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Short communication

Genetic variability and species identification within *Encephalartos* using Random Amplified Polymorphic DNA (RAPD) markers

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Abstract

Encephalartos is the second largest genus of the living cycads with 65 species and 2 sub-species. Most species of *Encephalartos* are morphologically very similar and therefore could benefit from additional tools for their correct identification. The present study aimed to study the genetic variability within 22 species of the genus *Encephalartos* using Random Amplified Polymorphic DNA (RAPD) markers. Seventeen RAPD primers were used to carry out the present study. These primers were used to amplify the genomic DNA of the 22 species of *Encephalartos*. Amplification products ranged in size from 250 bp to 2500 bp, while in number they ranged from 1 to 12. A total of 186 reproducible bands were scored of which 166 (88%) were polymorphic and 20 (12%) were monomorphic and 3 were unique bands. Within the species of *Encephalartos* a high degree of genetic variability exists, as the similarity index value ranged from 0.440 to 0.833. The lowest value of 0.440 was between *E. altensteinii* and *E. transvenosus,* while *E. bubalinus* and *E. princeps* exhibited maximum values (0.833), indicating their close genetic relatedness. Two primers generated unique bands in three species of *Encephalartos*, which could assist to identify these species at the molecular level. Three unique bands, OPC-02$_{\sim 300}$, OPC-02$_{\sim 500}$ and OPE-01$_{\sim 2300\ bp}$, were generated for *E. gratus, E. hilderbrandtii* and *E. inopinus*, respectively. The results suggest considerable potential of the RAPD approach for correct genetic identification of individual species as well as an efficient way of fraud prevention. Genetic variability within the species of *Encephalartos* can be ascertained using these primers.

Keywords: Cycads; Genetic variability; Molecular markers; Polymerase chain reaction

1. Introduction

Cycads are regarded as the oldest group of seed plants surviving on Earth (Brenner et al., 2003). Today they are restricted to the tropics and subtropics of both the Old and New Worlds. They flourished across the earth 265 million years ago (Osborne et al., 1999; Golding and Hurter, 2003). They have been included in a red data list owing to their limited occurrence (Golding and Hurter, 2003).

Encephalartos is the second largest genus of the order Cycadales with 65 species and two sub-species (Norstog and Nicholls, 1997). The species of *Encephalartos* are widely distributed across various climatic zones. Most of these species are morphologically very alike in appearance, therefore, it would be beneficial to study variability within the genus using molecular approaches. DNA analyses are suited for this purpose as it is highly conserved and is not affected by changing climatic conditions. DNA fingerprinting can distinguish species rapidly using small amounts of DNA and thus can assist in reliable, non-destructive identification of the phenotype. Various approaches are available for DNA fingerprinting such as amplified fragment length polymorphism (AFLP) (Zabeau and Vos, 1993), restriction fragment length polymorphism (RFLP) (Botstein et al., 1980), simple sequence repeats (SSRs) (Tautz, 1989) and randomly amplified polymorphic DNAs (RAPD) (Williams et al., 1990). RAPD markers have been extensively used for the identification of either species or cultivars in a wide range of plants (Martin et al., 1997; Mariniello et al., 2002; Koller et al., 1993; Esselman et al., 2000). Use of RAPD markers (10-mer oligonucleotides of arbitrary sequences) does not require prior knowledge about the genome of plants. Furthermore, they are simple to use, efficient

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Table 1
List of species of *Encephalartos* collected from DBG = Durban Botanical Garden, Durban and PMB = University of KwaZulu-Natal Botanical Garden, Pietermaritzburg

S.N.	Species	Voucher number and place of collection
1.	*E. arenarius*	SP74, 40, 82 (DBG)
2.	*E. bubalinus*	SP80, 84 (DBG)
3	*E. chimanimaniensis*	SP33, 86, 92, 94 (DBG)
4.	*E. ferox*	SP44, 54 (DBG) and SP85, 98, 103 (PMB)
5.	*E. gratus*	SP32, 49, 62, 78 (DBG)
6.	*E. hilderbrandtii*	SP34, 38, 51, 64 (DBG)
7.	*E. horridus*	SP39, 50 (DBG)
8.	*E. inopinus*	SP36, 42, 76, 99 (DBG) and 125 (PMB)
9.	*E. kisambo*	SP35, 83, 96, 108 (DBG)
10.	*E. latifrons*	SP81, 87, 89, 112 (DBG)
11.	*E. lebomboensis*	SP30, 43 (DBG) and SP95, 109 (PMB)
12.	*E. lehmannii*	SP45, 60 (DBG) and SP119, 121
13.	*E. manikensis*	SP65, 72, 113, 115 (DBG)
14.	*E. msinganus*	SP53 (PMB) and SP106, 110 (DBG)
15.	*E. munchii*	SP41, 71 (PMB) and SP120, 126 (DBG)
16.	*E. natalensis*	SP29 (DBG) and SP47, 55, 57 (PMB)
17.	*E. princeps*	SP26, 56, 59, 61, 66 (PMB)
18.	*E. senticosus*	SP75, 128, 130, 133 (DBG)
19.	*E. transvenosus*	SP48, 58 (PMB) and SP129, 131, 132 (DBG)
20.	*E. villosus*	SP46 (DBG) and SP63, 67 (PMB)
21.	*E. altensteinii*	SP70 (DBG) and SP134, 137, 139 (PMB)
22.	*E. woodii*	SP73, 88 (DBG)

Voucher specimens are deposited in University of Natal Herbarium (NU).

and provide a quick method for identification of plants at any developmental stage (Khasa and Dancik, 1996; Conner and Wood, 2001). Therefore, the present study was undertaken to study the genetic variability within 22 species of *Encephalartos* using RAPD markers.

2. Material and methods

Young leaves of 22 species of *Encephalartos* were collected from the Durban Botanical Garden, Durban and the University of KwaZulu-Natal Botanical Garden at Pietermaritzburg, South Africa (Table 1). Prior to analyses all material was stored at −70 °C.

To isolate the DNA from the collected leaves, the protocol described by Rogers and Bendich (1988) was adopted with minor modifications. Briefly, liquid nitrogen was used to grind 500 mg of freeze–dried tissue. The grounded powder was suspended in 3 ml CTAB extraction buffer [2% CTAB (Cetyl trimethyl ammonium bromide), 100 mM Tris–HCl (pH 8.0), 20 mM EDTA and 1.4 M NaCl] in 15 ml tubes. The suspension was incubated at 65 °C for 1 h and extracted with an equal volume of chloroform: *iso*-amyl alcohol (24:1) followed by centrifugation at 10,000 *g* for 10 min. The aqueous phase was transferred to a fresh tube and one-tenth volume of CTAB and NaCl (10% CTAB and 0.7 M NaCl) added. This was again extracted with an equal volume of chloroform:*iso*-amyl alcohol (24:1) and centrifuged as before. DNA was precipitated using an aqueous phase with 2–3 volumes of *iso*-propanol for 20 min at −20 °C. The DNA recovered after centrifugation was dissolved in high salt TE [10 mM Tris–HCl (pH 8.0): 0.1 mM EDTA (pH 8.0) and 1 M NaCl] and extracted with equal volumes of phenol:chloroform:*iso*-amyl alcohol (25:24:1). After centrifugation, the aqueous phase was collected and DNA was precipitated with 1/10 volume of 5 M NaCl and three volumes of absolute alcohol for 10–20 min at −20 °C. The pellet of DNA recovered after centrifugation was washed three times with 70% alcohol, and absolute alcohol and dissolved in 20 µl HPLC water. The DNA was quantified by means of UV spectrophotometry at 280 nm. Finally the DNA was diluted to a working solution of 10 ng/µl for PCR analyses. Bulk DNA analysis was carried out using pooled DNA of 4–5 genotypes for each species.

Thirty random 10-mer primers (Operon Technologies, California, USA) of the A to G series were used for RAPD analysis. The amplification reaction mixture (total volume of 25 µl) contained 50 ng genomic DNA, 3 µl of 10× assay buffer, 0.2 mM dNTPs (Roche Diagnostics), 4 µM primer and 1 U Taq DNA polymerase (Roche Diagnostics). All the amplifications were

Table 2
List of primers used for RAPD analysis and the number of scored bands in different species of *Encephalartos*

Primers	Sequences	Total bands	Polymorphic bands	Polymorphic bands (%)	No. of unique bands
OPA-11	5′-CAATCGCCGT-3′	6	6	100	0
OPB-01	5′-GTTTCGCTCC-3′	7	5	71.4	0
OPB-07	5′-GGTGACGCAG-3′	10	9	90	0
OPB-11	5′-GTAGACCCGT-3′	8	7	87.5	0
OPB-20	5′-GGACCCTTAC-3′	6	6	100	0
OPC-02	5′-GTGAGGCGTC-3′	15	13	86.6	2
OPC-07	5′-GTCCCGACGA-3′	12	10	83.3	0
OPD-02	5′-GGACCCAACC-3′	14	12	85.7	0
OPD-05	5′-TGAGCGGACA-3′	8	8	100	0
OPD-07	5′-TTGGCACGGG-3′	10	8	80	0
OPD-12	5′-CACCGTATCC-3′	7	6	85.7	0
OPD-17	5′-TTTCCCACGG-3′	14	14	100	0
OPD-20	5′-ACCCGGTCAC-3′	9	9	100	0
OPE-01	5′-CCCAAGGTCC-3′	14	13	92.8	1
OPE-14	5′-TGCGGCTGAG-3′	11	10	90.9	0
OPE-18	5′-GGACTGCAGA-3′	8	7	87.5	0
OPG-13	5′-CTCTCCGCCA-3′	9	7	77.7	0
Total		184	166		3

carried out in a DC-960G Gradient Thermal Cycler (Corbett Research, Australia) using the following programme. One cycle of initial denaturation at 94 °C for 1 min, 36 °C for 20 s and 72 °C for 2 min, followed by 45 cycles: 1 min at 94 °C, 1 min at 35 °C, 2 min at 72 °C with a final extension at 72 °C for 5 min. The amplified samples were analyzed by electrophoresis in 1.4% agarose gel after staining with ethidium bromide (0.5 μg/ml) and viewed under UV light. All the amplifications were repeated twice and only reproducible bands were scored for further analysis. The faintly stained bands not clearly resolved, were not considered for data collection. For the confirmation of unique bands generated by the bulk DNA of a species, DNA of all available genotypes of the particular species were used for PCR amplification.

Data was subjected to similarity matrix analysis using the Numerical Taxonomy and Multivariant Analysis System programme package for PC (NTSYS-pc version 2.11U (Rohlf, 1988). Percentage of polymorphic bands were defined as the percentage of polymorphic bands amplified by a single primer to that of the total number of bands produced by the same primer.

3. Results and discussion

A total of 30 decamer primers were screened with the bulk DNA (comprised of 3–4 genotypes) of 22 species of *Encephalartos.* Of the 30 primers, 17 gave reproducible polymorphic DNA amplification patterns. The number of polymorphic bands amplified by individual primers varied from 1 to 10, while the total number of amplified bands ranged from 1 to 12. The size of the amplified products was from 250 bp to 2500 bp. A total of 186 amplified products were generated using 17 decamer primers, with 88% (166 bands) polymorphism (Table 2). Among 186 amplified products, only 3 unique bands, specific for species could be identified. These unique bands were generated with primers OPC-02 (5′-GTGAGGCGTC-3′) and OPE-01 (5′-CCCAAGGTCC-3′).

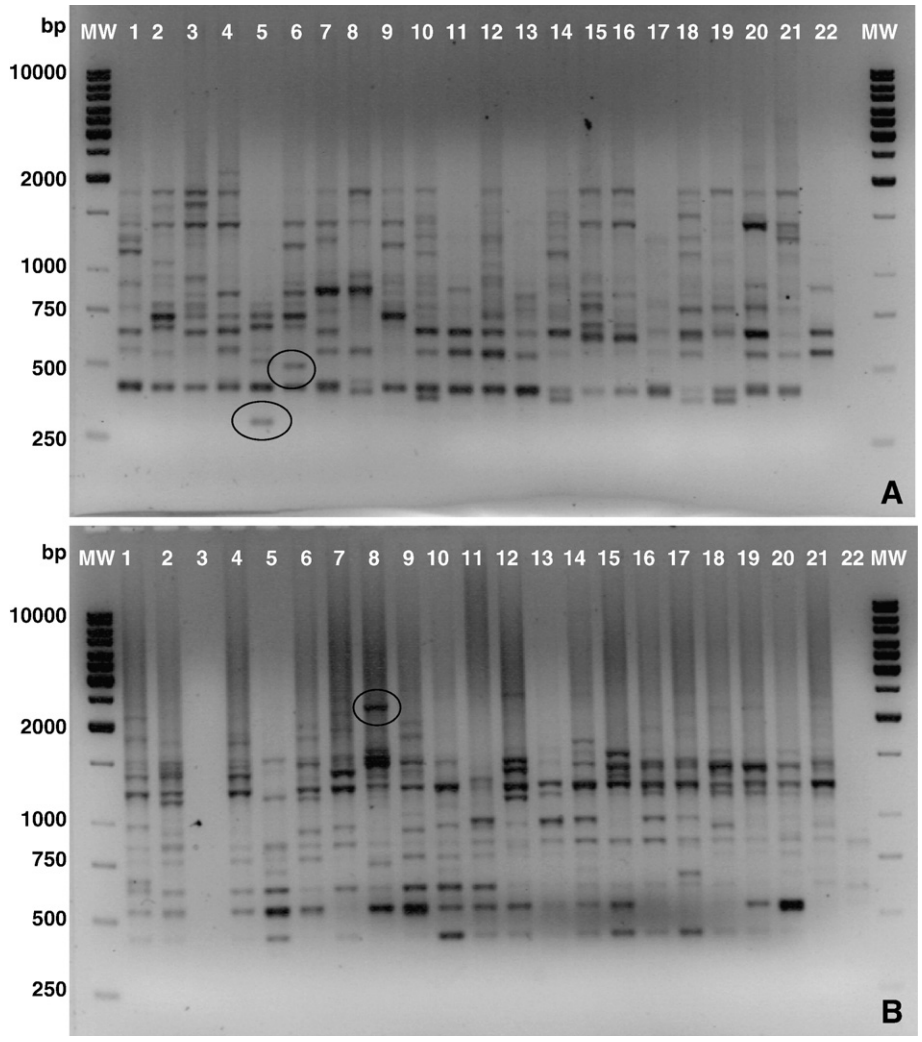

Fig. 1. RAPD profile of 22 species of *Encephalartos* with primers OPC-02 (A) and OPE-01 (B). The encircled bands indicate species-specific bands. MW: DNA ladder (1 Kb), 1: *E. arenarius*, 2: *E. bubalinus,* 3: *E. chimanimaniensis,* 4: *E. ferox,* 5: *E. gratus,* 6: *E. hilderbrandtii*, 7: *E. horridus,* 8: *E. inopinus,* 9: *E. kisambo,* 10: *E. latifrons,* 11: *E. lebomboensis,* 12: *E. lehmannii,* 13: *E. manikensis,* 14: *E. msinganus,* 15: *E. munchii*, 16: *E. natalensis,* 17: *E. princeps,* 18: *E. senticosus,* 19: *E. transvenosus,* 20: *E. villosus,* 21: *E. altensteinii,* 22: *E. woodii*. Three unique bands were generated, (A) lane 5, 300 bp marker for *E. gratus*; lane 6, 500 bp marker for *E. hilderbrandtii* and (B) lane 8, 2300 bp marker for *E. inopinus*.

E. gratus and *E. hildebrandtii* have been identified with primer OPC-02 with band sizes of ~300 bp, ~500 bp respectively, while primer OPE-01 generated an unique band of ~2300 bp to identify *E. inopinus* (Fig. 1).

Similarity index values for the RAPD data ranged from 0.440 to 0.833. This parameter serves to ascertain the degree of genetic relatedness among the 22 species screened. *E. altenstenii* and *E. transvenosus* were the most distantly related with the lowest similarity index value (0.440) while *E. bubalinus* and *E. princeps* were most closely related having a value of 0.833.

Routinely, morphological features are used to identify species. Polymerase Chain Reaction (PCR) based techniques using RAPD markers, are now widely adopted in plant systematics and population biology to solve discrepancies in species identification, their classification as well as their hierarchical positions (Hadrys et al., 1992; Wolfe and Liston, 1998). The banding patterns generated using RADP markers remains the same irrespective of the developmental stage of the plant (Swenson et al., 1995). This feature of RAPD loci has made them very useful for identifying rare and endemic populations or linages for conservation when other methods fail to detect variations or resolve relationships (Esselman et al., 2000). They have also been found useful in elucidating the relationships between sub- (Wolff and Morgan-Richards, 1998) as well as co-generic species (Spooner et al., 1997). In spite of their wide usage, RAPD markers have a few disadvantages. One is their sensitivity to change in reaction conditions, which might lead to differences in results from different laboratories. This could be overcome by thorough standardization of DNA isolation techniques and PCR reaction conditions. During the course of this study, PCR amplifications were carried out 4–5 times using two different thermocyclers to ensure the repeatability of banding patterns. Despite negative arguments regarding the use of RAPD markers for population genetic studies, several comparative studies between allozymes, RAPDs and AFLPs were carried out. These studies showed that RAPD markers have analytical efficiency similar to the other approaches (Sun and Wong, 2001; Kjølner et al., 2004). Therefore their use for such studies is still acceptable. However, as indicated above, care needs to be taken to ensure reproducibility of amplification profiles as well as using sensitive statistical software for analysis.

Of the 22 species analyzed, we have identified three species-specific markers, based on generated DNA fingerprints. In the past this approach had been successfully used to identify the species in both angiosperms such as olive (Belaj et al., 2001), persimmon (Yamagishi et al., 2005) and gymnosperms such as spruce (Khasa and Dancik, 1996) and pine (Nkongolo et al., 2002). To the best of our knowledge no such approach for identification of species is available for the genus *Encephalartos*. This technique can assist in the validation of species identity and to address *ex-situ* conservation issues that involve taxonomic identification and species admixture, however, more species and populations need to be analyzed before generalized deductions can be made.

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