Allozyme and DNA sequence comparisons of nine species of *Encephalartos* (Zamiaceae)

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Abstract

Phylogenetic relationships between *Encephalartos altensteinii* Lehmann, *E. arenarius* R.A. Dyer, *E. horridus* (Jacquin) Lehmann, *E. latifrons* Lehmann, *E. lehmannii* Lehmann, *E. longifolius* (Jacquin) Lehmann, *E. princeps* R.A. Dyer and *E. trispinosus* (Hooker) R.A. Dyer were studied, using *E. ferox* Bertoloni f. as outgroup. Three continuous and one discontinuous buffer systems were used and gene products of 14 enzyme coding loci were examined by horizontal starch gel-electrophoresis. Genetic variation was studied in a cultivated population of *E. lehmannii* and the average heterozygosity value for this population is 13.5%, which falls within the range reported for other cycad species. Fixed allele differences between the species studied was not found at any of the loci studied, which suggest that these species are closely related. DNA sequence analysis of *rbcL* and ITS 1 & 2 genes (1428 and 895 basepairs, respectively) confirmed the close genetic relationships between these taxa. According to ITS and *rbcL* sequences *E. altensteinii* and *E. princeps* are sibling taxa which form a sister group to *E. arenarius*, *E. horridus*, *E. latifrons*, *E. lehmannii*, *E. longifolius*, and *E. trispinosus*. The genetic distances between both groups were 0.12–0.47% for ITS and 0.08–0.16% for *rbcL* DNA. The results indicate recent (probably pleistocenic) speciation for this group of cycads, and the relationships are discussed with reference to affinities based on morphology and distribution. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Phylogenetic relationships; *Encephalartos*; Zamiaceae; Allozyme; DNA sequence; *rbcL*; ITS 1 & 2

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1. Introduction

The taxonomy of the Cycadales is problematic because really distinctive morphological characteristics are lacking in most groups, and species are usually defined in terms of sets of characteristics. Furthermore, the characteristics used for identification may not be of phylogenetic significance due to a high degree of convergence or divergence. It also seems reasonable to assume that at least some of the species in a given geographical area are closely related (i.e. derived from a common ancestor) due to slow reproduction rates (Tang, 1990) and absence of long-distance dispersal mechanisms for seeds. In the Eastern Cape Province of South Africa eight of the 13 known *Encephalartos* species are thought to be closely related. These are *Encephalartos arenarius* R.A. Dyer, *E. horridus* (Jacquin) Lehmann, *E. latifrons* Lehmann, *E. lehmannii* Lehmann, *E. longifolius* (Jacquin) Lehmann, *E. princeps* R.A. Dyer, and *E. trispinosus* (Hooker) R.A. Dyer, with *E. altensteinii* Lehmann thought to be somewhat more distantly related. These are the species used for this study. The remaining species, *E. caffer* (Thunberg) Lehmann, *E. cycadifolius* (Jacquin) Lehmann, *E. friderici-guilielmi* Lehmann, *E. ghellinckii* Lemaire, and *E. villosus* Lemaire, most certainly belong to other, more distantly related groups within the genus. The studied species have either or both very strongly dentate (almost dentate-lobed) leaflets and very glaucous leaves (Dyer, 1965a, b). Such dentate leaflets are not known anywhere else in the distribution area of the genus, and glaucous leaves are uncommon elsewhere (Vorster, 1993, 1999). Apart from these two characteristics, these eight species vary greatly in respect of growth form (development of an aerial trunk) and cone morphology. The development of an aerial trunk may well be a rather plastic (albeit at present genetically fixed) reaction to local environmental conditions, as the habitat becomes markedly drier further inland from the coast. The cones, being the reproductive structures, could be expected to provide a more reliable indication of phylogenetic relationships than vegetative characteristics. However, cone morphology varies considerably, and cannot be satisfactorily correlated with vegetative morphological features (Vorster, 1993).

The purpose of this study is to compare the eight species of *Encephalartos* listed above in respect of DNA sequence (ITS 1 & 2 and *rbcL*) and allozyme data to determine whether they form a phylogenetically coherent group and to determine the relationships between the species. *Encephalartos ferox* Bertoloni f. is included as outgroup species. We have also analysed a cultivated population of *E. lehmannii* to compare within- and between species allozymic variation.

2. Material and methods

2.1. Allozyme study

Electrophoretic data for 126 individuals, from nine species (Table 1), were compared. We used cultivated plants, firstly because of the remote locations of

Table 1
Locality description of the *Encephalartos* individuals studied

Sample	Species	Locality	Nos.
1–2 3–9	<i>E. altensteinii</i>	University of Pretoria KNBG	1406, 1444 Cultivated, AL58-247/16, AL209-69/ 94, Bathurst, Mkambati, Boesmans River Mouth, Grahamstad
1–6 7–8 9–10	<i>E. arenarius</i>	GLOW University of Pretoria KNBG	1429, 1464 1310/83 AR-2, 1310/83 AR-3
1–5 1–5 6–9	<i>E. ferox</i>	GLOW GLOW University of Pretoria	^a ^a 1386, 1454, 1520, 1494
10–13 1 2–4 5–9	<i>E. horridus</i> <i>E. latifrons</i>	KNBG GLOW University of Pretoria KNBG	HO-7, HO-10, HO-18, HO-20 ^a 3501, 3502, 1461 LF-1, LF-2, LF-9, LF-15, 48-13 directly north of LF-15
1–4 5–36 37–40 41–44 45–48	<i>E. lehmannii</i>	GLOW GLOW University of Pretoria Stellenbosch KNBG	^a ^b 1367, 1368, 1369, 1482 In Van der Bank et al. (1998) 573/82 LH-9, 573/82 LH-7, Olifantshoek, Christo/Jac
1–5 6–8 9–10 1–3 4–7 8–11	<i>E. longifolius</i> <i>E. princeps</i>	GLOW University of Pretoria KNBG GLOW University of Pretoria KNBG	^a 1455, 1466, gray type 574/52 LO-12, 53081 LO2 ^a 1394, 1414, 1490, 3464 571/82 PR-18, 571/82 PR-9, 571/182 TR-8, 571/82 PR-19
1–5 6–9 10–11	<i>E. trispinus</i>	GLOW University of Pretoria KNBG	^a 1397, 1436, 1495, 1497 110/83 TP-41, TP-38

GLOW, Lowveld National botanical Garden; KNBG, Kirstenbosch National Botanical Gardens.

^a Mature plants are not numbered at the GLOW.

^b Samples from seedlings.

natural populations, and secondly because plants in nature have become very scarce due to illicit collecting for the ornamental plant trade. We have, however, used only plants of undisputed identity, and with collection data where possible. When sampling different specimens of the same species, we took care not to sample a clone twice. Herbarium voucher specimens of the plants studied were deposited at the herbarium of the Botany Department at the University of Stellenbosch (STEU) and at the herbarium of the Lowveld National Botanical garden (GLOW). *Encephalartos ferox* was selected as outgroup because it has a number of characteristics not found in the Eastern Cape Province species. It has a soft-textured stem in contrast to the hard and compact stems of the Eastern Cape species: it has salmon-red cones which

are rare in *Encephalartos*, the male cones emerge in succession rather than simultaneously and are often borne on markedly long peduncles (a trait of tropical species), and it is geographically far separated from the Eastern Cape (Vorster, 1999).

In order to compare intra- and interspecific variation, we also used seedlings raised from field-collected seed of different plants of *E. lehmannii*, and grown at GLOW. The seedlings will eventually be planted in one of several gene banks at GLOW.

Leaf tissue extracts were prepared and analysed by starch gel electrophoresis (12% gels) using the extraction buffer, standard electrophoretic procedures, method of interpretation of gel banding patterns and locus nomenclature referred to by Van der Bank et al. (1998). The buffers used are: tris–EDTA–borate (A; pH 8.6; Goncharenko et al., 1992); histidine citrate (HC; pH 5.7; Kephart, 1990); tris–EDTA–borate (MF; pH 8.6; Markert and Faulhaber, 1965), and lithium–borate (electrode: pH 8.0) tris–citrate (gel: pH 8.7) (RW; Ridgway et al., 1970). Statistical analysis of allozyme data was executed using BIOSYS-1 (Swofford and Selander, 1981) and DISPAN (Ota, 1993). We have used GENEPOP (Raymond and Rousset, 1995) to determine if significant genotypic differentiation for all species studied could be found.

2.2. DNA sequence analyses

Plant material was identical to that used for allozyme analysis; in addition fresh material of *E. altensteinii*, and *E. lehmannii* (no. 2) was obtained from the Botanical Garden of Heidelberg University.

DNA was isolated from dried leaves of *Encephalartos* using the CTAB method (Doyle and Doyle, 1990). The marker genes were amplified by polymerase chain reaction (PCR) using the following primers: *rbcL*-N (forward) 5'-ATG TCA CCA CAA ACA GAR ACK AAA GC-3'; *rbcL*-R (reverse) 5'-CAA AAT TAA ATM SGA TCT CTT TCC ATA C-3'; ITS-S10 (forward) 5'-GAA TGA TCC GGT GAA GTG TTC GGA TCG-3'; ITS-AB 102 (reverse) 5'-ACT CTT CCC GGT TCG CTC GCC GTT AC-3'.

2.2.1. PCR conditions

A final volume of 50 µl contained 0.5–1 µg DNA, 5 µl 10 × Taq buffer (500 mM KCl, 100 mM Tris–HCl, 1% Triton × 100, pH 9.0), 3 µl 25 mM MgCl₂, 12.5 pmol primer, 1.5 µl dNTPs (10 mM), 0.75 U Taq-Polymerase (Pharmacia), 1 µl DMSO, and 1 µl 20 mg/ml BSA. PCR cycle for *rbcL*: 2 min at 94°C, then 30 cycles with 45 s at 94°C, 90 s at 45°C and 90 s at 72°C, and finally 5 min at 72°C; ITS: 2 min at 94°C, then 30 cycles with 45 s at 94°C, 90 s at 71°C and finally 20 min at 72°C.

PCR products were further amplified by Cycle-Sequencing using the “ThermoSequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP” (Amersham Life Science) according to the protocol of the manufacturer. Products were sequenced with an automatic sequencer ALFexpress (Pharmacia). Sequences will be deposited with GeneBank. Sequences were aligned manually or with use of CLUSTAL W (1.6) (gap-penalty 10). Aligned sequences were analysed

using the phylogeny program PAUP* 4.0 (Swofford, 1991). Methods employed were maximum parsimony (MP), neighbour-joining (NJ), and maximum likelihood (MLE). A bootstrap analysis (1000 replicates) was performed to estimate how good the furcations were supported by the data.

3. Results and discussion

3.1. Allozyme study

Locus abbreviations, enzyme commission numbers, and monomorphic loci are listed in Table 2. Allele products at the following loci were monomorphic: GPI-1, MDH, PGDH, PGM-1, -2, PEPD and PEP-LT1, and those of ACP and PER migrated cathodally. Allele frequencies for polymorphic loci are presented in Table 3, and allozyme phenotypes of putative heterozygotes were congruent with those expected on the basis of the quaternary structure of the enzyme (Ward, 1977).

3.1.1. Within species variation

Ellstrand et al. (1990) reported values of $P = 50\%$, $A = 1.61$ and $H = 0.08$; Byrne and James (1991) obtained values of 60% , 1.8 and 0.24 ; Sharma et al. (1998b) reported ranges of $P = 17.6\text{--}35.3\%$, $A = 1.2\text{--}1.4$ and $H = 0.02\text{--}0.11$; and Sharma et al. (1998a) obtained values of $P = 26\%$, $A = 1.3$ and $H = 0.06$. Therefore, the ranges of these values are from 17.6 to 60% , $1.2\text{--}1.8$ and $0.02\text{--}0.11$ respectively. We have obtained an H value of 0.135 , a P value of 35.7% , and an A value of 1.6 for the

Table 2

Locus abbreviations, enzyme commission numbers (E.C. No.) and buffers are listed after each enzyme. See Material and Methods for descriptions of buffers

Enzyme	Locus	E.C. no.	Buffer
Aspartate aminotransferase	AAT	2.6.1.1	A
Acid phosphatase	ACP	3.1.3.2	HC
Adenylate kinase	AK	2.7.4.3	RW
Esterase	EST	3.1.1.-	RW
Glucose-6-phosphate isomerase	GPI-1 ^a , -2	3.5.1.9	MF
Leucine aminopeptidase	LAP	3.4.11.1	A
Malate dehydrogenase	MDH ^a	1.1.1.37	HC
Peptidase		3.4.-.-	
Substrate: Phenylalanyl-proline	PEP-D ^a		RW
Leucyl-tyrosine	PEP-LT ^a		RW
Peroxidase	PER	1.11.1.7	MF
Phosphoglucomutase	PGM-1 ^a , -2 ^a	5.4.2.2	MF
6-Phosphogluconate dehydrogenase	PGDH ^a	1.1.1.44	A

^a Monomorphic loci.

Table 3

Allele frequencies at polymorphic loci for the *Encephalartos* species studied. See Table 4 for species designations

Locus allele		Species								
		1	2	3	4	5	6	7	8	9
AAT	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.917	1.000
	B								0.083	
EST	A				0.067				0.143	
	B	1.000	0.967	0.591	0.633	0.950	0.646	0.875	0.786	0.833
	C		0.033	0.364	0.200	0.050	0.293	0.125	0.071	0.167
	D			0.045	0.033					
	E				0.067		0.061			
ACP	A	1.000	0.967	0.950	0.893	0.950	0.900	0.917	0.917	0.909
	B		0.033	0.050	0.107	0.050	0.100	0.083	0.083	0.091
AK	A	0.700	0.567	0.778	0.584	0.917	0.625	0.750	0.400	0.693
	B	0.300	0.433	0.222	0.333	0.083	0.208	0.250	0.600	0.269
	C				0.083		0.167			0.038
GPI2	A	1.000	1.000	1.000	0.643	0.500	0.662	0.750	0.500	0.500
	B				0.143	0.500	0.309	0.250	0.500	0.500
	C				0.214		0.029			
LAP	A	1.000	0.882	1.000	0.600	1.000	1.000	1.000	1.000	1.000
	B		0.118		0.400					
PER	A	0.500	0.125	0.188	0.071		0.097	0.150		
	B	0.500	0.875	0.812	0.929	1.000	0.903	0.850	1.000	1.000

cultivated *E. lehmannii* population studied. These values fall within the ranges reported above for the *P* and *A* values, and it is 2.5% higher for the *H* value. The latter value is probably due to the fact that we have studied a cultivated population selected to include the maximum phenotypic variation of the parent plants. Nevertheless, our values are also less than corresponding values reported for other outbreeding plants (Gottlieb, 1981) and for 28 studies of gymnosperms (Hamrick et al., 1979). This is in agreement with the results of the above-mentioned authors on other cycad species.

A zymogram showing more phenotypic variation within than between species in allele products at the EST enzyme coding locus is presented in Fig. 1. Gene diversity analysis revealed that 85% of the differentiation was intraspecific and only 15% interspecific. This, together with the fact that the taxa all share the most common allele at all of the enzyme coding loci studied, indicate that they are very closely related and that their divergence must be relatively recent. It also demonstrates that sample error due to small sample sizes is a significant factor to consider. For example, Van der Bank et al. (1998) recommended that the results of their preliminary study should be verified by using larger sample sizes, and it is evident that they have over estimated the amount of genetic differentiation between *E. altensteinii* and *E. lehmannii*.

Fig. 1. Zymogram showing allelic variation in nine cycad taxa and genotypes for individual specimens at the esterase enzyme coding locus.

3.1.2. Between species differentiation

The taxonomic uses of allozyme electrophoretic data were reviewed by many authors, including Avise (1974), Thorpe (1982) and Thorpe and Solé-Cava (1994). Several statistically based measures of genetic distance are available to reduce genetic differentiation between populations over a range of enzyme loci to a single figure level. However, the Nei (1978) measure is now used predominantly (Thorpe and Solé-Cava, 1994). It is interesting that the consensus tree has got the same topology as the Nei (1978) tree (Figs. 2e and c).

The Nei (1978) unbiased genetic distance values (adapted for small sample sizes) between taxa are presented in Table 4. The mean genetic distance values between the ingroup taxa studied are 0.009 and 0.025 between the ingroup and outgroup taxa studied. These values were higher for the other genetic distance values reported in Table 4, but reflect similar groupings, and are included for comparisons with values listed in the literature. Phylogenetic relationships based on all four genetic distance value sets are depicted in Figs. 2a–d and a majority-rule consensus tree is shown in Fig. 2e. The cophenetic correlation value for the rooted Wagner trees in Fig. 2a is 95.1% the chord distance coefficient of (Cavalli-Sforza and Edwards (1967) and 94.9% for the Rogers (1972) genetic distance (Fig. 2d). Poor bootstrap values (average: 45.4, range: 22–79) between the branches were obtained (using DISPAN), and the only relative good values (65 and 79 in Figs. 2b and c) showed incongruent groupings between these trees. The consensus tree (Fig. 2d) showed that *E. altensteinii* is basal to the other ingroup taxa.

Shaklee et al. (1982), Thorpe (1982) and Thorpe and Solé-Cava (1994) showed the relationship between taxonomic divergence and genetic distance, and concluded that the genetic distance (Nei, 1972) average 0.05 (range: 0.002–0.07) for conspecific populations and 0.30 (range: 0.03–0.61) for congeneric species. The genetic distance values obtained in the present study (Table 4) between the congeneric species (0.025) falls within the lower limit for populations from the same species. The close relationships between the taxa studied is responsible for the low bootstrap values in Fig. 2b (range: 22–65; average: 43) and in Fig 2c (range: 35–79; average: 53). For morphological data, bootstrap values of 50–70 are considered adequate, but for molecular data higher values are required. This supports the results of the DNA analysis, which also indicated that the species are closely related and that recent divergence has occurred.

The Wright (1978) fixation (F) index is another measure to describe differentiation between populations. The amount of differentiation among subpopulations relative to the limiting amount under complete fixation, F_{ST} , averaged 0.150 for polymorphic loci (Table 5). This is an indication of little genetic differentiation

a) **b)**

Fig. 2. Dendrograms showing phylogenetic relationships based on the genetic distance values of: (a) Cavalli-Sforza and Edwards (1967) chord distance and the distance Wagner procedure (Farris, 1972), (b) Nei et al. (1983), (c) Nei (1978), (d) Rogers (1972) using the distance Wagner procedure, and (e) a consensus tree based on allozyme data (Table 4) for nine cycad taxa. Numbers at nodes in (b) and (c) are bootstrap values, and majority rule percentages in (e).

c)

d)

Fig. 2 (continued).

between the cycads studied. The mean value of the Wright (1978) fixation index of individuals relative to the total population, F_{IS} , is 0.500 and 0.575 for the total population and its subpopulations (F_{IT}). The values of F_{IS} are close to zero in most natural populations where random mating within subpopulations occur (Nei, 1986). The F_{IT} value is also indicative of limited barriers to gene flow between the taxa studied. The loci did not contribute much to intraspecific differences (F_{ST} values ranged from only 0.09 to 0.295) because the taxa shared the most common alleles at all loci (Table 3). Our average F_{IS} value is more than the values reported by other

e)

Fig. 2 (continued).

researchers for cycads (0.2–0.47). Conversely, the genetic distance values between the taxa in the present study are less than values obtained by other authors for cycads. For example, the Nei (1978) genetic distance values were between 0.014 and 0.250 (Sharma et al., 1998b), from 0.042 to 0.222 for six *Encephalartos* species (Van der Bank et al., 1998), and the Rogers (1972) genetic distances ranged from 0.023 to 0.072 (Sharma et al., 1998a). Walters and Decker-Walters (1991) also reported similar (low) overall variation in *Zamia pumila* L. *sensu lato* and high geographical differentiation. Our maximum genetic distance values were 0.038 between the outgroup species and *E. latifrons* (Nei, 1978) and 0.118 for the Rogers (1972) genetic distance (Table 4). These values are, therefore, comparable to the values for other cycad taxa, which indicate that the species are closely related.

Even though fixed allele mobility differences between species did not occur at any of the loci studied, significant ($P < 0.05$) differences in allelic distributions were obtained at the following loci: EST (between *E. arenarius* and *E. ferox*, *E. altensteinii* and *E. latifrons*; *E. lehmannii* and *E. altensteinii*, *E. latifrons* and *E. princeps* respectively), GPI-2 (between *E. horridus* and *E. altensteinii*; and *E. altensteinii* and *E. trispinosus*, respectively) and LAP (between *E. horridus* and all of the other taxa excluding *E. altensteinii* and *E. ferox*). In addition, significant ($P < 0.05$) heterogeneity differences was only found at the ACP locus (Table 6) for the combined analysis of all taxa studied. These differences may present insights into the relationships of the taxa studied.

3.1.3. Relationships

Encephalartos altensteinii was included in the study because it occurs in the same geographical area as the others and naturally hybridises with some of them. For example, it interbreeds freely with *E. trispinosus* where the two species are sympatric

Table 4

Matrix of Nei's (1978) unbiased genetic distance coefficient below diagonal, Cavalli-Sforza and Edwards' (1967) chord distance above diagonal, and Rogers (1972) and Nei et al. (1983) distances (bold values above and below diagonal, respectively)

Species		1	2	3	4	5	6	7	8	9
1	<i>E. ferox</i>	*****	0.106	0.135	0.223	0.198	0.191	0.135	0.219	0.207
2	<i>E. altensteinii</i>	0.008	*****	0.114	0.161	0.172	0.165	0.117	0.180	0.168
3	<i>E. arenarius</i>	0.013	0.011	*****	0.183	0.175	0.141	0.109	0.197	0.165
4	<i>E. horridus</i>	0.035	0.014	0.018	*****	0.195	0.143	0.170	0.189	0.175
5	<i>E. latifrons</i>	0.038	0.029	0.029	0.027	*****	0.130	0.093	0.130	0.066
6	<i>E. lehmannii</i>	0.026	0.017	0.008	0.011	−0.008	*****	0.098	0.155	0.094
7	<i>E. longifolius</i>	0.008	0.004	0.004	0.012	−0.015	0.003	*****	0.131	0.088
8	<i>E. princeps</i>	0.036	0.015	0.028	0.014	−0.008	0.005	0.004	*****	0.107
9	<i>E. trispinosus</i>	0.035	0.021	0.021	0.016	−0.019	0.002	−0.003	−0.004	*****
1		*****	0.049	0.059	0.118	0.094	0.093	0.06	0.118	0.092
2		0.014	*****	0.055	0.078	0.080	0.074	0.051	0.086	0.077
3		0.023	0.016	*****	0.085	0.083	0.051	0.044	0.104	0.073
4		0.061	0.032	0.041	*****	0.100	0.057	0.073	0.092	0.075
5		0.048	0.036	0.038	0.047	*****	0.061	0.048	0.056	0.026
6		0.045	0.034	0.025	0.025	0.021	*****	0.036	0.067	0.040
7		0.023	0.017	0.015	0.036	0.011	0.012	*****	0.068	0.036
8		0.059	0.040	0.048	0.044	0.021	0.030	0.021	*****	0.038
9		0.053	0.035	0.033	0.038	0.005	0.011	0.010	0.014	*****

Table 5

Summary of Wright's (1978) F -statistics at all loci

Locus	F_{IS}	F_{IT}	F_{ST}
AAT	1.000	1.000	0.075
EST	0.241	0.330	0.117
ACP	0.327	0.339	0.018
AK	0.590	0.627	0.090
GPI2	0.480	0.596	0.223
LAP	1.000	1.000	0.295
PER	0.593	0.674	0.198
Mean	0.500	0.575	0.150

(Dyer, 1965b; Vorster, 1986). Nevertheless, *E. altensteinii* differs from the other species in respect of its bright green, narrow, very moderately dentate leaflets, and its yellowish cones, and has been suspected of belonging to a different group of species. Not unexpectedly, in all the phenograms (Fig. 2) it occurs in a basal position.

Encephalartos arenarius closely resembles the other species on account of its short stem, its short and very rigid fronds of which the apices tend to recurve, and its more or less glaucous and very pronounced dentate leaflets. In all the phenograms it is basal to all the remaining ingroup species studied (*E. longifolius*, *E. horridus*, *E. lehmannii*, *E. trispinosus*, *E. latifrons* and *E. princeps*). Vegetatively it very strongly

Table 6
Contingency Chi-square analysis at polymorphic loci

Locus	No. of alleles	Chi-square	D.F. ^a	<i>p</i>
AAT	2	22.154	8	0.005
EST	5	64.521	32	0.001
ACP	2	2.451	8	0.964
AK	3	29.283	16	0.022
GP12	3	41.086	16	0.001
LAP	2	83.510	8	0.000
PER	2	21.001	8	0.007
Total	2	264.007	96	0.000

^aD.F., degrees of freedom.

resembles *E. latifrons* to such an extent that the two species are not always easy to distinguish in the absence of cones which are distinct in their morphology (Dyer, 1956). In *E. arenarius*, the female cone scale faces are smooth rather than warty, and the male cone scales have much less prominently produced apices, but as yet we cannot say what the phylogenetic significance of these differences is. It therefore comes as a surprise that these two species are so far apart in all the trees. Very glaucous variants of *E. arenarius* vegetatively also strongly resemble *E. horridus*, and the only obvious difference between the cones is the russet-brown indumentum in *E. horridus*. It is therefore also unexpected that these two species are so far apart. However, the small genetic distance values and low bootstrap numbers as a result of very similar allele frequencies, indicate that it closely resembles the other species, and that the relationships and variable positions of the other ingroup taxa are probably random.

Encephalartos longifolius is basal to *E. princeps*, *E. trispinosus* and *E. latifrons* in Figs. 2a and d, and basal to *E. horridus*, *E. lehmannii*, *E. trispinosus*, *E. latifrons* and *E. princeps* in Figs. 2b and c as well as the consensus tree. All these species have relatively short and very rigid fronds, and typically the very base of the frond has a sharply delineated pale-coloured zone which is also present in *E. arenarius*. This pale-coloured zone is also present in other species to as far north as Kenya (e.g. *E. kisambo* Faden and Beentje, 1989), but is usually not nearly as conspicuous as in these Eastern Cape species. However, the separation of *E. longifolius* is supported by its combination of usually an almost total absence of teeth on the leaflets and well-developed arborescent habit. Its basal position relative to *E. princeps*, *E. trispinosus* and *E. latifrons* in Figs. 2a and d corresponds with the drawn-out apices of the microsporophylls and the warty faces of the megasporophylls of all four species, and the very well-developed trunks of *E. longifolius*, *E. latifrons* and *E. princeps*. Of these four species, *E. princeps* is the only one that habitually produces multiple cones per stem. *Encephalartos longifolius* hybridises naturally with *E. horridus* where the two species come into contact near Uitenhage (Vorster, 1986), and where it also hybridises with *E. lehmannii* in the Paardepoort (JH, personal observation). It is

possible that hybridisation with *E. horridus* was previously more extensive, as suggested by occasional specimens exhibiting strongly dentate leaflets.

Encephalartos horridus is vegetatively a very distinctive species. In Fig. 2a, it is basal to *E. lehmannii*, *E. longifolius*, *E. princeps*, *E. trispinosus* and *E. latifrons*, and in Fig. 2b it is basal to all these species except *E. longifolius*. While this is plausible in terms of morphological traits, it is not particularly illuminating. However, in Figs. 2c–e it is grouped together with *E. lehmannii*. Superficially these two species are morphologically quite different, with the leaflets of *E. horridus* being heavily pungent-lobed and those of *E. lehmannii* being almost completely entire at maturity. Nevertheless, there are also close morphological resemblances between the two, including relatively poorly developed stems, hard and strongly glaucous fronds with apices more or less recurving, solitary green cones overlain with russet indumentum, microsporophylls with moderately projecting apices, and megasporophylls with mostly smooth exposed faces. The two species are geographically adjacent, and north of Uitenhage there is a morphologically intermediate population that may or may not be indicative of interbreeding between the two species. Occasional warty female cones in *E. horridus* may be indicative of past hybridisation with *E. longifolius*.

Encephalartos lehmannii in all the phenograms is well separated from *E. princeps*, which it remarkably resembles vegetatively although it differs profoundly in respect of morphology and number of both male and female cones. The two species are also geographically (and thus reproductively) well separated. In all the phenograms it occupies a position close to that of *E. horridus*.

Encephalartos trispinosus in Figs. 2a–e presents no real conflict with morphological data, falling in the group of species with drawn-out apices of microsporophylls and warty exposed faces of megasporophylls, but nevertheless we are surprised at its wide separation from *E. horridus*. Vegetatively, these two species can be very similar, both having abbreviated stems and heavily glaucous and lobed fronds. These results confirm that the two species are quite distinct.

Encephalartos latifrons, in all the phenograms, occupies unexpected positions relative to *E. arenarius* and *E. longifolius*. Its cone morphology (see above under *E. longifolius*) supports its grouping with *E. trispinosus*, *E. longifolius*, and *E. princeps*. The surprise is that it lies so far from *E. arenarius*, which it vegetatively resembles to a marked degree, even though the cones of the two species are quite different. It is also surprising that it lies so far from *E. longifolius* which it resembles by its thick and long trunk, recurved green leaves, drawn-out microsporophylls, and warty exposed faces of the megasporophylls. *Encephalartos longifolius* lacks the pungently lobed leaflets of *E. latifrons*, and occasionally heavily lobed leaflets are suspected to be indicative of past hybridisation with *E. horridus*.

Encephalartos princeps occupies a position basal to *E. trispinosus* and *E. latifrons* in Figs. 2a, c and e. This placement is supported by the similarity of its cone morphology to these species, but not really by its vegetative morphology. Again it is surprising that it lies so far from *E. lehmannii* which is vegetatively quite similar, although the two species are very different in their cone morphology and geographical distribution. Its vegetative similarity to *E. lehmannii* may well reflect convergent evolution, because there are conspicuous differences in the number and

Fig. 3. Molecular phylogeny of *Encephalartos* inferred from DNA sequences of the *rbcL* gene and ITS 1&2 sequences. (a) (b)=*rbcL* data: (a) MP phylogram; numbers refer to character changes, (b) NJ: bootstrap cladogram; number at bifurcation indicates the occurrence (in %) of the bifurcation in all trees. MP: CI = 1.0, RI = 1.0; (c) (d) = ITS data: (c) MP phylogram of a single most parsimonious tree; numbers refer to character changes, (d) NJ bootstrap cladogram; number at bifurcation indicates the occurrence (in %) of the bifurcation in all trees. MP: CI = 1.0, RI = 1.0.

morphology of the cones of these two species. Its varying positions in Fig. 2 raises the question of whether its phylogenetic affinities lie at all with the other species. Indeed, it is dissimilar to all the other species studied. It occurs further northwards than any of the other species, its well-developed trunk is more slender compared to the often massive trunks of the others, it has far narrower and softer textured leaflets (like *E. lehmannii* practically edentate though not pungent), and it habitually bears more than one cone per stem.

3.2. DNA sequence analyses

Using *E. ferox* as outgroup, phylogeny reconstructions from *rbcL* and ITS 1&2 DNA sequences (Appendix A) produced almost identical tree topologies,

Fig. 3 (continued).

independent of the method employed (MP, NJ or MLE). In Fig. 3, respective phylograms and cladograms for MP (Fig. 3 a, c) and NJ bootstrap (Fig. 3 b, d) trees are illustrated. Because *rbcL*- and nuclear ITS DNA produced similar trees, it indicates that data are not distorted by past hybridisation and reticulate evolution. Two clusters are evident: *E. altensteinii* and *E. princeps* cluster together as sibling species, and form a sister group to *E. arenarius*, *E. horridus*, *E. latifrons*, *E. lehmannii*, *E. longifolius*, and *E. trispinosus* which show no interspecific sequence variation. A single parsimony-informative character separates both groups of species, and genetic distances (p-distance) between both groups were 0.12–0.47% for ITS and 0.08–0.16% for *rbcL* DNA (Table 7).

The *rbcL* tree resolves into three groups (Fig. 3a). The group comprising *E. altensteinii* is supported by morphological evidence. The group comprising *E. princeps* is to a far lesser extent supported by morphological evidence, but geographical evidence lends credibility to its separation, presumably reflecting

Table 7

Uncorrected pairwise distances between taxa; 1.0 = 100% divergence. Above diagonal *rbcL*; below diagonal: ITS 1&2

Species	1	2	3	4	5	6	7	8	9
1 <i>E. ferox</i>	—	0.0016	0.0024	0.0016	0.0016	0.0016	0.0016	0.0008	0.0016
2 <i>E. altensteinii</i>	0.0165	—	0.0008	0.0000	0.0000	0.0000	0.0000	0.0008	0.0000
3 <i>E. arenarius</i>	0.0152	0.0035	—	0.0008	0.0008	0.0008	0.0008	0.0016	0.0008
4 <i>E. horridus</i>	0.0154	0.0035	0.0000	—	0.0000	0.0000	0.0000	0.0008	0.0000
5 <i>E. latifrons</i>	0.0155	0.0047	0.0012	0.0012	—	0.0000	0.0000	0.0008	0.0000
6 <i>E. lehmannii</i>	0.0151	0.0035	0.0000	0.0000	0.0012	—	0.0000	0.0008	0.0000
7 <i>E. longifolius</i>	0.0155	0.0036	0.0000	0.0000	0.0012	0.0000	—	0.0008	0.0000
8 <i>E. princeps</i>	0.0140	0.0023	0.0012	0.0012	0.0024	0.0012	0.0012	—	0.0008
9 <i>E. trispinosus</i>	0.0153	0.0035	0.0000	0.0000	0.0012	0.0000	0.0000	0.0012	—

longer genetic isolation than between species of the main group. The third (main) group comprising *E. trispinosus*, *E. arenarius*, *E. longifolius*, *E. horridus*, *E. lehmannii* and *E. latifrons* shows no further resolution, confirming morphological and geographical evidence that these species form a close group, with species equally well defined.

The ITS tree (Fig. 3c) which should provide a better resolution than cpDNA sequences also separates *E. altensteinii* and *E. princeps* from the other species. *Encephalartos altensteinii* and *E. latifrons* are well defined, as indicated by the relatively long branches on which they are situated, in contrast to *E. lehmannii*, *E. trispinosus*, *E. horridus*, and *E. arenarius*.

Encephalartos horridus has a fairly wide geographical distribution over which some genetic if not morphological variation could be expected, but plants from different origins had identical DNA sequences. Genetically, the species analysed are closely related. Using *rbcL* data, *E. altensteinii* and *E. princeps* show a genetic distance of 0.16–0.08% to the other *Encephalartos* species. For *rbcL* data we derived a calibration of 1% sequence divergence equalling 28 million years (Treutlein, 1998). Using this crude estimate, we can assume that divergence of the species studied started approximately 4–2 million years ago in the Late Pliocene/Early Pleistocene.

4. Conclusions

Encephalartos ferox, selected as outgroup, is not closely related to any of the other species investigated. However, the allozyme and DNA sequence data implicate close and recent evolutionary relationships between the ingroup taxa, and *E. altensteinii* is separated from the other Eastern Cape species studied by both allozyme and DNA data, as shown by its basal position in all the trees (Figs. 2 and 3).

Encephalartos arenarius, in spite of its very strong vegetative resemblance to *E. latifrons*, and having rare vegetative characteristics in common with *E. horridus*

and *E. trispinosus*, occupies a basal position relative to *E. longifolius*, *E. horridus*, *E. lehmannii*, *E. trispinosus*, *E. latifrons* and *E. princeps* in all the allozyme phenograms but not in the ITS and *rbcL* trees. From the allozyme data we conclude that it has the least recent evolutionary links to any of these species, and especially to those that it vegetatively most closely resembles.

Encephalartos longifolius also occupies a basal position in allozyme based trees relative to *E. trispinosus*, *E. latifrons* and *E. princeps* (Fig. 2) with which it shares conspicuously elongated apices to the microsporophylls and warty exposed faces of the megasporophylls.

Encephalartos horridus and *E. lehmannii* seem to be an offshoot of the previous group, rather than a separate lower branch. These two species are grouped close together in all the phenograms and DNA trees. We conclude that they are closely related, much closer than we previously suspected.

The grouping of *E. trispinosus* and *E. latifrons* is surprising. Vegetatively, they are quite different, and the only resemblance to each other is the cone characteristics common to both as well as to *E. longifolius* and *E. princeps*. The position of these species in all the phenograms does, however, confirm Dyer's decision to recognise *E. arenarius* as distinct from *E. latifrons* (Dyer, 1956), and *E. trispinosus* as distinct from *E. horridus* (Dyer, 1965a).

The position of *E. princeps* as basal to *E. trispinosus* and *E. latifrons* in allozyme analyses (Figs. 2a, c and e) agrees with the cone characteristics of these species. The grouping of *E. princeps* with *E. trispinosus* in Figs. 2b and d probably does not reflect its true phylogenetic position, because *E. princeps* deviates from the general pattern by its multiple cones, geographical distribution, and perhaps its somewhat softer-textured foliage. DNA data that places *E. princeps* as a sister taxon to *E. altensteini* conflicts between both data sets.

The genetic distance between these species is much smaller than normal for gymnosperms (Hamrick et al., 1979). The same was found in the Australian genus *Macrozamia* (Sharma et al., 1998a, b). Possible explanation for the small genetic distances between the species are: (a) these plants are slow to reach reproductive age (it may take 50 years to reach reproductive age under natural conditions; PV personal observation), (b) successful reproductive events are probably separated by a hundred years or more, and (c) the plants lack a mechanism for long distance dispersal of seeds (Norstog and Nicholls, 1997; Tang, 1990; Vorster, 1995). While the small genetic differentiation may cast doubt on the specific status of these presumed species, the separation into species is well supported by morphological and geographical data, and reproductive behaviour, the latter amongst others manifested by different associated (and probably pollinating) insect species (Oberprieler, 1995). It seems certain that the Eastern Cape is an evolutionary centre for *Encephalartos* (as manifested by the present-day abundance of species and individuals), and has likely been so for an evolutionarily significant time. Traits such as heavily dentate leaflets, glaucous foliage, russet indumentum on the cones, and the tendency to produce single cones per stem, seem to signify that these species are closely related to each other. Our impression is that the present array of species is the result of fairly recent radiation (as manifested by the small and closely spaced distribution areas).

Evidence of suspected past hybridization comprises phenomena like occasional heavily dentate leaflets in species with normally entire leaflets (like *E. longifolius* and *E. lehmannii*), which can only be explained by former different climatic conditions which allowed species to occupy geographical ranges different from the present, allowing them to come into contact with each other. Recent origin of some species is suggested by the polymorphism of for instance *E. trispinosus*.

The small genetic differences obtained in the present study are indicative of the close relationship between the species. The data confirm that, apart from *E. altensteinii*, the morphologically similar species (the other ingroup taxa) in the Eastern Cape Province form a phylogenetically closely coherent group. Preliminary DNA amplification fingerprinting (DAF) results (Coetzer et al., unpublished results) indicate a similar grouping of the taxa as reported in Fig. 2e, and it supports the conclusions from the present study (i.e. little differentiation indicating recent speciation).

In conclusion, this is the first attempt at using morphological, allozyme and DNA sequence data to elucidate evolutionary relationships between these species of *Encephalartos*, and although some meaningful relationships and recent speciation were noticed, it is obvious that much more data are needed before these complex relationships can be clarified satisfactorily.

Acknowledgements

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Appendix A

Nucleotide sequences of *rbcL* (A) and ITS 1&2 (B) for nine cycad species (“-” = gap; “?” = no information obtained)

(A) *rbcL* data:

28 <i>Encephalartos ferox</i>	GCACCTGCTGCCCTG??GCAAG?????AGCGAACTGTCGAATTTCGGTGCTTCGCGGCTGCTGCCGATACCTCGACCA---TCGA??CACA?CCGAGCGAATGTCTCGTTAG CACGACGACTTGTCTCTCGATATTTCGTTGCGGGGGGTCG?GCAGCGTCGCGGACCT?TCTCA?GTTTCG?TGTTTGGTG 314 <i>Encephalartos altensteinii</i> GCACCTGC??CCTGGTTGCA?GACAACGAGCGAACTGTCGAATTTCGGTGCTTCGCGGCTGCTGCCGATACCTCGACCGCGTCGAACGTACATCCGAGCGAATGTCTCGTTAG CACGACGACTTGTCTCTCCGATATTTCGTTGCGGGGGGTCGAGGACGCTCGCGGACCGGTCTCACCGTTTCGAATGTTTGGTG 75 <i>Encephalartos arenarius</i> GCACCTGCTGCCCTGGTTGCAAGACAACGAGCGAACTGTCGAATTTCGGTGCTTCGCGGCTGCTGCCGATACCTCGACCA---TCGAACGTACATCCGAGCGAATGTCTCGTTAG CACGACGACTTGTCTCTCGATATTTCGTTGCGGGGGGTCGAGGACGCTCGCGGACCGGTCTCACCGTTTCGAATGTTTGGTG 40 <i>Encephalartos horridus</i> GCACCTGCTG?CCTGGTTGCAAGACAACGAGCGAACTGTCGAAT?GGTGCTTCGCGGCTG??CGATACCTCGACCA---TCGAACGTACATCCGAGCGAATGT?CGTTAG CACGACGACTTGTCTCT?ATATTTCGTTGCGGGGGGTCGAGGACGCTCGCGGACCGGTCTCACCGTTTCGAATGTTTGGTG 90 <i>Encephalartos latifrons</i> ?????????????????AAGACA?GAGCGAACTGTCGAATTTCGGTGCTTCGCGGCTGCTGCCGATACC?CGACCA---TCGAACGTACATCCGAGCGAATGT?TCGTTAG CACGACGACTTGTCTCTCGATATT?GTTGCGGGG?GTCGAAGCAGGCTCGCGGACCGGTCTCACCGTTTCGAATGTTTGGTG 56 <i>Encephalartos lehmannii</i> ?CACCTGCTGCCCTGGTTGCAAGACAACGAGCGAACTGTCGAATTTCGGTGCTTCGCGGCTGCTGCCGATACCTCGACCA---TCGAACGTACATCCGAGCGAATGTCTCGTTAG CACGACGACTTGTCTCTCGATATTTCGTTGCGGGGGGTCGAGGACGCTCGCGGACCGGTCTCACCG?TTCGAATGTTTGGTG 235 <i>Encephalartos lehmannii</i> GCACCTGCTGCCCTGGTTGCAAGACAACGAGCGAACTGTCGAATTTCGGTGCTTCGCGG?TGCTGCCGATACCTCGACCA---TCGAACGTACATCCGAG?AATGTCTCGTTAG CACGACGACTTGTCTCTCGATATTTCGTTGCGGGGGGTCGAGGACGCTCGCGGACCGGTCTCACCGTTTCGAATGTTTGGTG 5 <i>Encephalartos longifolius</i> ?????????????????GACAACGAGCGAACTGTCGAATTTCGGTGCTTCGCGGCTGCTGCCGATACCTCGACCA---TCGAACGTACATCCGAGCGAATGTCTCGTTAG CACGACGACTTGTCTCTCGATATTGCTTCGCGGGGCTTCGAGGACGCTCGCGGACCGG?CTCACCGTTTCGAATGTTTGGTG 42 <i>Encephalartos princeps</i> GCACCTGCTGCCCTGGTTGCAAGACAACGAGCGAACTGTCGAATTTC??GC??CGCGGCTGCTGCCGATACCTCGACCA---TCGAACGTACATCCGAGCGAATGTCTCGTTAG CACGACGACTTGTCTCTCGATATTTCGTTGCGGGGGGTCGAGGACGCG?CGCGGACCGGTCTCACCGTTTCGAATGTTTGGTG 66 <i>Encephalartos trispinosus</i> GCACCTGCTGCCCTGGTTGCAAGACAACGAGCGAACTGTCGAATTTCGGTGCTTCGCGGCTGCTGCCGATACCTCGACCA---T?G?ACGTACATCCGAGCGAAT?TCTCGTTA GCACGACGACTTGTCTCTCGATATTTC?TTCGGGGGGTCGAGGACGCGT?GGCGACCGGTCTCAC??TTCGAATGTTTGGTG 68 <i>Encephalartos trispinosus</i> GCACCTGCTGC?CTGGTTGCAAGACAACGAGCGAACTGTCGAATTTCGGTGCTTCGCGGCTGCTGCCGATACCTCGACC?---TCGAACGTACATCCGAGCGAATGTCTCGTTAG CACGACGACTTGTCTC?CGATATTTCGTTGCGGGGGGTCGAGGACGCTCGCGGACCT?GCTCACCGTTTCGAATGTTTGGTG
28 <i>Encephalartos ferox</i>	TGTCGGGAATCGGTGCCTCGCGGGGCTCTGTGTTTCGGCTTCCCACGCGTTTCGCTTTGGTTTGTGCGAG-ACATGCGGGGATGGCGATCGAGGTTCCAGTTACACCTCTCGCGCT GGGGTGCAATCCGGCCGTTGCAACCGTGCCCT-CCCGCGCGGGGCTTCCGTTTGACGACCGTCAGCGAGGCGCGGCGCTG 314 <i>Encephalartos altensteinii</i> TGTCGGGAATCGGTGCCTCGCGGGGCTTGTGTTTCGGCTTCCAGGCGTTTCGCTTTGGTTTGTGCGAG-ACATGCGGGGATGGCGATCGAGGTTCCAGTTACACCTCTGCCACA GGGTGCAATCCGGCCGTTGCAACCGTGCCCT-CCCGCGCGGGGCTTCCGTTTGGCGACCGTCAGCGAGGCGCGGCGCTG 75 <i>Encephalartos arenarius</i> TGTCG?AATCGGTGCCTCGCGGGGCTTGTGTTTCGGCTTCCAGGCGTTTC?TTGGTTTGTGCGAG-ACATGCGGGGATGGCGATCGAGGTT?CAGTTACACCTCTGCCACA GGGGTGCAATCCGGCCGTTGCAACCGTGCCCT?TTCGCGCGGGGCTTCCGTTTGGCGACCGTCAGCGAGGCGCGGCGCTG 40 <i>Encephalartos horridus</i> TGTCGGGAATCGGTGCCTCGCGGGGCTTGTGTTTTCGGCTTCCAGGCGTTTCGCTTTGGTTTGTGCG?A-ACATGCGGGGATGGCGCAT--GGTTCCAGTTACACCTCTGCCACA GGGGTGCAATCCGGCCGTTGCAACCGTGCCCTTCCCGCGGGGCTTCCGTTTGGCGACCGTCAGCGAGGCGCGGCGCTG 90 <i>Encephalartos latifrons</i> TGTCGGGAAT?GGTGCTTCGCGGGGCTTGTGTTTTCGGCTTCCAGGCGTTTCGCTTTGGTTTGTGCGAGCACATGCGGGGATGGCGATCGAGGTTCCAGTTACACCTCTGCCACA GGGGTGCAATCCGGCCGTTGCAACCGTGCCCT-CCCGCGGGGCTTCCGTTTGGCGACCGTCAGCGAGGCGCGGCGCTG 56 <i>Encephalartos lehmannii</i> TGTCGGGAATCGGTGCCTTCGCGGGGCTTGTGTTTTCGGCTTCCAGGCGTTTCGCTTTGGTTTGTGCGAG-ACATGCGGGGATGGCGATCGAGGTTCCAGTTACACCTCTGCCACA GGGGTGCAATCCGGCCGTTGCAACCGTGCCCTTCCCGCGGGGCTTCCGTTTGGCGACCGTCAGCGAGGCGCGGCGCTG 235 <i>Encephalartos lehmannii</i> TGTCGGGA?TCG?TGCTTCGCGGGGCTTGTGTTTTCGGCTTCCAGGCGTTTCGCTTTGGTTTGTGCGAG-ACATGCGGGGATGGCGATCGAGGTTCCAGTTACACCTCTGCCACA GGGGTGCAATCCGGCCGTTGCAACCGTGCCCTTCCCGCGGGGCTTCCGTTTGGCGACCGTCAGCGAGGCGCGGCGCTG 5 <i>Encephalartos longifolius</i> TGTCGGGAATCGGTGCCTTCGCGGGGCTTGTGTTTTCGGCTTCCAGGCGTTTCGCTTTGGTTTGTGCGAG-ACATGCGGGGATGGCGATCGAGGTTCCAGTTACACCTCTGCCACA GGGGTGCAATCCGGCCGTTGCAACCGTGCCCTTCCCGCGGGGCTTCCGTTTGGCGACCGTCAGCGAGGCGCGGCGCTG 42 <i>Encephalartos princeps</i> TGTCGGGAATCGGTGCCTTCGCGGGGCTTGTGTTTTCGGCTTCCAGGCGTTTCGCTTTGGTTTGTGCGAG-ACATGCGGGGATGGCGATCGAGGTTCCAGTTACACCTCTGCCACA GGGGTGCAATCCGGCCGTTGCAACCGTGCCCT-CCCGCGGGGCTTCCGTTTGGCGACCGTCAGCGAGGCGCGGCGCTG 66 <i>Encephalartos trispinosus</i> TGTCGGGAAT?GGT?CCT?GC?GGGCTTGTGTTTTCGGCTTCCAGGCGTTTCGCTTTGGTTTGTGCGAG-ACATGCGGGGATGGCGATCGAGGTTCCAGTTACACCTCTGCCACA GGGGTGCAATCCGGCCGTTGCAACCGTGCCCT?TTCGCGCGGGGCTTCCGTTTGGCGACCGTCAGCGAGGCGCGGCGCTG 68 <i>Encephalartos trispinosus</i> TGTCGGGAAT??TGCTTCGCGGGGCTTGTGTTTTCGGCTTCCAGGCGTTTCGCTTTGGTTTGTGCGAG-ACATGCGGGGATGGCGATCGAGGTTCCAGTTACACCTCTGCCACA GGGGTGCAATCCGGCCGTTGCAACCGTGCCCTTCCCGCGGGGCTTCCGTTTGGCGACCGTCAGCGAGGCGCGGCGCTG

[illegible]

(B) ITS data:

28 <i>Encephalartos ferox</i>	????????????????????????AAGTGTGCGATTAAAGCTGGT????????TTACAGATTAACCTATTACACTCCTGAATATAAAACCAAAGATA
314 <i>Encephalartos altensteinii</i>	CCGATATCTTGGCAGCGTTCAGAG????TCCCAACCTGGGGTGCCCGCTGAGGAAGCGGGGGCTGCAGTAGCTGCTGAATCTTCCACTGGTACATGGAC
75 <i>Encephalartos arenarius</i>	????????????????????????AAGTGTGCGATTAAAGCTGGTGTAAAGATTACAGATTAACCTATTACACTCCTGAATATAAAACCAAAGATA
40 <i>Encephalartos horridus</i>	CCGATATCTTGGCAGCGTTCAGAGTAACCTCCCAACCTGGGGTGCCCGCTGAGGAAGCGGGGGCTGCAGTAGCTGCTGAATCTTCCACTGGTACATGGAC
90 <i>Encephalartos latifrons</i>	????????????????????????AAGTGTGCG????T?AAG?TG????????TTAC?GATTAACTTATTACACTCCTGAATATAAAACCAAAGATA
56 <i>Encephalartos lehmannii</i>	CCGATATCTTGGCAGCGTTCAGAGTAACCTCCCAACCTGGGGTGCCCGCTGAGGAAGCGGGGGCTGCAGTAGCTGCTGAATCTTCCACTGGTACATGGAC
235 <i>Encephalartos lehmannii</i>	????????????????????????AAGTGTGCG????T?AAG?TG????????TTAC?GATTAACTTATTACACTCCTGAATATAAAACCAAAGATA
5 <i>Encephalartos longifolius</i>	CCGATATCTTGGCAGCGTTCAGAGTAACCTCCCAACCTGGGGTGCCCGCTGAGGAAGCGGGGGCTGCAGTAGCTGCTGAATCTTCCACTGGTACATGGAC
42 <i>Encephalartos princeps</i>	????????????????????????AAGTGTGCGATTAAAGCTGGTGTAAAGATTACAGATTAACCTATTACACTCCTGAATATAAAACCAAAGATA
66 <i>Encephalartos trispinosus</i>	CCGATATCTTGGCAGCGTTCAGAGTAACCTCCCAACCTGGGGTGCCCGCTGAGGAAGCGGGGGCTGCAGTAGCTGCTGAATCTTCCACTGGTACATGGAC
28 <i>Encephalartos ferox</i>	????????????????????????AAGTGTGCGATTAAAGCTGGT????????TTACAGATTAACCTATTACACTCCTGAATATAAAACCAAAGATA
314 <i>Encephalartos altensteinii</i>	CCGATATCTTGGCAGCGTTCAGAG????TCCCAACCTGGGGTGCCCGCTGAGGAAGCGGGGGCTGCAGTAGCTGCTGAATCTTCCACTGGTACATGGAC
75 <i>Encephalartos arenarius</i>	????????????????????????AAGTGTGCGATTAAAGCTGGTGTAAAGATTACAGATTAACCTATTACACTCCTGAATATAAAACCAAAGATA
40 <i>Encephalartos horridus</i>	CCGATATCTTGGCAGCGTTCAGAGTAACCTCCCAACCTGGGGTGCCCGCTGAGGAAGCGGGGGCTGCAGTAGCTGCTGAATCTTCCACTGGTACATGGAC
90 <i>Encephalartos latifrons</i>	????????????????????????AAGTGTGCG????T?AAG?TG????????TTAC?GATTAACTTATTACACTCCTGAATATAAAACCAAAGATA
56 <i>Encephalartos lehmannii</i>	CCGATATCTTGGCAGCGTTCAGAGTAACCTCCCAACCTGGGGTGCCCGCTGAGGAAGCGGGGGCTGCAGTAGCTGCTGAATCTTCCACTGGTACATGGAC
235 <i>Encephalartos lehmannii</i>	????????????????????????AAGTGTGCG????T?AAG?TG????????TTAC?GATTAACTTATTACACTCCTGAATATAAAACCAAAGATA
5 <i>Encephalartos longifolius</i>	CCGATATCTTGGCAGCGTTCAGAGTAACCTCCCAACCTGGGGTGCCCGCTGAGGAAGCGGGGGCTGCAGTAGCTGCTGAATCTTCCACTGGTACATGGAC
42 <i>Encephalartos princeps</i>	????????????????????????AAGTGTGCGATTAAAGCTGGTGTAAAGATTACAGATTAACCTATTACACTCCTGAATATAAAACCAAAGATA
66 <i>Encephalartos trispinosus</i>	CCGATATCTTGGCAGCGTTCAGAGTAACCTCCCAACCTGGGGTGCCCGCTGAGGAAGCGGGGGCTGCAGTAGCTGCTGAATCTTCCACTGGTACATGGAC

28	<i>Encephalartos ferox</i>	?????????????????AAGTGTGCGAATTTAAAGCTGGT????????TTACAGATTAACTTTATTTACACTCTCTGAAATATAAAACCAAGAATACCGATATCTTGACAGCTTCCGAG????TCCCAAACTCGGGTGGCCGTGAGGAAGCGGGGTGCAGTAGCTGCTGAATCTTCCACTGTGTACATGATATCTGTAGAGATTTCGCAATCTCTCTGTTATTCCAAATTTTCCAAAGTCTCACTCAATGTTATCCAAAGTTAAAGAGAGATAATTTAAACAAATATGCGCC
314	<i>Encephalartos altensteinii</i>	TCCCTTATTTGGGATGACCATTAACCAAAATTTGGGTTTATCTGCCAAAACATTTGGTAGAGCATTTTACGATGCTCTGGTGGGATCTTGATTTTACC
75	<i>Encephalartos arenarius</i>	GTCTAGAAGATTTCGGAATTCCTCTGCTATTCTCAAACCTTTTAAAGTCACTCACTGATGTATCAAGTTGAAAGAGATAATTTAAACAAATATGCGCC
40	<i>Encephalartos horridus</i>	TCCCTTATTTGGGATGACCATTAACCAAAATTTGGGTTTATCTGCCAAAACATTTGGTAGAGCATTTTACGATGCTCTGGTGGGATCTTGATTTTACC
90	<i>Encephalartos latifrons</i>	GTCTAGAAGATTTCGGAATTCCTCTGCTATTCTCAAACCTTTTAAAGTCACTCACTGATGTATCAAGTTGAAAGAGATAATTTAAACAAATATGCGCC
56	<i>Encephalartos lehmannii</i>	TCCCTTATTTGGGATGACCATTAACCAAAATTTGGGTTTATCTGCCAAAACATTTGGTAGAGCATTTTACGATGCTCTGGTGGGATCTTGATTTTACC
235	<i>Encephalartos lehmannii</i>	GTCTAGAAGATTTCGGAATTCCTCTGCTATTCTCAAACCTTTTAAAGTCACTCACTGATGTATCAAGTTGAAAGAGATAATTTAAACAAATATGCGCC
5	<i>Encephalartos longifolius</i>	TCCCTTATTTGGGATGACCATTAACCAAAATTTGGGTTTATCTGCCAAAACATTTGGTAGAGCATTTTACGATGCTCTGGTGGGATCTTGATTTTACC
42	<i>Encephalartos princeps</i>	TCCCTTATTTGGGATGACCATTAACCAAAATTTGGGTTTATCTGCCAAAACATTTGGTAGAGCATTTTACGATGCTCTGGTGGGATCTTGATTTTACC
66	<i>Encephalartos trispinosus</i>	TCCCTTATTTGGGATGACCATTAACCAAAATTTGGGTTTATCTGCCAAAACATTTGGTAGAGCATTTTACGATGCTCTGGTGGGATCTTGATTTTACC
28	<i>Encephalartos ferox</i>	?????????????????AAGTGTGCGAATTTAAAGCTGGT????????TTTACAGATTAACTTTATTTACACTCTCTGAAATATAAAACCAAGAATACCGATATCTTGACAGCTTCCGAG????TCCCAAACTCGGGTGGCCGTGAGGAAGCGGGGTGCAGTAGCTGCTGAATCTTCCACTGTGTACATGATATCTGTAGAGATTTCGCAATCTCTCTGTTATTCCAAATTTTCCAAAGTCTCACTCAATGTTATCCAAAGTTAAAGAGAGATAATTTAAACAAATATGCGCC
314	<i>Encephalartos altensteinii</i>	TCCCTTATTTGGGATGACCATTAACCAAAATTTGGGTTTATCTGCCAAAACATTTGGTAGAGCATTTTACGATGCTCTGGTGGGATCTTGATTTTACC
75	<i>Encephalartos arenarius</i>	GTCTAGAAGATTTCGGAATTCCTCTGCTATTCTCAAACCTTTTAAAGTCACTCACTGATGTATCAAGTTGAAAGAGATAATTTAAACAAATATGCGCC
40	<i>Encephalartos horridus</i>	TCCCTTATTTGGGATGACCATTAACCAAAATTTGGGTTTATCTGCCAAAACATTTGGTAGAGCATTTTACGATGCTCTGGTGGGATCTTGATTTTACC
90	<i>Encephalartos latifrons</i>	GTCTAGAAGATTTCGGAATTCCTCTGCTATTCTCAAACCTTTTAAAGTCACTCACTGATGTATCAAGTTGAAAGAGATAATTTAAACAAATATGCGCC
56	<i>Encephalartos lehmannii</i>	TCCCTTATTTGGGATGACCATTAACCAAAATTTGGGTTTATCTGCCAAAACATTTGGTAGAGCATTTTACGATGCTCTGGTGGGATCTTGATTTTACC
235	<i>Encephalartos lehmannii</i>	GTCTAGAAGATTTCGGAATTCCTCTGCTATTCTCAAACCTTTTAAAGTCACTCACTGATGTATCAAGTTGAAAGAGATAATTTAAACAAATATGCGCC
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28 *Encephalartos ferox*
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 235 *Encephalartos lehmannii*
 5 *Encephalartos longifolius*
 42 *Encephalartos princeps*
 66 *Encephalartos trispinosus*

References

- Avise, J.C., 1974. The systematic value of electrophoretic data. *Syst. Zool.* 23, 465–481.
- Byrne, M., James, S.H., 1991. Genetic diversity in the cycad *Macrozamia riedlei*. *Heredity* 67, 35–39.
- Cavalli-Sforza, L.L., Edwards, A.W.F., 1967. Phylogenetic analysis: models and estimation procedures. *Evolution* 21, 550–570.
- Doyle, J.J., Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. *Focus* 12, 13–15.
- Dyer, R.A., 1956. A new cycad [*Encephalartos arenarius*] from the Cape province. *J. S. Afr. Bot.* 22, 1–4.
- Dyer, R.A., 1965a. New species and notes on type specimens of South African *Encephalartos*. *J. S. Afr. Bot.* 31, 111–121.
- Dyer, R.A., 1965b. The cycads of southern Africa. *Bothalia* 8, 405–515.
- Ellstrand, N.C., Ornduff, R., Clegg, J.M., 1990. Genetic structure of the Australian cycad, *Macrozamia communis* (Zamiaceae). *Amer. J. Bot.* 77, 677–681.
- Farris, J.S., 1972. Estimating phylogenetic trees from distance matrices. *Am. Nat.* 106, 645–668.
- Goncharenko, G.G., Padutov, V.E., Silin, A.E., 1992. Population structure, gene diversity and differentiation in a natural population of Cedar pines (*Pinus* subsect. *Cembrae*, Pinaceae) in SSR. *Pl. Syst. Evol.* 182, 121–134.
- Gottlieb, L.D., 1981. Electrophoretic evidence and plant populations. In: Reinhold, L., Harborne, J.B., Swain, T. (Eds.), *Progress in Phytochemistry*. Pergamon Press, New York, pp. 1–46.
- Hamrick, J.L., Mitton, J.B., Linhart, Y.B., 1979. Level of genetic variation in trees: influence of life history characteristics. In: Conke, M.T. (Ed.), *Symposium on Isozymes of North American Forest Trees and Forest Insects*, USDA Forest General Technical Report, pp. 35–41.
- Kephart, S.R., 1990. Starch gel electrophoresis of plant isozymes: a comparative analysis of techniques. *Am. J. Bot.* 77, 693–712.
- Markert, C.L., Faulhaber, I., 1965. Lactate dehydrogenase isozyme patterns of fish. *J. Exp. Zool.* 159, 319–332.
- Nei, M., 1972. Genetic distance between populations. *Am. Nat.* 106, 283–292.
- Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89, 583–590.
- Nei, M., 1986. Definition and estimation of fixation indices. *Evolution* 40, 643–645.
- Nei, M., Tajima, F., Tatenno, Y., 1983. Accuracy of estimated phylogenetic trees from molecular data. *J. Mol. Evol.* 19, 153–170.
- Norstog, K.J., Nicholls, T.J., 1997. *The biology of the cycads*. Ithaca, Cornell University Press.
- Oberprieler, R., 1995. The weevils (Coleoptera: Curculionidae) associated with cycads I Classification, relationships, and biology. In: Vorster, P. (Ed.), *Proceedings of the Third International Conference on Cycad Biology*. Stellenbosch, Cycad Society of South Africa, pp. 295–334.
- Ota, T., 1993. *DISPAN*. Pennsylvania State University, Philadelphia.
- Raymond, M., Rousset, F., 1995. *GENEPOP* (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* 86, 248–249.
- Ridgway, G., Sherburne, S.W., Lewis, R.D., 1970. Polymorphism in the esterases of Atlantic herring. *Trans. Amer. Fish. Soc.* 99, 147–151.
- Rogers, J.S., 1972. Measures of genetic similarity and genetic distance. *Studies in Genetics*, Univ. Texas Publ. Vol. 7213, pp. 145–153.
- Shaklee, J.B., Tamaru, C.S., Waples, R.S., 1982. Speciation and evolution of marine fishes studied by electrophoretic analysis of proteins. *Pac. Sci.* 36, 141–157.
- Sharma, I.K., Jones, D.L., Forster, P.I., Young, A.G., 1998a. Low isozymic differentiation among five species of the *Macrozamia heteromera* group (Zamiaceae). *Biochem. Syst. Ecol.* 27, 67–77.
- Sharma, I.K., Jones, D.L., Forster, P.I., Young, A.G., 1998b. The extent and structure of genetic variation in the *Macrozamia pauli-guilielmi* complex (Zamiaceae). *Biochem. Syst. Ecol.* 26, 45–54.
- Swofford, D.L., 1991. *PAUP: phylogenetic analysis using parsimony*, version 3.1.1. Computer program distributed by the Illinois Natural History Survey. Champaign, IL.
- Swofford, D.L., Selander, R.B., 1981. BIOSYS-1: A FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *J. Hered.* 72, 281–283.

- Tang, W., 1990. Maturity in cycads. *Encephalartos* 24, 24–26.
- Thorpe, J.P., 1982. The molecular clock hypothesis: biochemical evaluation, genetic differentiation and systematics. *Annu. Rev. Ecol. Syst.* 13, 139–168.
- Thorpe, J.P., Solé-Cava, A.M., 1994. The use of allozyme electrophoresis in invertebrate systematics. *Zool. Scripta* 23, 3–18.
- Treutlein, J., 1998. Molekulare Phylogenie der Cycadeen Thesis (Diplom). University Heidelberg, Germany.
- Van der Bank, F.H., Vorster, P., Van der Bank, M., 1998. Phylogenetic relationships, based on allozyme data, between six cycad taxa indigenous to South Africa. *S. Afr. J. Bot.* 64 (3), 182–188.
- Vorster, P., 1986. Hybridization in *Encephalartos*. *Excelsa* 12, 101–106.
- Vorster, P., 1993. Taxonomy of *Encephalartos* (Zamiaceae): taxonomically useful external characteristics. In: Stevenson, D.W., Norstog, K.J. (Eds.), *Proceedings of Cycad 90, the Second International Conference on Cycad Biology*. Palm and Cycad Societies of Australia, Australia, Milton, pp. 294–299.
- Vorster, P., 1995. Aspects of the reproduction of cycads. 2. An annotated review of known information. In: Vorster, P. (Ed.), *Proceedings of the Third International Conference on Cycad Biology*. Cycad Society of South Africa, Stellenbosch, pp. 379–389.
- Vorster, P., 1999. A review of the arborescent tropical species of *Encephalartos*. In: Chen, C.J. (Ed.), *Proceedings of the Fourth International Conference on Cycad Biology*. International Academic Publishers, Beijing, pp. 87–91.
- Walters, T.W., Decker-Walters, D.S., 1991. Patterns of allozyme diversity in the West Indies cycad *Zamia pumila* (Zamiaceae). *Am. J. Bot.* 78, 436–445.
- Ward, R.D., 1977. Relationships between enzyme heterozygosity and quaternary structure. *Biochem. Genet.* 15, 123–135.
- Wright, S., 1978. *Evolution and the Genetics of Populations*, Vol. 4. Variability Within and Among Natural Populations. University of Chicago, Chicago.