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Short communication

The genetic relationship between *Encephalartos natalensis* and *E. woodii* determined using RAPD fingerprinting

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Abstract

The second largest cycad genus *Encephalartos,* consists of 50 living species all endemic to Africa. *Encephalartos natalensis*, a common variety found in KwaZulu-Natal is similar in morphology to *E. woodii* of which only a single clump has been found in the wild. This enigmatic species is represented by a single male plant. Although the phylogenetic relationship between *E. natalensis* and *E. woodii* is unknown, the ability of these species to hybridise suggests a close relationship. The aim of this study was to determine the genetic variability between *E. natalensis* and *E. woodii*. Genetic fingerprints were generated using the RAPD technique and data analysed using distance methods. Based on RAPD fingerprints, the intraspecific genetic variation among different *E. natalensis* plants is similar to the interspecific variation between *E. natalensis* and *E. woodii*. These data confirm the close relationship between *E. natalensis* and *E. woodii*.

Keywords: Cycadales; Cycad; Genetic relationship; *Encephalartos*; RAPDs; *E. woodii*; *E. natalensis*

1. Introduction

Members of the Cycadales, commonly referred to as cycads, have grown on Earth for the last 250 million years (Jones, 1993; Donaldson et al., 2003). The genus *Encephalartos* Lehm., possibly the largest in the Cycadales, consists of over 50 described species all indigenous to Africa (Donaldson, 2003). Species of *Encephalartos* are dioeceous and take several years to reach maturity. However, for one species, *E. woodii* Sander, only a solitary male has been found in nature, discovered in 1895 as a single clump (Donaldson, 2003). Despite *E. woodii* only consisting of a single original individual, this species reproduces asexually through the production of basal suckers and even though considered extinct, this enigmatic species is well represented in botanical collections in various countries around the world (Jones, 1993; Osborne, 1995). Other cycad species are commonly found in the area where *E. woodii* was first discovered (Jones, 1993). These include *E. natalensis* R.A. Dyer and I. Verd. and *E. villosus* Lem. It is known that hybridization can occur between different species of *Encephalartos* including *E. natalensis* and *E. woodii*. Thus given the unusual circumstance of *E. woodii*, it is possible that this species is a result of interspecies hybridization.

With the advent of molecular techniques it has become possible to determine genetic relationships based on the DNA between closely related individuals (Karp et al., 1996). Although molecular studies have been done on cycads, these have concentrated on higher order relationships (Rai et al., 2003; Chaw et al., 2005). Van der Bank et al. (2001) used ITS and *rbc*L sequences to determine the phylogeny of *Encephalartos*, but did not include *E. natalensis*, *E. villosus* or *E. woodii*, and report that species in this genus are closely related. For many taxa in the latter study, the pairwise genetic distance was 0.00 for both ITS and *rbc*L regions indicating that the level of polymorphism for these regions is not sufficient to determine the relationship between closely related taxa or individuals. In a further study, Treutlein et al. (2005) used ITS and *rbc*L sequences as well as a single ISSR (inter-simple sequence repeats) to resolve the phylogenetic history of the genus *Encephalartos*. Although *E. woodii* and *E. natalensis* grouped together with other taxa in larger clades, the genetic variability

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Table 1
Locality and description of plant material used in this study

Species	Origin
E. woodii (Ew)	Botanical Gardens, Durban
E. natalensis (En1)	Botanical Gardens, Durban
E. natalensis (En2)	Botanical Gardens, Durban
E. natalensis (En3)	University of Natal, Pietermaritzburg
E. natalensis (En4)	University of Natal, Pietermaritzburg
E. natalensis x *E. woodii* (NW1)	Cycad Improvement Center
E. natalensis x *E. woodii* (NW5)	Cycad Improvement Center
E. villosus (Ev)	Botanical Gardens, Durban

across the whole genus was found to be extremely low, resulting in unresolved clades.

Many molecular techniques such as RAPDs (random amplified polymorphic DNA markers) and AFLPs (amplified fragment length polymorphisms) do not require prior knowledge of the germplasm being studied and are able to distinguish between closely related individuals, making them ideal for use in taxa where insufficient sequence information is available (Liu and Cordes, 2004). Thus the aim of this preliminary study was to use RAPDs to examine the genetic relationship between *E. natalensis* and *E. woodii* using *E. villosus*, a closely related species capable of interbreeding with *E. natalensis*, as outgroup (Van der Bank et al., 1998; Treutlein et al., 2005).

Plant material was collected from eight individuals of *E. natalensis*, *E. woodii* and *E. villosus* as well as *E. natalensis* x *E. woodii* hybrids (Table 1). Only specimens of undisputed identity were used. Leaflets were wiped with chloroform to remove surface debris and immediately frozen in liquid nitrogen. The leaflets were stored at −70 °C. Leaflet material was homogenized in a mortar and pestle in liquid nitrogen and the powder re-suspended in extraction buffer (8 M urea, 0.1 M Tris-HCl pH 8, 0.05 M EDTA pH 8, 0.5 M NaCl and 1% SDS). The homogenate was stored on ice for 20 min and then incubated at 65 °C for 20 min. After centrifugation for 20 min at 10 krpm, 1.5 M potassium acetate was added to the supernatant and kept on ice for 30 min. After a second centrifugation for 20 min at 10 krpm the nucleic acids were precipitated by the addition of 0.6 (v/v) 2-propanol. The precipitate was collected by spooling the DNA, washing with 70% ethanol and re-suspended in 500 ul sterile distilled H_2O. Nucleic acids were further purified by the addition of 1% CTAB, 1 M NaCl and incubation at 65 °C for 1 h. Thereafter, chloroform extractions were performed until the inter-phase was clean. This was followed by a final precipitation

Table 2
Matrix of genetic distances for eight cycad taxa from RAPD data

Population	Ew	En1	En2	En3	En4	NW1	NW2	Ev
Ew	–							
En1	0.2183	–						
En2	0.2183	0.1590	–					
En3	0.2183	0.1590	0.0000	–				
En4	0.1882	0.0247	0.1306	0.1306	–			
NW1	0.1882	0.0762	0.1306	0.1306	0.1030	–		
NW2	0.1882	0.0762	0.1306	0.1306	0.1030	0.0000	–	
Ev	0.8361	0.6573	0.6573	0.6573	0.6149	0.7007	0.7007	–

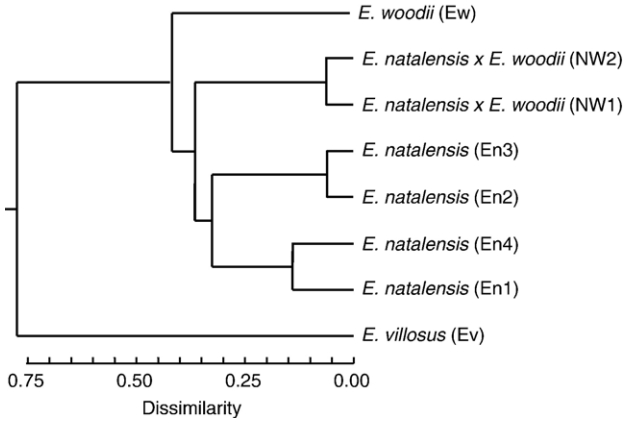

Fig. 1. Dendrogram of Euclidean pairwise genetic relationships of *E. natalensis*, *E. woodii*, *E. natalensis* x *E. woodii* and *E. villosus* using UPGM clustering analysis. The cophenetic correlation of the tree indicating goodness of fit is 0.99.

with the addition of 0.6 (v/v) 2-propanol. The DNA was spooled, washed with 70% ethanol and re-dissolved in sterile distilled H_2O. The DNA concentration was quantified using the Gene-Quant II (Pharmacia Biotech, Athens, Greece).

RAPD PCR reactions were performed using the Operon primers (Operon Technologies, Inc., Alameda CA). One hundred and forty 10-base primers were evaluated from which 10 most informative primers were used (OPAO2, OPA06, OPA09, OPA07, OPB07, OPC07, OPC16, OPC20, OPD12, OPD16). PCR amplification followed the procedure of Williams

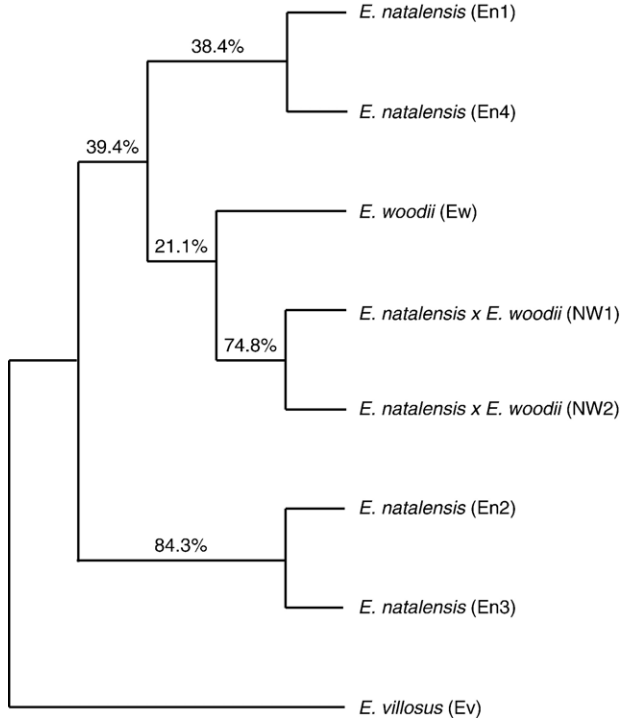

Fig. 2. NJ tree of the genetic relationship amongst *E. natalensis*, *E. woodii*, *E. natalensis* x *E. woodii* and *E. villosus* using UPGM clustering analysis. Both Phylip 3.6 and MEGA 2.1 produced similar trees with only a slight variation in branch lengths. The percentage above a branch represents the bootstrap support for that node (Phylip 3.6).

et al. (1990) with some modification. Each reaction mixture (25 ul) contained 2.5 ul of 10X reaction buffer, 2 mM $MgCl_2$, 2.5 mM each of dGTP, dATP, dTTP, dCTP, 5 pmol primer, 20 ng DNA and 0.9 U of *Taq* polymerase (Boehringer Manheim). Amplification was carried out using one initial denaturation cycle at 95 °C for 5 min followed by 45 cycles at 95 °C for 10 s, 40 °C for 10 s, and 72 °C for 2 min. Amplification products were analyzed by gel electrophoresis on a 1.5% agarose gel. Approximately 20 ul of sample was loaded on the gel and run at 80 V for 4 to 6 h. Reproducibility of bands was determined by comparing replicated experiments. RAPD data was coded for the presence (1) and absence (0) of bands. The binary data was analyzed using the NCSS 2000 statistical package (Hintz, 1998) using the Euclidean distance method and UPGMA (unweighted pair group of arithmetic averages) and cophenetic correlation of the dendrogram calculated as a measure of "goodness of fit". The Neighbor-Joining (NJ) method of Saitou and Nei (1987) and the UPGMA method of clustering was used to construct a tree by successive clustering using Phylip 3.6 (Felsenstein, 2004) and MEGA 2.1 (Kumar et al., 2001). The bootstrap test with 10 k replicates was performed to determine node support.

An average of 8.2 data points were generated per primer selected. The genetic distance values between taxa are presented in Table 2. The mean genetic distance between the ingroup taxa is 0.1607, 0.2033 between *E. woodii* and *E. natalensis*, and 0.7470 between ingroup taxa and *E. villosus* (outgroup). The genetic distance between *E. natalensis* and the *E. natalensis* x *E. woodii* hybrids was 0.1034, and 0.1882 between *E. woodii* and *E. natalensis* x *E. woodii*. The clustering of taxa using Euclidean and NJ distances grouped *E. woodii*, *E. natalensis* and *E. woodii* x *E. natalensis* hybrids together (Figs. 1 and 2). Bootstrap values within the ingroup were not significant and ranged between 21.1% and 84.3%. The *E. natalensis* x *E. woodii* hybrids grouped together with a 74.8% bootstrap, and *E. woodii* grouped with the hybrids with a low bootstrap (21.4%).

The small genetic distance of 0.2033 between *E. natalensis* and *E. woodii* shows that there is relatively little differentiation between these species, and is comparable to the within species value for *E. natalensis* of 0.1607. Although the mean genetic distance between *E. woodii* and *E. natalensis* falls just outside the genetic distance within *E. natalensis*, the close relationship between these species is indicated by the clustering of *E. woodii* within the *E. natalensis* group. These data suggest that *E. woodii* and *E. natalensis* share a common genetic origin. Furthermore, it is difficult to attribute the single occurrence of *E. woodii* in the wild to extinction when considering the climatic and geographic adaptability of *E. woodii* (Jones, 1993). We therefore suggest that *E. woodii* is possibly a product of hybridization, which would explain its single occurrence in nature, rather than extinction. The genetic distance between *E. natalensis* x *E. woodii* hybrids and *E. natalensis* and *E. woodii* allow a comparison of the effect of hybridization. Although *E. woodii* clusters with the hybrids, *E. natalensis* does not form a single cluster that is distinct to *E. woodii* and the *E. natalensis* x *E. woodii* hybrids. Thus the close relationship of the hybrids to *E. natalensis* suggests that *E. natalensis* or an *E. natalensis*-like progenitor has possibly contributed to a hybridization event giving rise to *E. woodii*.

The genetic relationship based on RAPD data between the outgroup, *E. villosus* and ingroup, *E. natalensis* is comparable to the findings of Van der Bank et al. (1998). Their study based on allozyme data, found that *E. villosus* was closer related to *E. natalensis* than *E. transvenosus*, a species morphologically similar to *E. natalensis* but geographically distinct. Futhermore, the study of Treutlein et al. (2005) determined that *E. villosus* falls within a separate but closely related group to *E. natalensis* and *E. woodii*. Thus the choice of *E. villosus* as outgroup in the comparison of *E. natalensis* and *E. woodii* is justified.

E. natalensis and *E. woodii* are closely related taxa based on genetic distances from RAPD data. The close genetic relationship between *E. natalensis* and *E. woodii* is further supported by geographical and morphological data (Jones, 1993). The close genetic relationship between *E. natalensis* and *E. woodii*, together with the enigmatic nature of *E. woodii* suggests that this species is possibly a result of interspecies hybridization between an *E. natalensis*-like progenitor and another closely related species. Treutlein et al. (2005) make a similar generalized observation for species of *Encephalartos*. We recommend that more taxa be analyzed for clarification. Although these data suggest that *E. woodii* is not a genetically unique taxon, it will continue to have great novelty in cycad collections.

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