anatomical comparison here, there are similarities in the leaflets with midribs of *Chigua,* an extant South American genus thought to be closely related to *Zamia,* and those of *Stangeria.* The chromosome number of *Stangeria* is 2n = 16, a number found elsewhere only in *Zamia* and *Ceratozamia* (Norstog & Nicholls, 1997).

D. *LEPIDOZAMIA* IS CLOSELY RELATED TO *ENCEPHALARTOS*

The sequence data firmly place *Macrozamia, Lepidozamia,* and *Encephalartos* on a clade together. These three genera have a number of shared features, including large cones, sporophylls with diamond-shaped tips (shields), and ovules that develop fully even in the absence of pollination, and these similarities have been used to place them in a separate tribe, Encephalarteae of Zamiaceae (Stevenson, 1992; Norstog & Nicholls, 1997). *Encephalartos* is confined to Africa, while *Macrozamia* and *Lepidozamia* occur in Australia and have been placed together in the subtribe tribe Macrozamiinae (Stevenson, 1992). The two species of *Lepidozamia* were at one time included in *Macrozamia,* but the leaflets of *Lepidozamia* are inserted differently on the rachis, lack the colored callus of the leaflets of *Macrozamia,* and have different sporophyll

characteristics. Both sets of sequence data clearly associate *Lepidozamia* with *Encephalartos* instead of with *Macrozamia* as might be expected. This suggests the intriguing possibility that *Lepidozamia* diverged from a common ancestor with *Encephalartos,* perhaps in Gondwana or Antarctica before Africa and Australia became separated. It also suggests a need to look more closely at morphological and anatomical similarities between *Lepidozamia* and *Encephalartos* and to look for supporting fossil evidence.

E. SEQUENCE VARIATION IN *CERATOZAMIA* IS VERY LOW

Ceratozamia is a genus of 9–12 species found in Mexico, Guatemala, and Belize. The species of *Ceratozamia* often have short trunks, relatively long leaves and articulated leaflets (drop off separately) and have distinctive sporophylls with pairs of sharp "horns" or spurs. The species of *Ceratozamia* share with *Zamia, Chigua,* and *Microcycas* characters such as the articulated leaflets, absence of terminal leaflets in seedlings and adults, vestigial stipules, and peltate megasporophylls, and these genera have all been included in subfamily Zamioideae of the family Zamiaceae (Stevenson, 1992). The sequence data presented here support a relationship of *Ceratozamia* to *Zamia* and *Microcycas,* but the relationship is not as close as expected, as indicated by the low bootstrap support. This low level of support may reflect a long period of isolation from *Zamia,* possible misalignment of sequences, or indicate the need for additional molecular markers. A general description of the species of *Ceratozamia* was provided by Stevenson et al. (1986), in which two groups of species were informally recognized based on leaf shape (i.e., large and thin vs. narrow and thick), leaflet tips, and cone size. The sequence variation presented here does not provide evidence either for or against these groups. There was essentially no variation in *trn*L intron sequences and only a few variable bases in ITS2.

F. *MICROCYCAS* AND *ZAMIA* ARE CLOSELY RELATED

Microcycas is a genus with only one species, *M. calocoma,* restricted to Cuba and rare in cultivation. *Microcycas* has an erect trunk and leaves with characteristically drooping leaflets. The leaflets are articulated as in *Zamia* and *Ceratozamia.* The terminal leaflets are about as long as the others, giving the leaves a sweeping, chopped-off appearance. The cones are rather large and have notched sporophylls, somewhat like *Ceratozamia.* The female gametophyte of *Microcycas* produces a large number of archegonia (200+), and the male gametophyte produces up to 16 gametes in each pollen tube. This unusual trait has been considered a "primitive" condition by some, although the molecular data certainly do not support this idea. Congruent with the results of cpDNA studies of Caputo et al. (1991) and the morphological studies of Stevenson (1990a), the sequence data clearly indicate a close, sister taxon relationship between *Microcycas* and *Zamia.* Support from the ITS2 sequences is especially strong, with 26 shared substitutions and high bootstrap values. At the same time, *Microcycas* is distinguished by 26 unique molecular characters, about as many as *Ceratozamia,* which suggests a long period of isolation from *Zamia.*

G. MOLECULAR DATA PROVIDE USEFUL INFORMATION ABOUT SPECIES RELATIONSHIPS IN *ZAMIA*

Zamia is a widespread, diverse genus with many species. According to Stevenson et al. (1986), there are 47 recognized species, and several new ones have been added since that time. It is difficult to say exactly how many species there are because some of the species are poorly understood taxonomically. For example, the 40 or so named species of *Zamia* in the Caribbean have been both reduced to a single species, *Z. pumila,* by Eckenwalder (1980) or treated as 5–6 species (Stevenson, 1986; Sabato, 1990). Clearly, more work is needed on the taxonomy of

these species. *Zamia* is widely distributed from Florida and the West Indies, Mexico, and Central America, to South America, where new species continue to be found (Sabato, 1990). Structurally, *Zamia* is very diverse. The stems are often subterranean or very shortly caulescent, but some rain-forest species have trunks that reach several meters in height. At least one species, *Z. pseudoparasitica,* is a true epiphyte in the Panamanian rain forest, with pendant leaves, large cones, and mucilaginous, sticky seeds that may aid in seed dispersal. *Zamia* leaves vary greatly in the size and width of the leaflets. The cones are usually compact, with hexagonal sporophyll ends arranged in straight rows. *Zamia* is diverse cytologically, with chromosome numbers ranging from 2n = 16 to 2n = 28, perhaps related to active evolution and speciation (Caputo et al., 1996; Norstog & Nicholls, 1997).

Evidence from both *trn*L intron and ITS2 data quite firmly supports the close relationship between *Zamia* and *Microcycas,* congruent with previous phylogenetic studies. Variation in *trn*L intron sequences was low and provided little resolution when used alone. The ITS2 sequences were more variable and supported a close relationship between *Z. loddigesii* and *Z. spartea,* which had 8 shared characters. These two Mexican species are considered to be part of the same complex, with a chromosome number of 2n = 18, and apparently form hybrids where their ranges overlap and in cultivation (Jones, 1993; Norstog & Nicholls, 1997). There is less support for the relationship between *Z. standleyi* and *Z. pseudoparasitica,* but the data at least indicate that *Z. standleyi* is distinct from *Z. loddigesii,* two species that have been confused in the past (Jones, 1993). It is interesting to see that the molecular data indicate a relatively isolated position for *Z. pumila.* This is a taxonomically confusing species that is apparently undergoing active radiation in the Caribbean Basin (Norstog & Nicholls, 1997). The specimen included in this study was the native Florida population, which is often called *Z. integrifolia.* The results of this study demonstrate the potential of using molecular data to answer difficult questions about species relationships in *Zamia.*

Chigua is a relatively new genus from South America described by Stevenson (1990b), distinguished mainly by the leaflets, which have definite midribs and low-angled branch veins. The cones are slender and develop a long peduncle. Although specimens of *Chigua* were not available for this study, recent cpDNA RFLP studies in cycads indicated that *Chigua* is closely related to *Zamia* (Caputo et al, 1991).

VI. Conclusions

Results of this study demonstrate the utility of using DNA sequencing techniques to study the phylogeny and classification of cycads. The two gene regions sequenced here both provided valuable information about relationships at different levels of divergence. The genera were, in general, well resolved by the molecular data, although more work is clearly needed to verify these results. The divergent position of *Cycas* was reinforced, lending support to its ancient age and possibly primitive megasporophyll morphology. The position of *Dioon* indicates that it is not closely related to other New World cycads, as previously inferred from its morphology, but *Dioon* is isolated from the Old World genera as well. The molecular data indicate a surprisingly close relationship between *Lepidozamia* and *Encephalartos. Stangeria* does not seem particularly close to *Bowenia* but may have ancient connections to *Zamia.* The results of this study will prove to be very useful in planning future molecular studies of cycad phylogeny.

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