A Phylogeny for Two Cycad Families (Stangeriaceae and Zamiaceae) based on Chloroplast DNA Sequences

Pattamon Sangin^{1,3}, Paul I Forster², Mingkwan Mingmuang¹ and Goro Kokubugata³

 ¹ Department of General Science, Faculty of Sciences, Kasetsart University, Bangkok, 10900, Thailand
 ² Queensland Herbarium, Environmental Protection Agency, Brisbane Botanic Gardens, Mt Coot-tha Road, Toowong, Queensland, 4066, Australia
 ³ Department of Botany, National Museum of Nature and Science, 4–1–1 Amakubo, Tsukuba, 305–0005, Japan E-mail: gkokubu@kahaku.go.jp

Abstract Sequences of the trnS-trnG noncoding region chloroplast DNA from 40 species of the Zamiaceae and Stangeriaceae families and 3 out group species (Cycadaceae) were used to reconstruct phylogenetic trees using distance and parsimony methods. The distance tree was similar in topology to the parsimony tree and both indicated that *Encephalartos* had a relationship closer to *Lepidozamia* than *Macrozamia*. *Encephalartos*, *Lepidozamia* and *Macrozamia* comprised a monophyletic tribe, while *Dioon* was a basal-sister of the subfamily Encephalartoideae clade. These two analyses also revealed a close relationship between *Microcycas* and *Zamia* with high bootstrap support. Although *Stangeria* and *Ceratozamia* have been placed in different families, they were closer to each other, rather than *Bowenia* which is contrary to previous morphologically based classifications. The current study provides new molecular evidence that *Stangeria* and *Bowenia* are not sister taxa and reinforces the close relationship between *Encephalartos* and *Lepidozamia*.

Key words: Bowenia, Chloroplast DNA, Cycadales, Encephalartos, Lepidozamia, Macrozamia, Stangeria, Stangeriaceae, Zamia, Zamiaceae.

Introduction

The cycads (Order Cycadales; Stevenson, 1992; Norstog and Nichols 1998; Schneider et al., 2002) are a group of seed plants with ancient origins that have been often termed as 'living fossils', insomuch as the extant species are of lineages little changed since their first occurrence in the early Permian (Mamay, 1969; Zhu and Du, 1981; Gao and Thomas, 1989). They are distributed across the subtropical and tropical regions of the world, i.e., Africa, Asia, Central America and Australia with one species extending to Japan. On the basis of morphology, cycads have been classified into 3 families (Cycadaceae, Stangeriaceae and Zamiaceae), with 11 genera (Stevenson, 1992) and over 300 known species (Hill et al., 2007). Whilst Cycadaceae is unequivocally unique (cf. Brenner et al., 2003a, 2003b), the distinction between Stangeriaceae and Zamiaceae remains unclear when molecular characters are analyzed, rather than those purely from morphology (Hill *et al.*, 2003; Chaw *et al.*, 2005).

In the past decade, chloroplast genes have been used extensively to elucidate relationships in seed plants. Chloroplast genes are the consequences of an endosymbiotic event between a eukaryotic host cell and an ancestor of the cyanobacteria hence they have a slower mutation rates in comparison with the nuclear genes (Curtis and Clegg, 1984; Raven and Allen, 2003) with their conserved nature useful for testing relationships among genera thought to be closely related (Gielly and Taberlet, 1994). In recent studies using molecular markers such as the chloroplast *matK* gene, *trnL* intron and ITS2 rDNA sequences (Treutlein and Wink, 2002; Hill *et al.*, 2003; Bogler and Francisco-Ortega, 2004; Chaw et al., 2005) the intrafamilial classification of cycads has been slightly modified, particularly in the Zamiaceae family, where the genera Encephalartos (endemic to Africa) and Lepidozamia (endemic to Australia) have been found to be closer to each other rather than Macrozamia (endemic to Australia). These molecular phylogenetic trees were not congruent with the Stevenson (1992) classification that was based solely on morphological characters and which included Lepidozamia and Macrozamia in the subtribe Macrozamiinae D.Stevenson and Encephalartos in the subtribe Encephalartinae Benth. et Hook.f.; subtribes comprising the tribe both Encephalarteae Miq. within the subfamily Encephalartoideae D.Stevenson.

Currently, noncoding sequences of the chloroplast genome have been used as a new major focus for studying plant molecular evolution. Shaw *et al.* (2005) evaluated the relative level of variability among 21 noncoding chloroplast DNA regions in seed plants and the noncoding regions (Tier 1) that provided the greatest numbers of PICs (Potential Informative Characters), were identified as trnD-trnT, rpoB-trnC, trnStrnG, trnS-trnfM and trnT-trnL. In the current study we focus on the use of the trnS-trnG sequence (previously unused with cycads) to reconstruct phylogenetic trees for developing relationship hypotheses for within and between genera in the Zamiaceae and Stangeriaceae families.

Material and Methods

DNA extraction and PCR

Total genomic DNA of nine genera and 40 cycad species (Table 1) was extracted from plants cultivated in Tsukuba Botanical Garden. Voucher specimens for each species are deposited in the Herbarium of the National Museum of Nature and Science (TNS). Three *Cycas* species, namely *C. revoluta, C. wadei* and *C. media*, were included in the present analysis to serve as out groups following Hill *et al.* (2003) and in recognition of the basal position of this genus in the Cycadales

(Brenner, 2003a).

In the present study, Plant DNeasy Mini Kit (Quigen) was used for extracting DNA following the manufacturer's protocol. The trnS-trnG intergenic spacer region was amplified with primer trnS 5' GCCGCTTAGTCCACTCAGC 3' and trnG 5' GAACGAATCACACTTTTACCAC 3' (Hamilton, 1998). The polymerase chain reaction (PCR) amplification was performed in 5 μ l of the reaction with the following components: 2.5 μ l of 5×AmpDirect (Shimazu), 0.5 unit of Ex taq (Takara), 10 μ M of each primer and 1 μ l of genomic DNA. Amplifications were made in a Perkin Elmer 9700 thermocycler with an initial denaturing step of 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C, 1 min at 72°C and a final extension of 7 min at 72°C. PCR products were subjected to 1% agarose gel and DNA bands were visualized by ethidium bromide staining. The PCR products were purified using the ExoSAP-IT kit (United States Biochemical). Purified PCR product were sequenced with the ABI Big Dye Terminator Cycle Sequencing Kit V3.1 and run on 3130X/ Genetic Analyzer.

Data analysis

Sequences were edited and assembled using the program ATGC var. 4 (GENETYX Co.) and were initially aligned using Clustal W (Thompson et al., 1994). The resulting data was imported into the GENEDOC 2.6 program (Nicholas et al., 1997) following by a manual adjustment. Two phylogenetic reconstruction methods (maximum parsimony (MP) and neighbor-joining (NJ) method) were performed, using the program MEGA 4 (Tamura et al., 2007). The MP analyses were conducted with heuristic searches (close-neighbor interchange) using the random addition trees. Bootstrap analyses used 1,000 replicates for this method. For the NJ method, analyses were constructed with the nucleotide substitution model (Maximum Composite Likelihood Method) with the number of bootstrap replicates was set to 1,000. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option).

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Cycas Cycas media R. Br. GK 59 Cycas revoluta Thunb. GK 7136						Zamia furfuracea L. f.	GK 10481	AB434463
GK 7136	Cycadaceae				Cycas	Cycas media R. Br.	GK 59	AB43446 [,]
						Cycas revoluta Thunb.	GK 7136	AB43446

Region	Alignment length	Base				Transition	Transversion
Region	(base pairs)	А	С	G	Т	Transition	Transversion
trnS-G	903	30.3	17.6	20.3	31.9	4.102	3.106

Table 2. Average base frequencies for trnS-G intergenic spacer of 43 Cycad species

The sequences obtained were submitted to the DDBJ/EMBL/GenBank databases (Table 1).

Results and Discussion

Sequence characteristics

The length of the trnS-trnG intergenic spacer averaged 815 base pairs (bp) and the nucleotide frequencies were 30.3% (A), 17.6% (C), 20.3% (G) and 31.9% (T). This region showed a low GC content (37.9%), while it represented a high AT content (62.2%) which is characteristic of the chloroplast genome (Morton, 1995). The transition (Ts)/transversion (Tv) ratio was 1.32 (Table 2).

The sequence alignment of this region was 903 bp and appeared with 22 indels (insertion/deletion). These results indicated that the trnS-trnG region had a high level of variation among cycad genera.

Phylogenetic analyses

MP was reconstructed using the close-neighbor-interchange (CNI) with the random addition tree 100 replications producing 188 equally most parsimonious trees with trees length of 540. The consistency index was 0.630556, the retention index was 0.819048 and the composite index was 0.617319 (0.516455) for all sites and parsimonyinformative sites (in parentheses). There were a total of 725 positions in the final dataset, out of which 174 were informative. A consensus of these 74 trees is presented in Fig. 1. NJ (Fig. 2) was reconstructed by the Maximum Composite Likelihood method and was in the units of the number of base substitutions per site. The MP and NJ trees supported monophyly of each genus with high bootstrap values. In the MP tree clades consisted of ((Stangeria, Ceratozamia), ((Microcycas, Zamia), (Bowenia, (Dioon, (Macrozamia, (Encephalartos, Lepidozamia)))))) (Figs. 1, 2).

Both the MP and NJ analyses support the hypothesis that the tribe Encephalarteae (Encephalartos, Macrozamia and Lepidozamia) and tribe Zamieae (Microcycas and Zamia) are respectively monophyletic with high bootstrap values agreeing with Stevenson (1992), and then Dioon (endemic to Central America) was taken up as a sister group to this Encephalartoideae clade with a bootstrap value. The current results indicate that Encephalartos is more closely related to Lepidozamia than to Macrozamia therefore disagreeing with Stevenson's (1992) classification of tribe Encephalarteae based only on morphology but in accordance with previous molecular results (Treutlein and Wink, 2002; Hill et al., 2003; Bogler and Francisco-Ortega, 2004; Chaw et al., 2005). These results reinforce the conclusion that Lepidozamia and Encephalartos shared a common ancestry in Gondwana (200-135 MYA) and separated from each other before Africa and Australia were isolated (Bogler and Francisco-Ortega, 2004). As noted by Chaw et al. (2005), the relationship between these two widely disjunct genera is enigmatic; however, unlike some species of Cycas (Dehgan and Yuen, 1983), no species of Lepidozamia or Encephalartos possess seeds with special adaptations that enable dispersal for long distances, especially on the continental scale. As with the allied Macrozamia (Snow and Walter, 2007), all extant species of Lepidozamia and Encephalartos had large and heavy seeds that are locally or barely dispersed. These essentially localized distributions indicate dispersal limited distribution (Primack and Miao, 1992) not conducive to long range colonization.

Bowenia (endemic to Australia) and *Stangeria* (endemic to Africa) were classified in the family

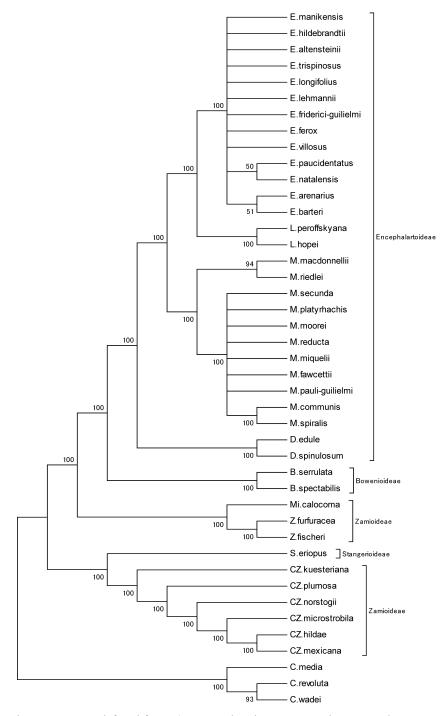


Fig. 1. The consensus tree inferred from 74 most parsimonious trees was shown. Branches corresponding to partitions reproduced in less than 50% trees were collapsed. The percentage of parsimonious trees in which the associated taxa clustered together were shown next to the branches.

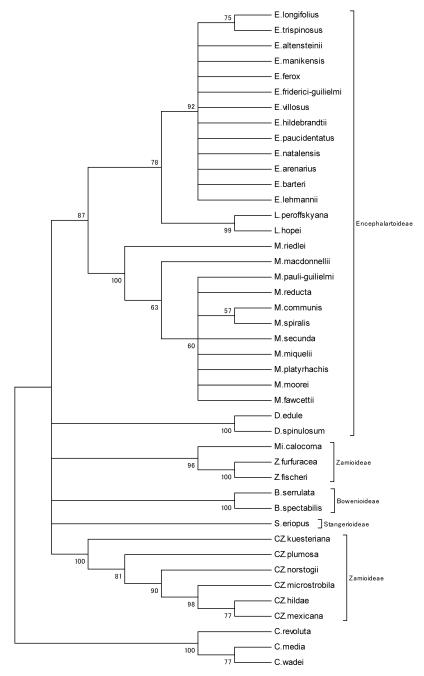


Fig. 2. Neighbor-Joining tree produced by analysis of trnS-trnG sequence data from Zamiaceae and Stangeriaceae families. The optimal tree with the sum of branch length=0.81275044 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches (> 50%).

Stangeriaceae by Stevenson (1992), albeit in separate subfamilies. However, most previous studies have implied that Bowenia and Stangeria were not closely related, and did not support the family Stangeriaceae, and most molecular based phylogenetic studies have concluded that Stangeria and Zamia were sister taxa (Rai et al., 2003; Bogler and Francisco-Ortega, 2004; Chaw et al., 2005), rather than Stangeria and Ceratozamia as found in the current study. Previously it was reported that the chromosome numbers of Stange*ria* and *Ceratozamia* are both 2n=16, whereas for Bowenia it is 2n=18; and Stangeria and Ceratozamia have similar karyotypes (Kokubugata et al., 2000, 2001, 2004). Furthermore, the florescence in situ hybridization using 45S and 5S ribosomal (rDNA) probes elucidated that Stangeria (S. eriopus) and Ceratozamia (C. hildae, C. kuesteriana, C. mexicana and C. norstogii) had similar distribution patterns of 45S and 5S rDNA sites on the somatic chromosome complements, with the conclusion that Stangeria could be closer to Ceratozamia than the other cycad genera (Kokubugata and Kondo, 1998; Kokubugata et al., 2002, 2004). The present molecular study supports the previous cytotaxonomic hypothesis.

Data from the trnS-trnG noncoding region of chloroplast DNA appears to be highly informative for determining relationship hypotheses in the Cycadales, which largely colloborate other molecular studies. On the other hand, there is not enough data to explain intrageneric phylogenies in each genus, and thus further taxon sampling and analyses based on the other sequences are required, particularly for the New World genera such as Ceratozamia and Zamia. Ultimately a rigorous classification for the Cycadales should be based on a dataset that incorporates information from a range of molecular studies, together with morphological characters and that acknowledges aspects of the pollination biology and dispersal abilities of these plants. Ultimately a rigorous classification for the Cycadales should be based on a dataset that incorporates information from a range of molecular studies, together with morphological characters and that acknowledges

aspects of the pollination biology and dispersal abilities of these plants. In spite of their lineage, a general consensus is that many of the extant species of cycads are relatively recent in derivation, possibly radiating since the Pleistocene (Treutlin and Wink, 2002; Vovides et al., 2007), albeit with a set of biological characteristics of putatively ancient origins. Their dependence on insect pollinators (usually in dependent obligate mutualisms (cf. Terry et al., 2005)) and limited ability to disperse due to an apparent loss of dedicated dispersal agents (Snow and Walter, 2007) all indicate that the extant cycads have limited options for long range colonization. Molecular and morphological differences between the extant genera of cycads (particularly those that are sister taxa such as Encephalartos and Lepidozamia) are likely to have arisen prior to their having been separated by continental separation events, such as the splitting up of Gondwana.

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References

- Bogler, J. D. and J. Francisco-Ortega. 2004. Molecular systematic studies in Cycads: evidence from *trnL* intron and ITS2 rDNA sequence. *The Botanical Review* 70: 260–273.
- Brenner, E. D., D. W. Stevenson and R. W. Twigg. 2003a. Cycads: evolutionary innovations and the role of plantderived neurotoxins. *Trends in Plant Science* 8: 446–452.
- Brenner, E. D., D. W. Stevenson, R. W. McCombie, M. S. Katari, S. A. Rudd, K. F. X. Mayer, P. M. Palenchar, S. J. Runko, R. W. Twigg, G. Dai, R. A. Martienssen, P. N. Benfey and G. M. Coruzzi. 2003b. Expressed sequence tag analysis in *Cycas*, the most primitive living seed plant. *Genome Biology* 4: R78.
- Chaw, S. M., T. W. Walters, C.-C. Chang, S.-H. Hu and S.-H. Chen. 2005. A phylogeny of cycads (Cycadales) inferred from chloroplast *matK* gene, *trnK* intron, and nuclear rDNA ITS region. *Molecular Phylogenetics*

and Evolution 31: 214-234.

- Curtis, E. S. and M. T. Clegg. 1984. Molecular evolution of chloroplast DNA sequences. *Molecular Phylogenetics and Evolution* 1: 291–301.
- Dehgan, B. and C. K. K. Yuen. 1983. Seed morphology in relation to dispersal, evolution, and propagation of *Cycas L. Botanical Gazette* 144: 412–418.
- Gao, Z. and B. A. Thomas. 1989. A review of fossil cycad megasporophylls, with new evidence of *Crossozamia pomel* and its associated leaves from the lower Permian of Taiyuan, China. *Review of Palaeobotany and Palynology* **60**: 205–223.
- Gielly, L. and P. Taberlet. 1994. The use of chloroplast DNA to resolve plant phylogeny: Noncoding versus *rbcL* Sequences. *Molecular Phylogenetics and Evolution* 11: 769–777.
- Hamilton M. B. 1998. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* 8: 393–401.
- Hill, K. D., M. W. Chase, D. W. Stevenson, H. G. Hills and B. Schutzman. 2003. The families and genera of cycads: a molecular phylogenetic analysis of Cycadophyta based on nuclear and plastid DNA sequences. *International Journal of Plant Sciences* 164: 933–948.
- Hill, K. D., D. W. Stevenson and R. Osborne. 2007. The world list of cycads. *Memoirs of the New York Botanic Garden* 97: 454–483.
- Kokubugata, G. and K. Kondo. 1998. Comparative karyotype analysis of *Ceratozamia mexicana* and *Microcycas calocoma* (Zamiaceae) using fluorochrome banding (CMA/DAPI) and fluorescence *in situ* hybridization of ribosomal DNA. *Plant Systematics and Evolution* 210: 41–50.
- Kokubugata, G., K. Kondo, G. W. Wilson, L. M. Randall, A. van der Schans and D. K. Morris. 2000. Comparison of karyotype and rDNA-distribution in somatic chromosomes of *Bowenia* species (Stangeriaceae, Cycadales). *Australian Systematic Botany* 13: 15–20.
- Kokubugata, G., K. D. Hill, G. W. Wilson, K. Kondo and L. M. Randall. 2001. A comparison of chromosome number and karyotype in somatic chromosome of Stangeriaceae (Cycadales). *Edinburgh Journal of Botany* 58: 457–481.
- Kokubugata, G., K. D. Hill and K. Kondo. 2002. Ribosome DNA distribution in somatic chromosome of *Stangeria eriopus* (Stangeriaceae, Cycadales) and molecular-cytotaxonomic relationships to some other cycad genera. *Brittonia* 54: 1–5.
- Kokubugata, G., A. P. Vovides and K. Kondo. 2004. Mapping 5S ribosomal DNA on somatic chromosome of four species of *Ceratozamia* and *Stangeria eriopus* (Cycadales). *Botanical Journal of the Linnean Society* 145: 499–504.
- Mamay, S. H. 1969. Cycads: fossil evidence of late Paleozoic origin. *Science* 164: 295–296.
- Morton, R. B. 1995. Neighboring base composition and tranversion/translation bias in a comparison of rice and maize chloroplast noncoding region. *Proceedings of*

National Academic of Sciences, USA 92: 9717-9712.

- Norstog, K. J. and T. J. Nichols. 1998. The Biology of the Cycads. Cornell University Press, Ithaca.
- Nicholas, K. B., H. B. Jr. Nicholas and D. W. Deerfield 1997. GeneDoc: Analysis and Visualization of Genetic Variation. Embnew. News **4**: 14.
- Primack, R. B. and S. L. Miao. 1992. Dispersal can limit local plant distribution. *Conservation Biology* 6: 513–519.
- Raven, J. A. and J. F. Allen. 2003. Genomics and chloroplast evolution; what did cyanobacteria do for plants. *Genome Biology* 4: 209.
- Rai, H. S., H. E. O'Brien, P. A. Reeves, R. G. Olmstead and S. W. Graham. 2003. Inference of higher-order relationship in the cycads from a large chloroplast data set. *Molecular Phylogenetics and Evolution* 29: 350–359.
- Schneider, D., M. Wink, F. Sporer and P. Lounibos. 2002. Cycads: their evolution, toxins, herbivores and insect pollinators. *Naturwissenschaften* 89: 281–294.
- Shaw, J., E. B. Lickey, J. T. Beck, S. B. Farmer, W. Liu, J. Miller, K. C. Siripun, C. T. Winder, E. E. Schilling and R. L. Small. 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* **92**: 1199–1209.
- Snow, E. L. and G. H. Walter. 2007. Large seeds, extinct vectors and contemporary ecology: testing dispersal in a locally distributed cycad, *Macrozamia lucida* (Cycadales). *Australian Journal of Botany* 55: 592–600.
- Stevenson, D. W. 1992. A formal classification of the extant cycads. *Brittonia* 44: 220–223.
- Tamura, K., J. Dudley, M. Nei and S. Kumar. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software Version 4.0. *Molecular Phylogenetics and Evolution* 24: 1596–1599.
- Terry, L. I., G. H. Walter, J. S. Donaldson, E. Snow, P. I. Forster and P. J. Machin. 2005. Pollination of Australian *Macrozamia* cycads: effectiveness and behavior of specialist vectors in a dependent mutualism. *American Journal of Botany* **92**: 931–940.
- Thompson, J. D., D. J. Higgins and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, posision-specific gap penalties and wight matrix choice. *Nucleic Acids Research* 22: 4673–4680.
- Treutlin, J. and M. Wink. 2002. Molecular phylogeny of cycads inferred from *rbcL* sequences. *Naturwis*senschaften 89: 221–225.
- Vovides, A. P., J. González-Astorga, M. A. Pérez-Farrera, D. González, C. Bárcenas and C. Iglesias. 2007. The cycads of Mexico: 25 years of research and conservation. *Memoirs of the New York Botanic Garden* 97: 611–641.
- Zhu, N. and X. Du. 1981. A new cycad: *Primocycas chinensis* gen. et sp. nov. discovered from the Lower Permian in Shanxi, China and its significance. *Acta Botanica Sinica* 23: 401–404.