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Sex identification in *Encephalartos natalensis* (Dyer and Verdoorn) using RAPD markers

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Abstract All cycads are strictly dioecious with a long juvenile stage. Currently, there is no method available to determine the sexuality of seedlings prior to the onset of cone formation. This study aimed to develop a sex specific Random Amplified Polymorphic DNA (RAPD) marker for *Encephalartos natalensis*. Initially, 140 primers were used to amplify the bulk DNA of five individuals each of known male and female sexuality. While a high degree of polymorphism was observed in the amplification profiles of male and female plants, only primer OPD-20 generated a specific band (~850 bp) in female DNA. To validate this observation, this primer was re-tested with 69 individuals of *E. natalensis*. The ~850 bp DNA band was present in all 38 female individuals tested and it was consistently absent in all 31 male plants tested. The result offers a rapid and simple test to determine sexuality of *E. natalensis* seedlings at early stages of development, before the onset of reproductive maturity thereby saving time and economic resources when cultivating these specimens.

Keywords Dioecious · Sex specific · Cycads · Bulk segregant analysis

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Introduction

The Cycads are the oldest group of plants surviving on Earth (Brenner et al. 2003). They flourished throughout the Mesozoic (from Triassic to the Cretaceous; 265 million years ago), but are now restricted to the tropics and subtropics of both the Old and New Worlds (Golding and Hurter 2003). Cycads in general exhibit a very high degree of endemism (Dyer 1965; Schneider et al. 2002). They are rare and are included in the red data book (Golding and Hurter 2003). They are seed-bearing plants similar to angiosperms, but unlike these, the seeds are naked, and are not enclosed in the fruit.

All the living cycads are included in the order Cycadales. Among the genera, *Encephalartos* is the second largest with 65 species and two subspecies (Norstog and Nicholls 1997). *Encephalartos* species are widely distributed across various climatic zones of the African continent. All *Encephalartos* species are strictly dioecious with

$2n$ = 18 chromosome numbers (Whitelock 2002). There is no recorded evidence of any monoecious genotypes. The plants of two sexes are morphologically alike and sexuality of the plants only becomes clear once the first cones appear. At present there is no way to predict sex in the cycads at the juvenile stage (Whitelock 2002). In higher plants about 4% of the flowering species are dioecious (Ainsworth 2000). In the majority of these cases, it is impossible to ascertain the sex of the plant at the seedling stage. This poses a considerable problem to horticulturists for various breeding programmes. DNA based markers provide a useful tool to indicate dimorphism of the genome corresponding to the dimorphic genders. Random Amplified Polymorphic DNA (RAPD) is an inexpensive and rapid method not requiring any information regarding the genome of the plant. This is based on the use of small arbitrary sequences, which are employed to amplify the genomic DNA of the plant (Williams et al. 1990).

The present study aimed to identify male and female specific RAPD markers for *Encephalartos natalensis* (Dyer and Verdoorn) so that the sex of individual plants can be determined before the appearance of the first cone on the plants, which only happens after 10–12 years (http://plantnet.rbgsyd.gov.au).

Materials and methods

Young leaves of confirmed male (31) and female (38) individuals of *E. natalensis* (Dyer and Verdoorn) were collected from areas adjoining the University of KwaZulu-Natal Botanical Garden at Pietermaritzburg, and the Durban Botanical Garden, Durban.

Genomic DNA was isolated from these leaves according to Rogers and Bendich (1988) with minor modifications: the use of phenol/chloroform/isoamyl-alcohol (25:24:1) for organic extraction and precipitation of DNA from the aqueous phase with iso-propanol. The DNA pellet was dissolved in sterile HPLC water and quantified with an UV spectrophotometer. The stock DNA was diluted with sterile HPLC water to a working solution of 10 ng/µl for PCR analysis.

Random 10-mer primers (Series B, C, D, E, F, G and J; OPERON Technologies, California, USA) were used for RAPD analysis of genomic DNA. Bulk segregant analysis (BSA; Paran and Michelmore 1993) was carried out by pooled DNA of five male and five female individuals. Amplification reactions were carried out in a volume of 25 µl containing 50 ng of genomic DNA, 3 µl of 10× PCR buffer (Roche Diagnostics), 0.2 mM dNTPs (Roche Diagnostics), 4 µM primer and 1 U of Taq DNA polymerase (Roche Diagnostics) using the DC-960G Gradient Thermal Cycler (Corbett Research, Australia) under the following conditions: an initial cycle of 94°C for 1 min, 36° C for 20 sec and 72° C for 2 min, followed by 45 cycles: 10 sec at 94° C, 20 sec at 35° C, 2 min at 72° C with a final extension at 72° C for 5 min. The products were separated by electrophoresis on 1.4% agarose gel in 1× TAE buffer and visualized with ethidium bromide (0.5 µg/ml) under the UV light. The 1 Kb ladder (MBI Fermentas, Hanover, USA) was used as a molecular size marker. To verify the absence of contamination a negative control reaction was carried out along with each PCR amplification in which DNA was replaced by water. The amplifications were independently repeated numerous times using the same procedure in order to ensure that the amplifications obtained with the primers were reproducible and consistent.

Results and discussion

Initially 140 RAPD primers were used to amplify the bulk DNA of male and female individuals. Of these, 25 primers generated reproducible amplification patterns with a high degree of polymorphism in the amplification profiles. Random decamer OPD-20 (5′-ACCCGGTCA-3′) produced a unique band of ~850 base pairs from bulked female DNA; this band was absent among products from bulked male DNA. In addition to this, many other bands were generated in both male and female specimens. To confirm the female specificity of band ~850 bp, 31 male and 38 female individuals were tested independently. Interestingly this band was amplified only in females (Fig. 1 **A, B & C**).

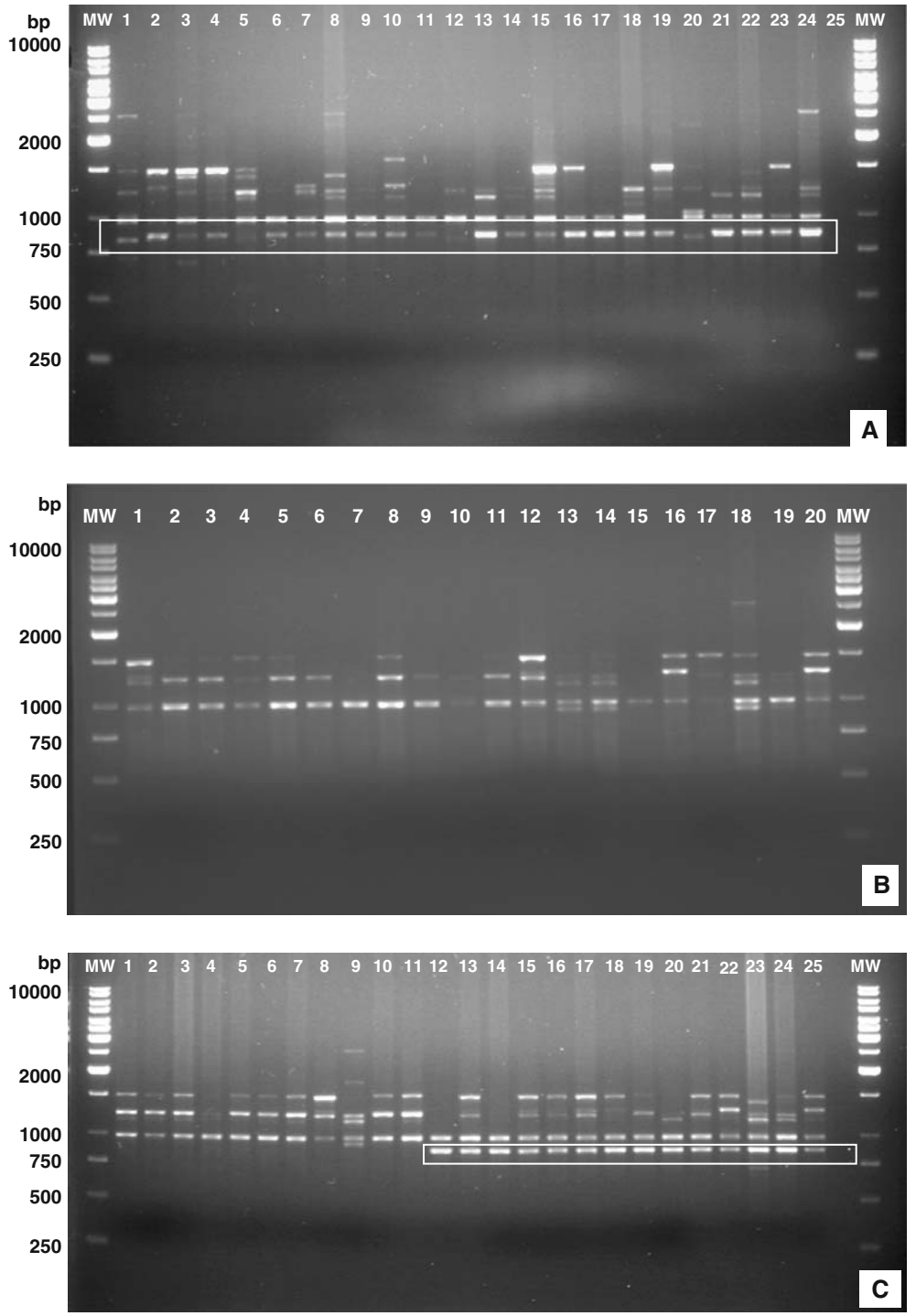

Fig. 1 Sex identification in *Encephalartos natalanesis*. **A–B**. Amplification profiles generated by a RAPD marker (OPD-20) in individuals collected from the areas adjoining the University of KwaZulu-Natal, Pietermaritzburg. PCR amplification profiles of genomic DNA from 24 female (**A**) and 20 male individuals (**B**). Lane 25 (**A**) is a negative control, where DNA was replaced with water. (**C**) PCR amplification profiles of 25 individuals collected from the Durban Botanical Garden, Durban. Lanes 1–11 represent male individuals; Lanes 12–25 are of female individuals. The DNA band indicated by a white box represents the unique female sex-linked band

The literature contains many reports highlighting the use of molecular markers such as RAPD, RFLP, AFLP, SSR, SCAR for gender identification in higher plants. Hormaza et al. (1994) reported a 945 bp band unique to females of *Pistachio vera*, on amplification with OPA-08. In *Piper longum*, two RAPD bands of 905 and 757 bp generated by OPA-10 and OPC-12, respectively were male-specific (Banerjee et al. 1999). Shirkot et al. (2002) reported six female specific and two male specific RAPD markers in *Actinida deliciosa*. Generation of sex-specific bands has not been restricted to RAPD primers, as male-specific bands were generated using AFLP primers in *Ficus fulva* and *Rumex nivalis* (Parrish et al. 2004; Stehlik and Blattner 2004).

To the best of our knowledge this is the first report, describing a method for sex identification in cycads. The results of the present work would be helpful to ascertain the sexuality of *E. natalensis* at the early seedling stage by commercial cycad growers as well as in formulating future breeding strategies. The approach adopted during this investigation could be applied to other *Encephalartos* species. Further investigations are underway to develop SCAR (Sequence Characterized Amplified Regions) markers using more species of *E. natalensis* from various geographical regions as well as a wide range of *Encephalartos* species.

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