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

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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.; both subtribes comprising the tribe 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, trnS-trnG, 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 μl of the reaction with the following components: 2.5 μl of 5×AmpDirect (Shimazu), 0.5 unit of Ex *taq* (Takara), 10 μM of each primer and 1 μl 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).

Table 1. Forty-three cycad species analyzed following Stevenson (1992)

Family	Subfamily	Tribe	Subtribe	Genus	Species	Voucher	DDBJ
Stangeriaceae	Stangerioideae			*Stangeria*	*Stangeria eriopus* (Kunze) Baill.	GK 10226	AB434424
	Bowenioideae			*Bowenia*	*Bowenia serrulata* (W. Bull) Chamb.	GK 10228	AB434425
					Bowenia spectabilis Hook. ex Hook. f.	GK 10229	AB434426
Zamiaaceae	Encephalartoideae	Diooeae		*Dioon*	*Dioon edule* Lindl.	GK 10249	AB434427
					Dioon spinulosum Dyer	GK 10246	AB434428
		Encephalarteae	Encephalartinae	*Encephalartos*	*Encephalartos altensteinii* Lehm.	GK 10250	AB434429
					Encephalartos arenarius R. A. Dyer	GK 10247	AB434430
					Encephalartos barteri Carruth. ex Miq.	GK 10237	AB434431
					Encephalartos ferox Bertol. f.	GK 10239	AB434432
					Encephalartos friderici-guilielmi Lehm.	GK 10233	AB434433
					Encephalartos hildebrandtii A. Braum et Bouche	GK 10242	AB434434
					Encephalartos lehmannii Lehm.	GK 10234	AB434435
					Encephalartos longifolius (Jacq.) Lehm.	GK 10235	AB434436
					Encephalartos manikensis (Gilliland)	GK 10240	AB434437
					Encephalartos natalensis R. A. Dyer et I.Verd.	GK 10236	AB434438
					Encephalartos paucidentatus Stapf et Burtt Davy	GK 10480	AB434439
					Encephalartos trispinosus (Hook.) R. A. Dyer	GK 10238	AB434440
					Encephalartos villosus Lem.	GK 10241	AB434441
			Macrozamiinae	*Macrozamia*	*Macrozamia communis* L. A. S. Johnson	GK 10219	AB434442
					Macrozamia fawcettii C. Moore	GK 10248	AB434443
					Macrozamia macdonnellii (F. Muell. ex Miq.) A. DC.	GK 10217	AB434444
					Macrozamia miquelii (F. Muell. ex Miq.) A. DC.	GK 10245	AB434445
					Macrozamia moorei F. Muell.	GK 10216	AB434446
					Macrozamia pauli-guilielmi W. Hill et F. Muell.	GK 10483	AB434447
					Macrozamia platyrhachis F. Muell.	GK 10479	AB434448
					Macrozamia reducta K. D. Hill et D. L. Jones	GK 10218	AB434449
					Macrozamia riedlei (Gaudich.) C. A. Gardner	GK 10480	AB434450
					Macrozamia secunda C. Moore	GK 10244	AB434451
					Macrozamia spiralis (Salisb.) Miq.	GK 10243	AB434452
				Lepidozamia	*Lepidozamia hopei* Regel	GK 10231	AB434453
					Lepidozamia peroffskyana Regel	GK 10230	AB434454
	Zamioideae	Ceratozamioideae		*Ceratozamia*	*Ceratozamia hildae* G. P. Landry et M. C. Wilson	GK 10221	AB434455
					Ceratozamia kuesteriana Regel	GK 10222	AB434456
					Ceratozamia mexicana Brongn.	GK 10223	AB434457
					Ceratozamia microstrobila Vovides et J. D. Rees	GK 10220	AB434458
					Ceratozamia norstogii D. W. Stev.	GK 10225	AB434459
					Ceratozamia sp ‘plumosa’	GK 10224	AB434460
		Zamieae	Microcycadinae	*Microcycas*	*Microcycas calocoma* (Miq.) A. DC.	—	AB434461
			Zamiinae	*Zamia*	*Zamia fischeri* Miq.	GK 10482	AB434462
					Zamia furfuracea L. f.	GK 10481	AB434463
Cycadaceae				*Cycas*	*Cycas media* R. Br.	GK 59	AB434464
					Cycas revoluta Thunb.	GK 7136	AB434465
					Cycas wadei Merrill	GK 10483	AB434466

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

Region	Alignment length (base pairs)	Base				Transition	Transversion
		A	C	G	T		
trnS-G	903	30.3	17.6	20.3	31.9	4.102	3.106

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 parsimony-informative 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

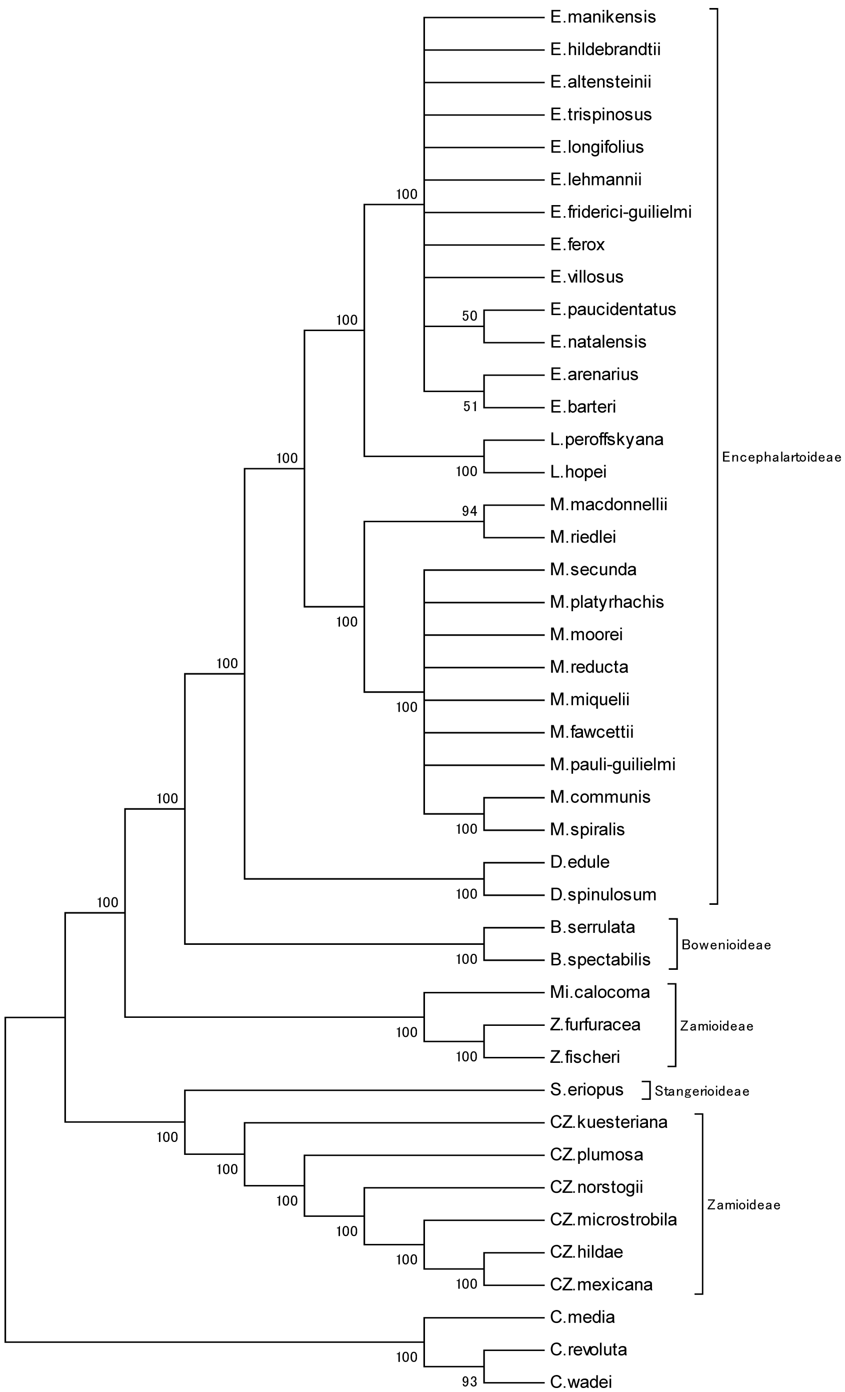

Fig. 1. The consensus tree inferred from 74 most parsimonious trees was shown. Branches corresponding to partitions reproduced in less than 50% trees were collapsed. The percentage of parsimonious trees in which the associated taxa clustered together were shown next to the branches.

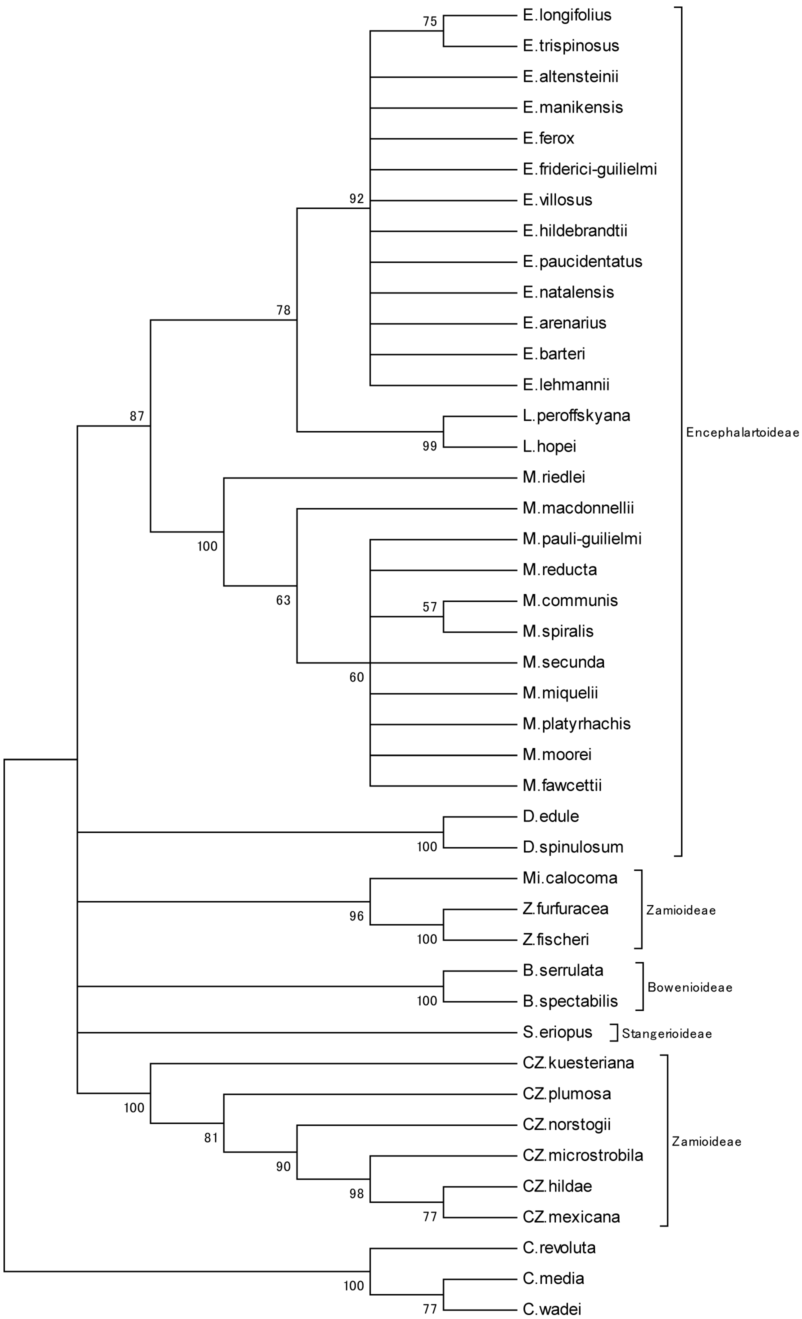

Fig. 2. Neighbor-Joining tree produced by analysis of trnS-trnG sequence data from Zamiaceae and Stangeriaceae families. The optimal tree with the sum of branch length=0.81275044 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates)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