Kasetsart J. (Nat. Sci.) 41 : 43 - 60 (2007)

Molecular Identification of *Encephalartos* (Zamiaceae) Species and Their Relationships to Morphological Characters

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ABSTRACT

RAPD and morphological characteristics were used to evaluate genetic similarity among twenty species of *Encephalartos* geographically distributed in several African countries. Eight out of sixty primers, i.e. OPA-05, OPA-07, OPB-07, OPB-08, OPB-11, OPJ-07, OPJ-13, and OPJ-15 could produce polymorphic and specific band patterns to identify these cycads. The primers generated 240 reproducible bands at the size of 0.15 - 2.20 kb. Polymorphism of the DNA profiles appraised by NTSYS program gave the similarity coefficient in the range of 0.53-0.80. Phylogenetic tree was constructed and twenty *Encephalartos* species were grouped into six clusters. The grouping corresponded well to the nine morphological characters (stem, color of rachis, leaf curvation, spiny leaflet, number of prickle, leaflet shape, keel, upper and lower margins of leaflet) as well as to their original geographic distribution.

Key words: *Encephalartos*, RAPD, phylogenetic tree, morphological characters, geographic distribution

INTRODUCTION

Cycads are among the most primitive living seed-plants. The order Cycadales comprises 3 families, 11 genera and 185 species. The genus *Encephalartos* belongs to the family Zamiaceae which consists of 52 known species (Jones, 1993). A number of new species of *Encephalartos* have been reported over the last twenty-five years, but largely fragmentary as the results of species complexes and the collections from isolated areas. To clarify the true identity of *Encephalartos* and group them based on their origins and relationships to morphological characteristics, an effective organization of these precious plants is needed.

Osborne and Grobbelaar (1993) used the numerical phenetic technique to study intragenic relationships in *Encephalartos lehm*. They used 86 combinations of morphological characteristics to construct phenogram and could separate 52 cycad species into five groups based on geographic distribution and morphology. However, the phylogeny of Cycadales is still ambiguous because distinctive morphological characteristics are lacking in most groups, and species are usually defined in terms of sets of characteristics. Furthermore, the species in a given geographical area are closely related due to slow reproduction rates (Tang, 1990) and absence of long distance dispersal mechanism for seeds. Van der Bank and

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Received date : 15/03/06 Accepted date : 24/11/06

Vorster (1998) used allozyme to investigate similarity among six cycad species in South Africa and suggested that *E. altensteinii* and *E. natalensis* did not share the same gene pool. However, the genetic distance was found to be only 0.042 between these two species, compared to the mean genetic distance value of 0.222 for the other ingroup taxa. Using allozymes also restricts the inquiry to specific parts of DNA coding for enzymes. Besides, the plant tissue used for allozyme studies has to be processed shortly after harvest since proteins are quite unstable. In contrast, DNA based methods allow a longer time span between harvest and processing (Weising *et al*., 1995).

Random amplified polymorphic DNA (RAPD) is a technique used to identify polymorphic DNA patterns analyzed by gel electrophoresis, after polymerase chain reaction (PCR) amplification, using arbitrary oligonucleotide primers (Williams *et al*., 1990). RAPD is more simple and less expensive than restriction fragment length polymorphism (RFLP) (Ragot and Hoisington, 1993). RAPD analysis has also been used for finding genetic relationships between species in several plants (Sala *et al*., 1998; Nakajima *et al*., 1998; Esselman *et al*., 2000) including the cycads of *Cycas* species (Sangin *et al*., 2006).

This project aimed at identifying the genetic similarity among *Encephalartos* species by using RAPD markers compared to their morphological characters and to assess the organization level of genetic diversity through phylogenetic tree.

MATERIALS AND METHODS

Plant materials

Young leaves of twenty *Encephalartos* species at Nongnooch Tropical Garden, Chonburi province, Thailand, were collected. The scientific names and their major geographic distribution are shown in Table 1 and Figure 1.

Morphological observation

Observations were made for each species of *Encephalartos* based on their characteristics of stem (aborescent, acaulescent), leaf (length, number per plant, and color), rachis (color, curvation, and twisting), petiole (prickle, spine, and leaf base collar), leaflet (shape , keel, overlapping type, insertion of angle, upper and lower margins, hairy, and concave). These leaf characteristics were collectively drawn and classified as described by Hill (2002) and shown in Figure 2 as follows:-

Prickle = modified leaflet into spine-like structure

Spine = completely modified form of leaflet having short and sharp structure

Collar = leaf base area having distinctive color

Dentate = leaflet outline having small saw-like structure

Lobe = leaflet outline having sharp curl structure

Insertion of angle;

horizontal = parallel attachment of leaflets to petiole

acute = < 45^{o} attachment of leaflets to petiole

obtuse = > 45^{o} attachment of leaflets to petiole

Overlapping upward = upward superimpose of leaflets alignment

Overlapping downward = downward superimpose of leaflets alignment

Overlapping upward and downward = both upward and downward superimpose of leaflets alignment

Keel; horizontal = parallel alignment of leaflets on horizontal plane

slightly keel = plane of leaflets alignment slightly upward

moderately keel = plane of leaflets

Table 1 *Encephalartos* cycad species and their geographic distribution.

No.	Scientific name	Geographic location
1.	*E. sclavoi* De Luca, D.W. Stev.& A. Moretii	Tanzania
2.	*E. chimanimaniensis* R.A. Dyer & I. Verd.	Mozambique, Zimbabwe
3.	*E. princeps* R.A. Dyer	South Africa (E. Cape)
4.	*E. cerinus* Lavranos & D.L. Goode	South Africa (KwaZulu-Natal)
5.	*E. trispinosus* (Hook.) R.A. Dyer	South Africa (E. Cape)
6.	*E. barteri ssp. barteri*	Ghana
7.	*E. septentrionalis* Schweinf.	Sudan, Uganda
8.	*E. aplanatus* Vorster	Swaziland
9.	*E. concinnus* R.A. Dyer & I. Verd.	Zimbabwe
10.	*E. munchii* R.A. Dyer & I. Verd.	Mozambique
11.	*E. ituriensis* Bamps & Lisowski	Dem. Rep. of Congo, Uganda
12.	*E. pterogonus* R.A. Dyer & I. Verd.	Mozambique
13.	*E. macrostrobilus* S. Jones & Wynants	North Uganda
14.	*E. nubimontanus* P.J.H. Hunter	South Africa (Limpopo)
15.	*E. caffer* (Thunb.) Lehm.	South Africa (E. Cape)
16.	*E. ngoyanus* I. Verd.	South Africa (KwaZulu-Natal)
17.	*E. equatorialis* P.J.H. Hunter	Uganda
18.	*E. arenarius* R.A. Dyer	South Africa (E. Cape)
19.	*E. laurentianus* De Wild	Angola, Dem. Rep. of Congo
20.	*Encephalartos sp.*	Zimbabwe

alignment moderately upward

strongly keel = plane of leaflets alignment totally upward

Leaflet margin;

flat = a monotonous margin

revolute = rolled backward or downward at the margins

Hairy = leaflet having many hairlike outgrowth from an epidermal cell

Concave = leaflet having a hollow and curve like the inside of a bowl

DNA extraction

DNA was extracted from young leaflets using modified CTAB method (Doyle and Doyle, 1990). Approximately 0.3 g of young leaflets was ground to fine powder in liquid nitrogen, followed by the addition of 1 ml preheated (65°C) 4X CTAB isolation buffer (4% CTAB, 1.4 mM NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0 and 10 mM 2-mercaptoethanol). The homogenate was incubated at 65°C for 1 h and was further extracted using chloroform: isoamyl alcohol (24:1). The mixture was centrifuged at 10,000 g for 10 min at room temperature. The aqueous phase was collected and mixed with 1 equal volume of absolute ethanol. The nucleic acid pellet was air-dried and resuspended in TE buffer (10 mM Tris, 1mM EDTA, pH 7.0). RNaseA was added to the sample at the final concentration of 10 ng/µl. After incubating at 37°C for 30 min, the sample was extracted with phenol: chloroform (1:1). Absolute ethanol was added with 2X volume of supernatant solution to make DNA precipitated. The DNA pellet was washed with 70% ethanol, air-dried and resuspended in 30 µl TE buffer. DNA concentration was determined both by 1% agarose gel electrophoresis and UV spectrophotometry.

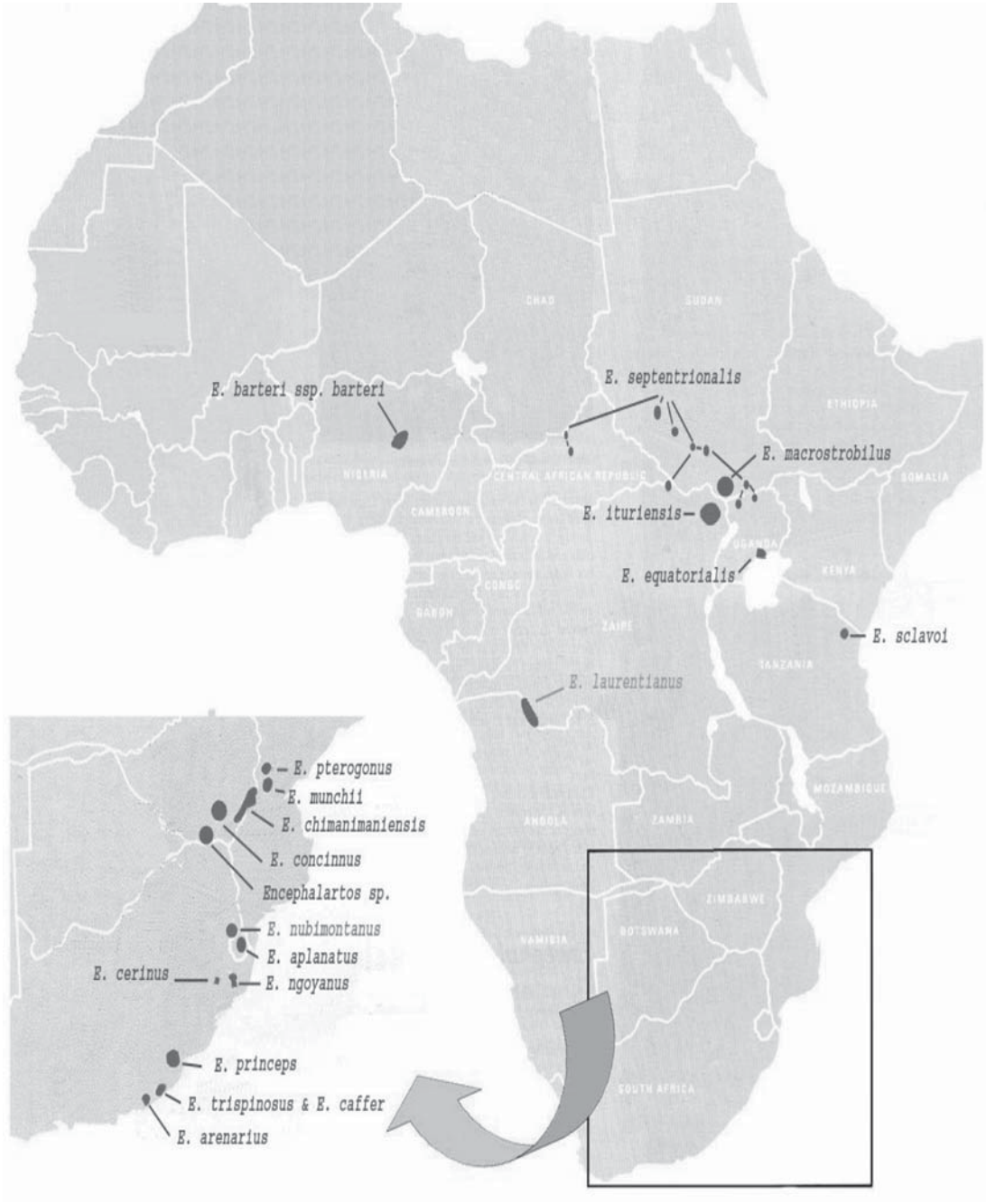


Figure 1 Geographic distribution of twenty *Encephalartos* species in Africa.
Source: modification from the map of Jones (1993)

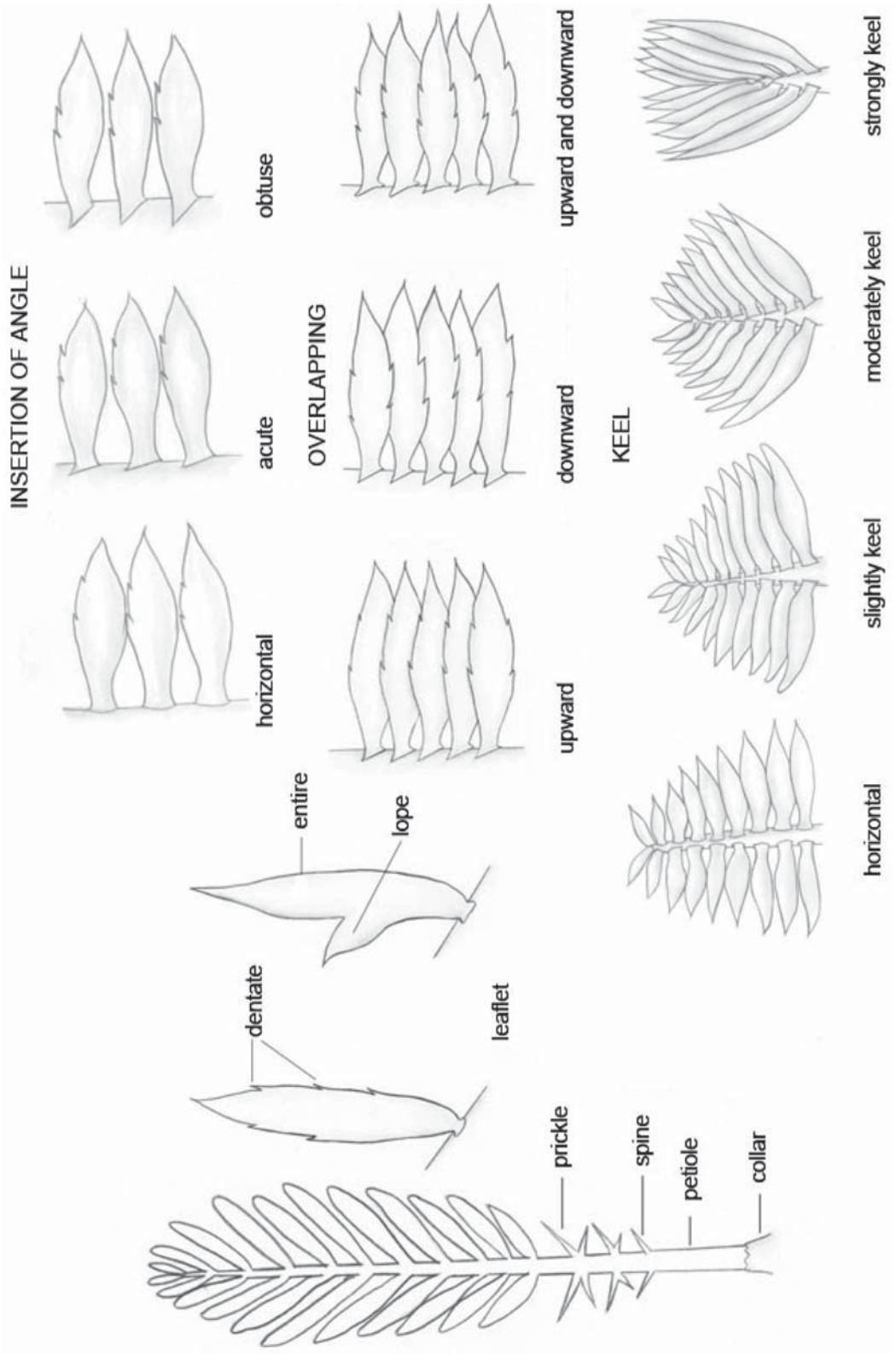


Figure 2 Drawing of morphological characteristics of leaf setting as seen in *Encephalartos*.

RAPD analysis

For initial primer screening, 25 µl reaction mixtures containing PCR buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 0.1% Triton X-100), 0.2 µM random primer, 200 µM dNTP, 2.0 unit of Dynazyme (Finnzymes), 1.5 mM $MgCl_2$ and 100 ng of DNA template were used. The amplification reactions were performed in a Perkin Elmer 9600 thermocycler for the following conditions: initial denaturation for 1 min at 94°C, 45 cycles of denaturation for 1 min at 94°C each, annealing for 1 min at 36°C, an extension of 2 min at 72°C, followed by a final extension at 72°C for 5 min. Sixty random primers (Operon Technologies Inc.: Kit OPA1-20, OPB 1-20 and OPJ 1-20) were subjected to preliminary screening. Eight primers that gave reproducible results in three independent DNA extractions were chosen for further analysis. These were OPA-05 (5′AGGGGTCTTG3′), OPA-07 (5′GAAACGGGTG3′), OPB-07 (5′GGTGA CGCAG3′), OPB-08 (5′GTCCACACGG3′), OPB-11 (5′GTAGACCCGT3′), OPJ-07 (5′ CCTCTCGACA3′), OPJ-13 (5′CCACACTACC 3′) and OPJ-15 (5′TGTAGCAGGG3′). The amplification products and a size marker (100 bp DNA ladder) were separated on a 1% agarose gel. The electrophoresis patterns were qualitatively determined for the presence (1) or absence (0) of the bands using NTSYS-PC software, version 2.0. Estimates of genetic similarity among all species were also calculated using Nei and Li (Nei and Li, 1979) coefficient of similarity between two individuals (i and j), according to the formula: Nei and Li coefficient = 2Xi,j/(Xi+Xj) where Xi,j is the number of shared bands present in both samples i and j, Xi is the total number of bands of individual i, and Xj is the total number of bands of individual j. The resulting similarity matrix was used to construct dendrograms by means of UPGMA (unweighted pair-group method with arithmetic average) as described by Sneath and Sokal (1973).

RESULTS AND DISCUSSION

Morphological characteristics

Seventeen species of *Encephalartos* showed the same aborescent type of stem (high above the ground) but three species (*E. caffer, E. ngoyanus* and *E. aplanatus*) contained acaulescent type (underground stem) (Figure 3 A and B). The leaf length, however, varied from 1.0 m as seen in the *E. caffer* to 7.0 m of *E. laurentianus.* The number of leaves per plant also varied with the lowest number of 11 per plant in *E. chimanimaniensis* to the highest of 60 per plant found in *E. trispinosus* (Table 2). Leaflet colors were found to be green of different shades, except *E. princeps* and *E. trispinosus* were in silver blue (Figure 3 C to H). Keel types (the alignment of leaflets) were found to be horizontal in *E. chimanimaniensis* and *E. barteri ssp.barteri* while the rest of them were in different degrees of plane alignment (Table 2). The majority of rachis color in *Encephalartos* was green but *E. sclavoi* was yellow and those of *E. princeps* and *E. trispinosus* were blue. Degrees of rachis curvature were varied in different types, i.e. gentle curve, recurve, and straight (Figure 4), but the leaf curvature was distinctively identified as twisting or non-twisting forms (Figure 5 A and B).

Number of prickle on the petiole was found to be 1-6 per petiole for most of *Encephalartos* with the exception of *E. equatorialis* having more than 12 per petiole and seven *Encephalartos* species (*E. ngoyanus, E. nubimontanus, Encephalartos sp, E. princeps, E. trispinosus, E. arenarius, E. caffer, E. cerinus*) contained no prickle on the petiole. Modified form of leaflet to spine comparing to the non-reduced leaflet were also shown (Figure 5 C and D). The presence or absence of leaf-base collar was accounted for (Figure 5 E, F, G, and H). Those *Encephalartos* having collar at leaf-base also showed the distinctive color at the leaf-base (Figure 5 E and F). As for leaflet characters, the

Table 2 Morphological characteristics of *Encephalartos.*

Cycad species	Stem	Leaf			Rachis			Petiole			Leaflet								
		Length (m)	No.	Curvature	Color	Curve	Twisting	Prickle	Spine	Leaf-base collar	Shape	Overlapping	Insertion angle	Keel	Margin	Margin upper	Margin lower	Hairy	Concave
E. sclavoi	aborescent	2.00	17	light green	yellow	straight	-	1-6	+	+	lanceolate	downward	obtuse	s.keel	revolute	dentate	dentate	-	+
E. chimanimaniensis	aborescent	1.50	11	dull green	green	straight	+	1-6	+	-	lanceolate	non-overlapping	horizontal	horizontal	revolute	dentate	dentate	-	-
E. laurentianus	aborescent	7.00	36	dark green	green	gentle curve	+	1-6	+	+	lanceolate	non-overlapping	obtuse	s.keel	flat	dentate	dentate	-	+
E. septentrionalis	aborescent	1.60	13	dull green	green	gentle curve	+	1-6	+	-	lanceolate	upward&downward	obtuse	m.keel	revolute	dentate	dentate	+	+
E. barteri ssp.barteri	aborescent	1.80	20	dull green	green	straight	-	1-6	+	-	lanceolate	non-overlapping	horizontal	horizontal	revolute	dentate	dentate	-	+
E. ngoyanus	acaulescent	1.25	17	dull green	green	straight	+	-	+	-	linear	non-overlapping	horizontal	st.keel	flat	entire	entire	+	+
E. nubimontanus	aborescent	1.40	12	blue green	green	straight	+	-	+	+	lanceolate	upward	obtuse	st.keel	flat	dentate	dentate	-	-
Encephalartos sp.	aborescent	1.60	30	dark green	green	gentle curve	+	1-6	+	-	lanceolate	upward&downward	obtuse	s.keel	revolute	dentate	dentate	-	+
E. concinnus	aborescent	2.00	14	dark green	green	straight	+	1-6	+	+	lanceolate	upward&downward	acute	s.keel	revolute	dentate	dentate	-	+
E. munchii	aborescent	1.30	32	dull green	green	straight	+	1-6	+	-	lanceolate	upward&downward	acute	m.keel	revolute	dentate	dentate	-	-
E. equatorialis	aborescent	4.00	30	light green	green	straight	-	>12	+	+	lanceolate	upward&downward	acute	m.keel	flat	dentate	dentate	-	+
E. ituriensis	aborescent	3.00	49	dark green	green	straight	+	1-6	+	+	lanceolate	non-overlapping	acute	s.keel	flat	dentate	dentate	-	+
E. pterogonus	aborescent	1.50	30	dull green	green	straight	+	1-6	+	-	lanceolate	upward&downward	acute	s.keel	revolute	dentate	dentate	-	-
E. macrostrobilus	aborescent	2.20	16	dull green	green	gentle curve	-	1-6	+	+	lanceolate	upward&downward	acute	s.keel	flat	dentate	dentate	+	+
E. princeps	aborescent	1.30	34	silver blue	blue	gentle curve	+	-	-	+	lanceolate	upward	acute	st.keel	flat	entire	entire	-	-
E. trispinosus	aborescent	1.25	60	silver blue	blue	recurve	+	-	-	+	lanceolate	upward	obtuse	st.keel	flat	entire	lobe	-	-
E. arenarius	aborescent	1.50	45	blue green	green	recurve	-	-	-	+	ovate	downward	acute	st.keel	revolute	entire	lobe	-	-
E. caffer	acaulescent	1.00	19	dull green	green	straight	+	-	+	-	linear	non-overlapping	horizontal	st.keel	flat	entire	entire	+	+
E. cerinus	aborescent	1.20	34	blue green	green	straight	-	-	+	-	linear	non-overlapping	obtuse	m.keel	revolute	entire	entire	-	+
E. aplanatus	acaulescent	1.50	12	dull green	green	straight	-	1-6	+	-	lanceolate	downward	acute	s.keel	flat	entire	dentate	-	+

No. = number of leaf; s.keel = slightly keel, m.keel = moderately keel, st.keel = strongly keel; + = presence, - = absence

shape was either lanceolate, linear or ovate, while they are either overlapping upward, downward, both upward and downward, or non-overlapping at all (Figure 6 A, B, C, and D). Insertion angle of leaflet was found in several forms, i.e. obtuse, horizontal, acute (Figure 2) while the margin curvatures of whole leaflet were either flat or revolute (Table 2). Leaflet margin was in one of these forms, i.e. dentate, lobe, or entire and there were some combination of these forms at upper or lower margin as well (Figure 3 C to H). The presence or absence of hairy and concave leaflet were also observed in *Encephalartos* (Table 2).

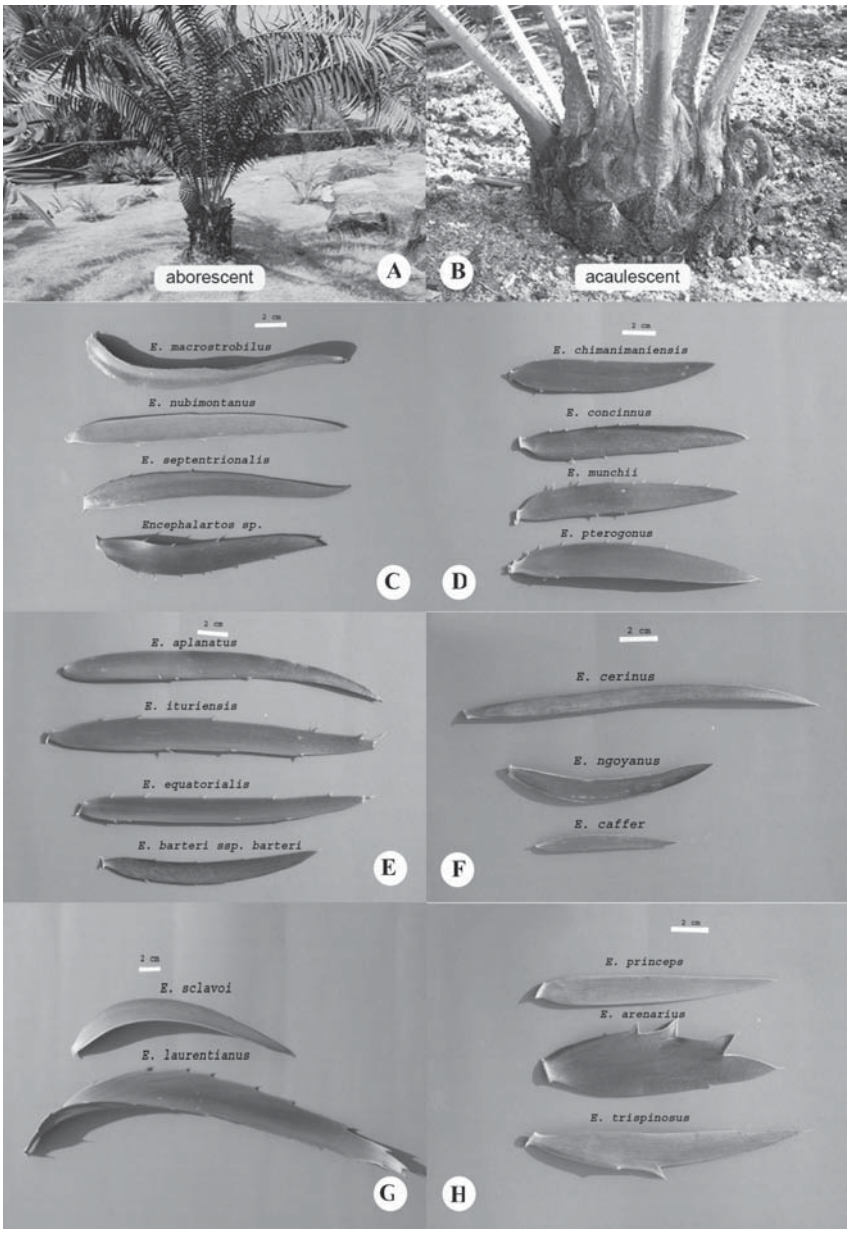


Figure 3 Morphological characters of *Encephalartos* A) aborescent type of stem, B) acaulescent type, C) to H) showing the color and concave shape of leaflet.

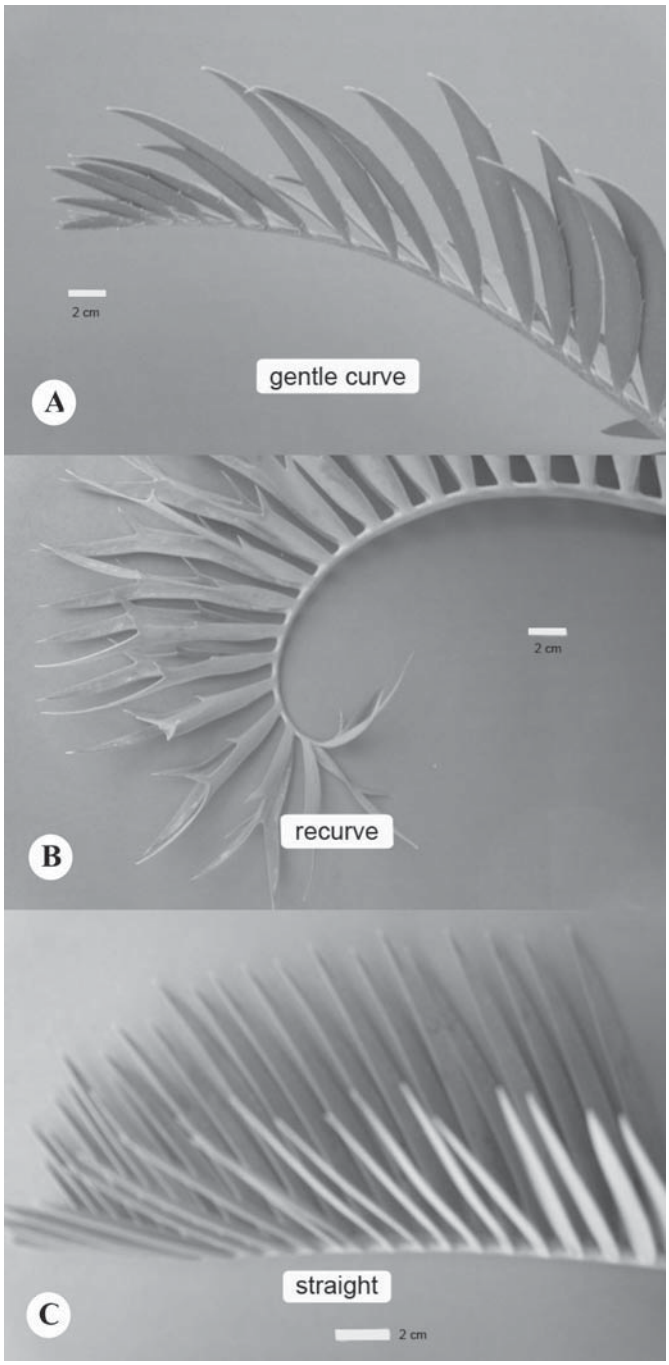


Figure 4 Rachis curvature of *Encephalartos* leaf A) gentle curve of *Encephalartos sp.* B) recurve of *E. trispinosus* C) straight leaf of *E. nubimontanus.*

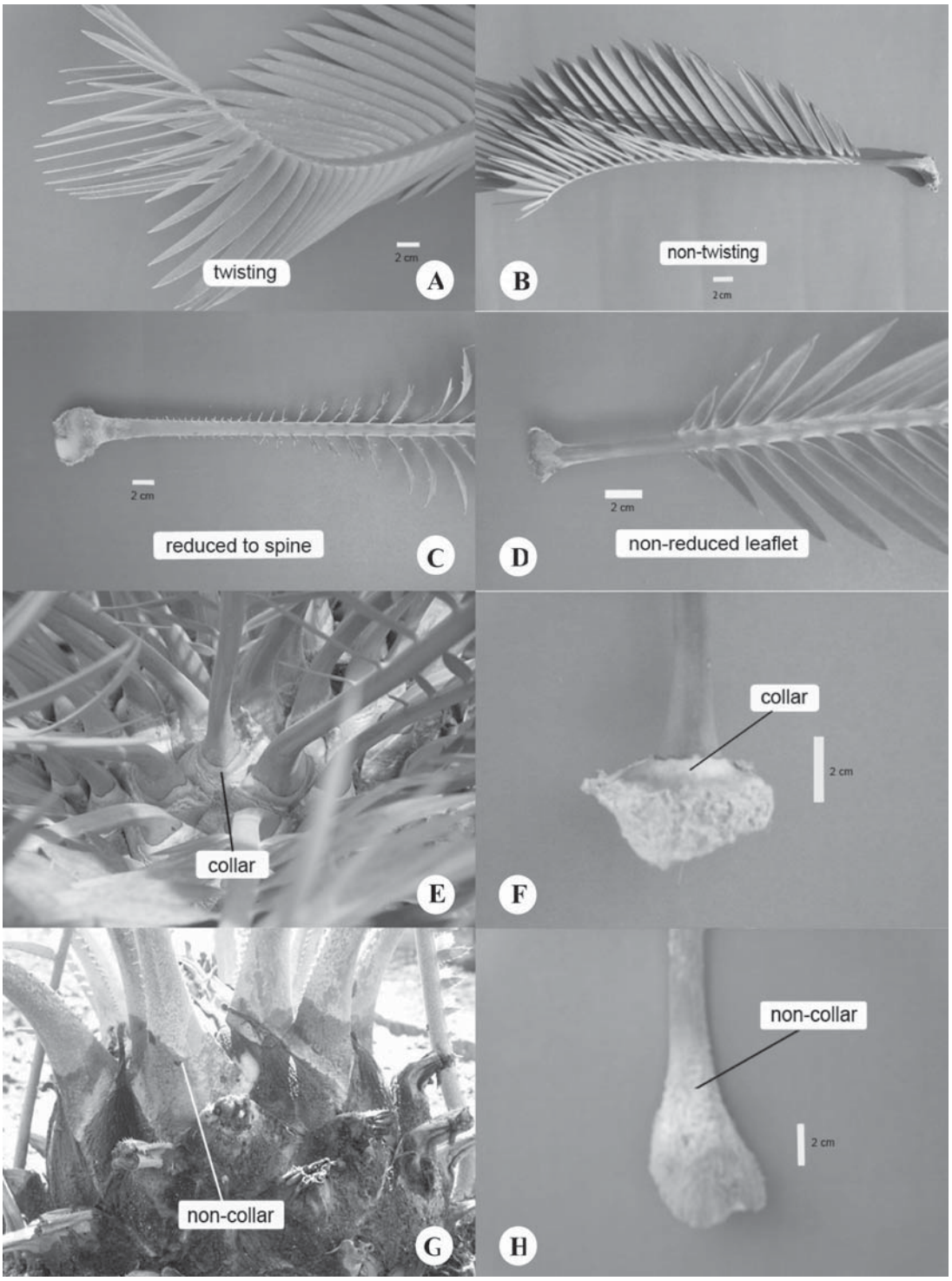


Figure 5 Morphological characters of *Encephalatos* A) twisting, B) non-twisting of leaf, C) leaflets reduced to spine, D) non-reduced leaflets, E) and F) collar at the leaf-base with distinct color, G) and H) non-collar leaf-base having no color.

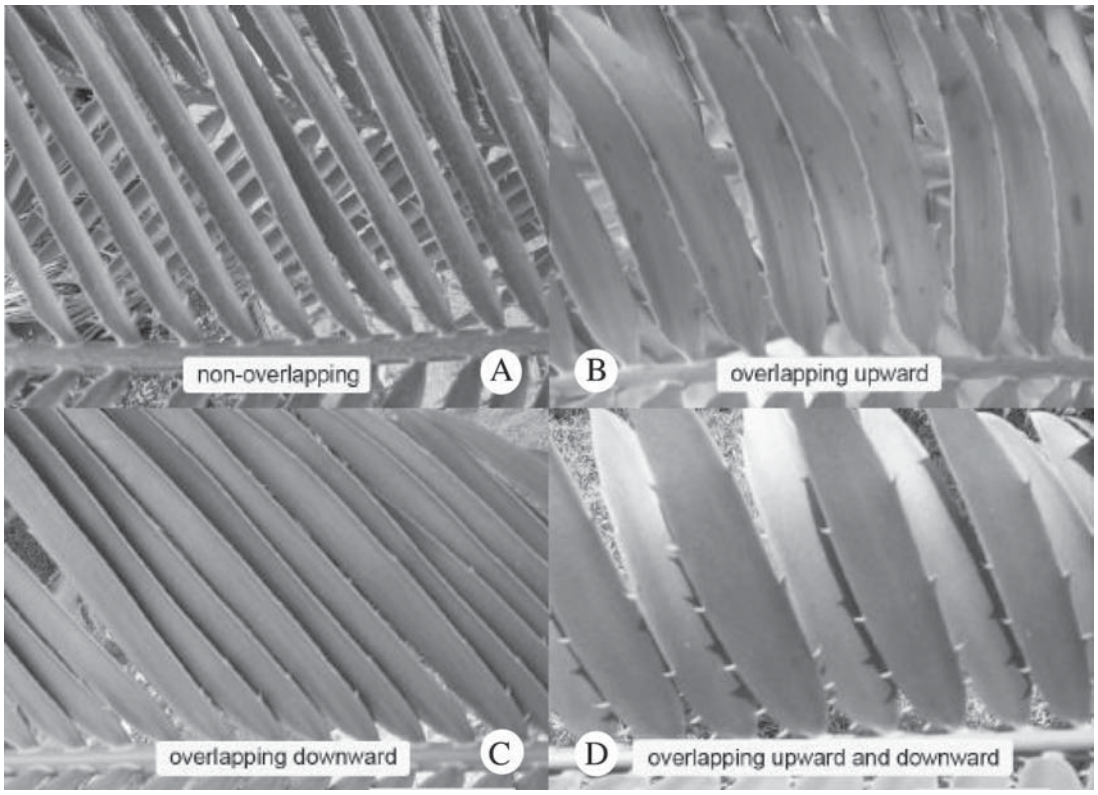


Figure 6 Leaflets overlapping types of Encephalartos A) non-overlapping, B) overlapping upward, C) overlapping downward, D) overlapping upward and downward.

Primer selection for DNA synthesis

To select the primers for DNA synthesis of *Encephalartos,* 60 primers of 10 nucleotides long (OPA 1-20, OPB 1-20 and OPJ 1-20; Operon Technology, USA) were used with DNA extracted from *E. sclavoi, E. chimanimaniensis, E. princeps, E. cerinus, E. trispinosus,* and *E. barteri ssp. barteri*. Sixteen out of these 60 primers could make DNA synthesis while 13 primers gave polymorphic DNA patterns among 20 species of *Encephalartos* (data not shown). However, only 8 primers, i.e. OPA-05, OPA-07, OPB-07, OPB-08, OPB-11, OPJ-07, OPJ-13, and OPJ-15 were selected based on their distinctive band patterns and specificity which gave band sizes of 0.15-2.20 kb (Table 3).

The results showed that primer OPA-05 gave a 900 bp band in most species of *Encephalartos* except in *E. sclavoi, E. munchii, E. ituriensis, E. nubimontanus* and *E. arenarius,* while a 700 bp band was found in 10 species of *Encephalartos* but not in *E. princeps, E. cerinus, E. trispinosus, E. barteri ssp. barteri, E. aplanatus, E. concinnus, E. caffer, E. ngoyanus, E. laurentianus,* and *Encephalartos sp.* (Figure 7A).

Primer OPA-07 gave a 350 bp in all 20 species of *Encephalartos,* a 700 bp in most species except in *E. princeps, E. aplanatus, E. caffer* and *E. ngoyanus* and also a 600 bp in 14 species but not in *E. princeps, E. aplanatus, E. ituriensis, E. caffer, E. ngoyanus,* and *E. equatorialis* (Figure 7B).

With proper selection of primers, this result showed the feasibility of using RAPD as a tool to illustrate the polymorphic DNA of *Encephalartos,* thereby, identify the character of them at molecular level with high accuracy. It is known that polymorphic DNA in each type of

Table 3 Numbers and sizes of DNA bands of 20 *Encephalartos* species using RAPD and eight selected primers.

Primers	Number of synthesized DNA	DNA size (kb)
OPA-04	16	1.70, 1.60, 1.20, 1.10, 1.00, 0.90, 0.85, 0.80, 0.71, 0.65, 0.60, 0.55, 0.51, 0.50, 0.45, and 0.30
OPA-05	27	1.60, 1.45, 1.30, 1.25, 1.20, 1.10, 1.05, 0.95, 0.90, 0.85, 0.81, 0.75, 0.70, 0.65, 0.61, 0.60, 0.58, 0.55, 0.51, 0.50, 0.45, 0.41, 0.40, 0.35, 0.31, 0.30, and 0.25
OPA-07	16	1.60, 1.50, 1.40, 1.30, 1.20, 1.10, 0.95, 0.85, 0.80, 0.70, 0.65, 0.60, 0.55, 0.45, 0.35, and 0.25
OPA-08	18	1.30, 1.20, 1.10, 1.00, 0.95, 0.90, 0.85, 0.75, 0.71, 0.70, 0.65, 0.60, 0.55, 0.45, 0.40, 0.35, 0.30, and 0.25
OPB-07	23	2.20, 2.10, 2.00, 1.50, 1.40, 1.30, 1.25, 1.20, 1.10, 1.05, 1.00, 0.95, 0.90, 0.85, 0.75, 0.70, 0.65, 0.60, 0.55, 0.50, 0.45, 0.42, and 0.40
OPB-08	18	1.50, 1.25, 1.20, 1.10, 1.05, 1.00, 0.95, 0.90, 0.85, 0.80, 0.70, 0.65, 0.60, 0.55, 0.50, 0.45, 0.41, and 0.35
OPB-10	19	1.70, 1.60, 1.40, 1.30, 1.25, 1.20, 1.10, 1.00, 0.90, 0.85, 0.80, 0.75, 0.70, 0.65, 0.60, 0.55, 0.51, 0.45, and 0.32
OPB-11	21	1.30, 1.25, 1.20, 1.10, 1.00, 0.90, 0.85, 0.81, 0.80, 0.75, 0.70, 0.65, 0.61, 0.55, 0.52, 0.50, 0.45, 0.42, 0.40, 0.35, and 0.30
OPJ-07	18	1.40, 1.20, 1.00, 0.90, 0.85, 0.80, 0.75, 0.70, 0.65, 0.60, 0.55, 0.52, 0.45, 0.40, 0.38, 0.35, 0.25, and 0.21
OPJ-13	20	1.10, 0.95, 0.90, 0.85, 0.80, 0.75, 0.72, 0.70, 0.65, 0.62, 0.60, 0.55, 0.52, 0.50, 0.45, 0.42, 0.40, 0.35, 0.30, and 0.25
OPJ-15	15	1.70, 1.60, 1.20, 1.10, 0.95, 0.85, 0.75, 0.70, 0.55, 0.52, 0.50, 0.48, 0.45, 0.35, and 0.30
OPJ-16	17	1.30, 1.20, 1.10, 1.05, 1.00, 0.95, 0.90, 0.85, 0.80, 0.75, 0.65, 0.55, 0.50, 0.45, 0.40, 0.38, and 0.31
OPJ-18	12	1.50, 1.20, 1.10, 0.95, 0.90, 0.85, 0.75, 0.72, 0.65, 0.62, 0.51, and 0.41

organism could be the results of several factors, i.e., changing of one base at primer annealing site or an insertion or deletion of DNA sequence (Williams *et al.*, 1990). Not only their phylogenetic tree could be constructed from these polymorphic DNA bands to see their evolutionary relationships, some common bands which were found in most or in all species of *Encephalartos,* i.e., 350 bp from OPA-07, 650 bp from OPB-07, 600 bp from OPB-08, 550 bp from OPB-11, and 850 bp from OPJ-15 (Table 3), could be used as genetic marker for that specific variety of plant as also shown in the *Citrus* (Nicolosi *et al.*, 2000).

Phylogenetic and morphological relationships

All 240 bands obtained from the use of RAPD to identify twenty species of *Encephalartos* were subjected to NTSYS analysis and UPGMA (Sneath and Sokal, 1973). Their relationships in terms of similarity coefficient were calculated as shown in Table 4, while a phylogenetic tree (dendrogram) showing their evolutionary

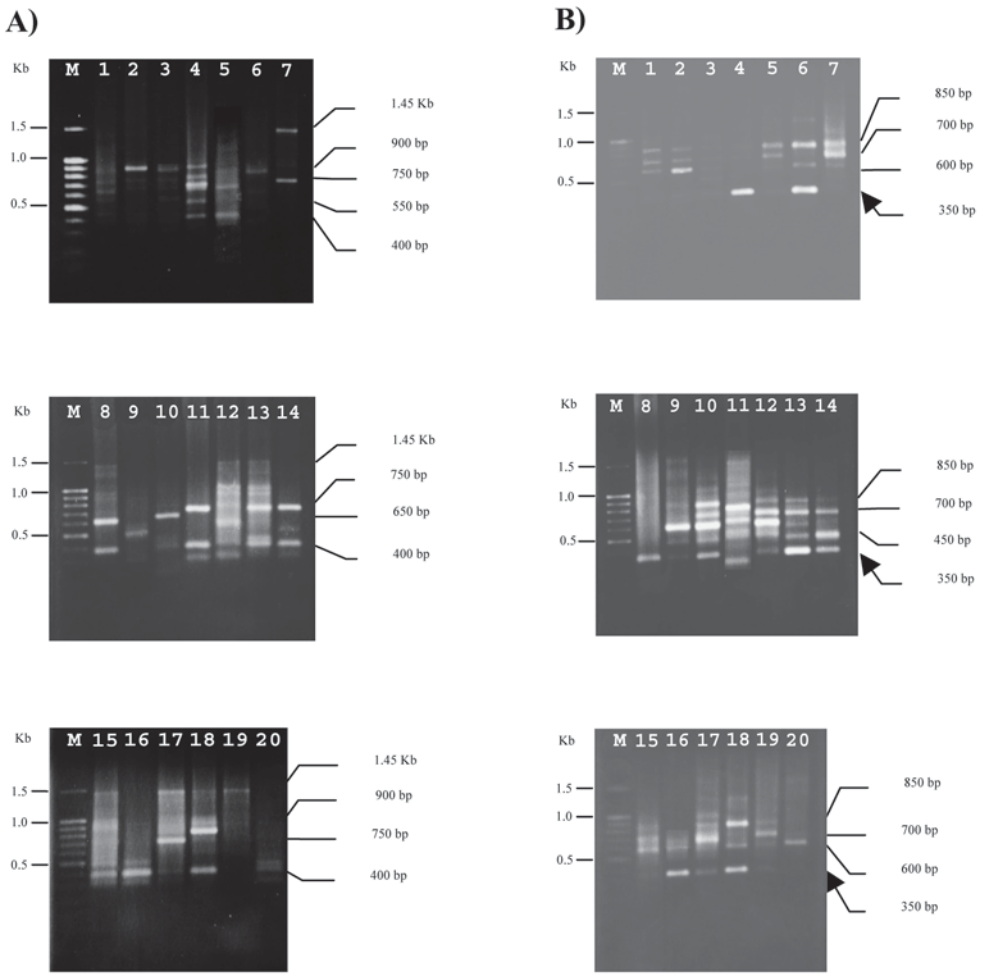


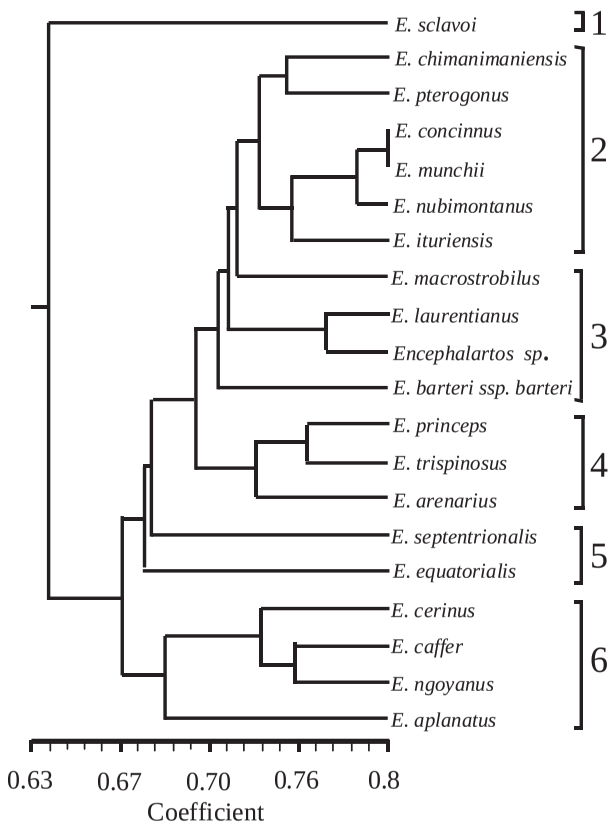
Figure 7 DNA patterns of twenty species of *Encephalartos* using RAPD synthesized by different primers: A) OPA-05, B) OPA-07; M = marker of 100 bp DNA ladder; arrow indicated the common band seen in all twenty species of *Encephalartos*; 1-20 were species of *Encephalartos* as indicated in Table 1.

relationship as determined from their genetic make-up was constructed (Figure 8). The similarity coefficient was found in the range of 0.53-0.80 which could group them into six clusters. It is interesting to find that molecular identification and grouping determined by RAPD technique agreed with their morphological characters (Figure 8) as follows:-

Cluster 1 – *E. sclavoi* was the only *Encephalartos* belonged to this group with unique yellow color of rachis. The stem was aborescent while the leaf was straight with spine on petiole. There were 1-6 prickles per leaf, and lanceolate leaflet. Both upper and lower margins of leaflet were dentate. Insertion of angles was obtuse. The difference of *E. sclavoi* from the rest of *Encephalartos* as seen from its DNA pattern supported the finding of Osborne and Grobbelaar (1993) based on its morphology aspect having *E. sclavoi* separated from other *Encephalartos* as also seen from the geographic isolation of *E. sclavoi* in the northeastern of Tanzania (Figure 1).

Table 4 Similarity coefficient of twenty *Encephalartos* species analyzed by NTSYS.

	E. sclavoi	*E. chimanimaniensis*	*E. princeps*	*E. cerinus*	*E. trispinosus*	*E. barteri ssp.barteri*	*E. septentrionalis*	*E. caffer*	*E. ngoyanus*	*E. equatorialis*	*E. arenarius*	*E. laurentianus*	*Encephalartos. sp.*	*E. aplanatus*	*E. concinnus*	*E. munchii*	*E. ituriensis*	*E. pterogonus*	*E. macrostrobilus*	*E. nubimontanus*
E. sclavoi	1.00																			
E. chimanimaniensis	0.63	1.00																		
E. princeps	0.58	0.68	1.00																	
E. cerinus	0.58	0.66	0.66	1.00																
E. trispinosus	0.62	0.70	0.76	0.70	1.00															
E. barteri ssp.barteri	0.61	0.68	0.74	0.69	0.70	1.00														
E. septentrionalis	0.61	0.66	0.68	0.64	0.68	0.70	1.00													
E. caffer	0.53	0.64	0.62	0.72	0.59	0.65	0.60	1.00												
E. ngoyanus	0.56	0.65	0.68	0.74	0.61	0.75	0.65	0.75	1.00											
E. equatorialis	0.57	0.66	0.59	0.61	0.62	0.65	0.63	0.59	0.68	1.00										
E. arenarius	0.67	0.69	0.70	0.59	0.76	0.63	0.65	0.58	0.64	0.63	1.00									
E. laurentianus	0.63	0.75	0.68	0.66	0.73	0.73	0.71	0.64	0.72	0.69	0.72	1.00								
Encephalartos sp.	0.56	0.70	0.68	0.66	0.69	0.68	0.66	0.63	0.70	0.66	0.72	0.77	1.00							
E. aplanatus	0.54	0.63	0.65	0.67	0.66	0.64	0.64	0.65	0.70	0.59	0.61	0.70	0.64	1.00						
E. concinnus	0.63	0.76	0.73	0.64	0.73	0.77	0.65	0.64	0.73	0.70	0.73	0.73	0.77	0.63	1.00					
E. munchii	0.64	0.73	0.73	0.64	0.69	0.71	0.62	0.63	0.70	0.65	0.70	0.72	0.75	0.60	0.81	1.00				
E. ituriensis	0.61	0.69	0.65	0.62	0.62	0.65	0.65	0.61	0.68	0.72	0.66	0.66	0.68	0.61	0.75	0.77	1.00			
E. pterogonus	0.63	0.75	0.68	0.65	0.68	0.71	0.65	0.64	0.71	0.68	0.70	0.73	0.68	0.59	0.76	0.72	0.74	1.00		
E. macrostrobilus	0.63	0.73	0.66	0.66	0.68	0.69	0.68	0.61	0.64	0.71	0.67	0.73	0.66	0.62	0.70	0.70	0.71	0.73	1.00	
E. nubimontanus	0.59	0.72	0.73	0.68	0.68	0.72	0.66	0.68	0.75	0.62	0.67	0.70	0.68	0.63	0.79	0.79	0.73	0.73	0.73	1.00



	Stem	Color of rachis	Rachis Curvature	Spine	Number of prickle	Shape of leaflet	Upper margin	Lower margin	Keel
1	aborescent	yellow	straight	+	1-6	lanceolate	dentate	dentate	slightly keel
2	aborescent	green	straight	+	1-6	lanceolate	dentate	dentate	horizontal
	aborescent	green	straight	+	1-6	lanceolate	dentate	dentate	slightly keel
	aborescent	green	straight	+	1-6	lanceolate	dentate	dentate	slightly keel
	aborescent	green	straight	+	1-6	lanceolate	dentate	dentate	moderately keel
	aborescent	green	straight	+	-	lanceolate	dentate	dentate	strongly keel
	aborescent	green	straight	+	1-6	lanceolate	dentate	dentate	slightly keel
3	aborescent	green	gentle curve	+	1-6	lanceolate	dentate	dentate	slightly keel
	aborescent	green	gentle curve	+	1-6	lanceolate	dentate	dentate	slightly keel
	aborescent	green	gentle curve	+	1-6	lanceolate	dentate	dentate	slightly keel
	aborescent	green	straight	+	1-6	lanceolate	dentate	dentate	horizontal
4	aborescent	blue	gentle curve	-	-	lanceolate	entire	entire	strongly keel
	aborescent	blue	recurve	-	-	lanceolate	entire	lobe	strongly keel
	aborescent	green	recurve	-	-	ovate	entire	lobe	strongly keel
5	aborescent	green	gentle curve	+	1-6	lanceolate	dentate	dentate	moderately keel
	aborescent	green	straight	+	>12	lanceolate	dentate	dentate	moderately keel
6	aborescent	green	straight	+	-	linear	entire	entire	moderately keel
	acualescent	green	straight	+	-	linear	entire	entire	strongly keel
	acualescent	green	straight	+	-	linear	entire	entire	strongly keel
	acualescent	green	straight	+	1-6	lanceolate	entire	dentate	slightly keel

Figure 8 Morphological and genetic relationships of twenty *Encephalartos* species as determined by RAPD and NTSYS analysis.

Cluster 2 – This group consisted of *E. pterogonus, E. concinnus, E. munchii, E. nubimontanus* and *E. ituriensis.* Petiole was green while the stem was aborescent. The leaf was also straight with spine on petiole. There were 1-6 prickles per leaf, and lanceolate leaflet. Both upper and lower margins of leaflet were dentate. Insertion of angles varied from horizontal to acute. The grouping of these six species of *Encephalartos* agreed with morphological classification done by Osborne and Grobbelaar (1993) and also agreed with their geographic distribution found in the eastern part of Zimbabwe and western of Mozambique. In addition, *E. chimanimaniensis, E. pterogonus, E. concinnus,* and *E. munchii* were all classified as Manikensis complex (Hill, 1999). The dendrogram also indicated a close relationship (>80%) between *E. concinnus* and *E. munchii* but slightly different from that of *E. nubimontanus* in the insertion of angle of leaflet and the number of prickle.

Cluster 3 – This group included *E. macrostrobilus, E. laurentianus, Encephalartos sp.,* and *E. barteri ssp. barteri.* They were all very similar morphologically having aborescent stem, green petiole, the same type of straight or gentle curved leaf with spine. The number of prickle was 1-6 per leaf, with lanceolate shape and dentate margins. Their insertions of leaflet angle were acute except in *E. barteri ssp. barteri* having a horizontal type. However, they were found to distribute themselves in different countries of Sudan, Zaire, Zimbabwe, and Nigeria (Figure 1).

Cluster 4 – Three species of *Encephalartos,* i.e. *E. princeps, E. trispinosus,* and *E. arenarius* belonged to this group. Their stems were all above the ground (aborescent type). Petioles were blue or green in color, while the leaves were gentle curved, or recurved (Figure 4 A and B). They had neither spine nor prickle on petiole. Leaflets were either lanceolate or oval in shape. Upper margin of leaflet was smooth while lower margin was dentate. Their insertions of leaflet angle were acute. These three species were also classified into the same Lehmanii group based on their morphology alone as reported by Osborne and Grobbelaar (1993). It should be noted here that phylogenetically *E. princeps* and *E. trispinosus* showed closer relationship with each other than with *E. arenarius* (Figure 8) which also confirmed by their geographic distribution at Eastern Cape of South Africa, while that of *E. arenarius* was found further down to the south (Figure 1) . The similar morphology of *E. princeps* and *E. trispinosus* as seen from their blue petiole and lanceolate leaflet was also different from green petiole and oval-shaped leaflet of *E. arenarius.* This result of grouping among the three species agreed with the result shown by Van der Bank *et al.* (2001) using allozyme technique.

Cluster 5 – Only *E. septentrionalis* and *E. equatorialis* belonged to this group. Both had aborescent type of stem. Petioles were green, while the leaves were gentle curved or straight. Spines were also found. The number of prickle was 1-6 per leaf in *E. septentrionalis* but more than 12 per leaf in *E. equatorialis.* Leaflets were lanceolate in shape having dentate margins for both upper and lower parts. Their insertions of leaflet angles were all acute. Although their genetic and morphological make-up were quite close but they were from two different countries in Africa (Figure 1).

Cluster 6 – There were four species of *Encephalartos,* i.e. *E. cerinus, E. ngoyanus, E. caffer,* and *E. aplanatus* belonged to this group as determined by their DNA patterns. The unique character of this group was the underground stem of acualescent type except that of *E. cerinus.* The underground stem was not found at all in other clusters of *Encephalartos.* However, considering other characters beside stem type, *E. cerinus, E. caffer,* and *E. ngoyanus* were very similar in morphology. They all had green petiole, straight leaf, spiny petiole, no prickle, needle-like leaflet, smooth upper and lower margins, while their

leaves varied in keel, i.e. slightly, moderately and strongly keel. On the other hand, *E. aplanatus* was found to be different in having 1-6 prickles per petiole, lanceolate leaflet, dentate margin, and slightly keel. In addition, only *E. caffer* was found at Eastern Cape of South Africa while the remaining three species of the same cluster were found in the close-by area of eastern KwaZulu-Natal and Swaziland (Figure 1).

CONCLUSION

Nine morphological characters of *Encephalartos*, i.e. stem, petiole color, leaf curvation, spiny petiole, number of prickle per leaf, leaflet shape, upper and lower margin of leaflet, and insertion of angle were identified. Eight primers (OPA-05, OPA-07, OPB-07, OPB-08, OPB-11, OPJ-07, OPJ-13, and OPJ-15) could be used to synthesize DNA with polymorphic band patterns. All 240 bands were analyzed using NTSYS to obtain their similarity coefficient and constructed the phylogenic tree. The twenty *Encephalartos* species were grouped into six clusters based on their genetic make-up and evolutionary line. The molecular identification confirmed their grouping based on morphological characters which agreed with geographic distribution as well.

ACKNOWLEDGEMENTS

This research was financially supported by Kasetsart University Research and Development Institute (KURDI) and Public-Private Technology Transfer Center. The authors would like to thank Mr. Kampon Tansacha and Mr. Anders J. Lindstrom at Nongnooch Tropical Garden for providing *Encephalartos* specimens.

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