Population genetics and conservation of critically small cycad populations: a case study of the Albany Cycad, *Encephalartos latifrons* (Lehmann)

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Declining populations of less than 250 mature individuals are symptomatic of many Critically Endangered cycads, which, globally, comprise the most threatened group of organisms as a result of collecting and habitat loss. Survival plans focus on law enforcement, reintroduction, and augmentation programmes using plants from the wild and botanical gardens. Augmentation is one of the few remaining options for cycad populations, although the assumed benefits remain untested and there is a possibility that augmentation from different sources could compromise the genetic integrity of existing populations, especially when garden plants have no provenance data. We studied *Encephalartos latifrons*, a South African endemic, which is a typical Critically Endangered cycad. We studied the extent and structure of genetic diversity in wild and *ex situ* populations to assess the potential benefits and risks associated with augmentation programmes. We examined 86 plants using amplified fragment length polymorphisms (AFLPs). The 417 AFLP markers thus generated yielded a unique DNA ‘fingerprint’ for each plant. Wild populations retain high levels of genetic diversity and this is reflected among the *ex situ* holdings at the Kirstenbosch Botanical Garden. No population differentiation is evident, indicating a single panmictic population, consistent with moderately high levels of gene flow between subpopulations and a sexual mode of reproduction. Bayesian clustering identified four genotype groups in the wild, as well as a genotype group only found in *ex situ* collections. Our results indicate that *E. latifrons* would benefit from augmentation programmes, including the use of undocumented collections, and careful management of breeding plants would increase the heterogeneity of propagules. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, 105, 293–308.

ADDITIONAL KEYWORDS: AFLP – genetic diversity – population assignment – population structure – South Africa.

INTRODUCTION

Cycads comprise a relatively small group of gymnosperms with 304 extant species distributed in tropical, subtropical, and warm temperate regions (Jones, 2002; Hill, Stevenson & Osborne, 2007). They are currently ranked as the most threatened group of organisms on the planet, with 62% of the known species (in Africa, Asia, Australia, South, and Central America) threatened with extinction (Hoffmann et al., 2010). Although the main reasons for their decline (habitat loss and collecting) are well known, the impacts of small population size and the implications for the survival and recovery of cycad populations are not well understood. Yet this is becoming an increasingly important issue in cycad conservation, with at
least 28 species comprising fewer than 250 mature individuals in the wild (Donaldson, 2003).

The IUCN/SSC Cycad Action Plan (Donaldson, 2003) identified a number of possible interventions for the management of very small cycad populations, including the introduction of seedlings from parent stocks in botanical gardens, artificial pollination of wild plants, and the translocation of plants to create viable subpopulations with balanced sex ratios. These interventions are based on the hypothesis that augmentation of wild populations will result in greater resilience to the impacts of small population size (Donaldson, 2003). This hypothesis assumes that augmentation will increase genetic variability and that there is a low risk, or at least a manageable risk, associated with the disruption of existing population genetic structure or the possible effects of outbreeding depression (Schaal & Leverich, 2004).

Small and restricted populations may not always result in extinction. Hamrick (2004) hypothesized that tree species may be resilient to the impact of small population size for two reasons. First, much of the genetic variation within tree species is found within rather than among populations so that even the extinction of some populations would result in relatively little overall loss of genetic diversity. Second, the longevity of individual trees and the potential for high rates of pollen flow should make tree species more persistent and less vulnerable to the loss of genetic diversity when populations decline. Consistent with this hypothesis, several studies of long-lived perennials have shown that species with small population sizes may have unexpectedly high levels of genetic variation (e.g. Castanopsis pumila var. pumila: Fu & Dane, 2003; Antirhea aromatica: González-Astorga & Castillo-Campos, 2004; Agave victoriae-reginae: Martínez-Palacios, Eguiarte & Furnier, 1999; Leucopogon obtectus: Zawko et al., 2001).

Although cycads are generally long-lived (Vovides, 1990; Raimondo & Donaldson, 2003) and could have relatively high rates of pollen flow associated with insect pollination (Donaldson, 1997; Terry, 2001; Terry et al., 2005, 2007), studies of genetic variation and genetic structure in cycad populations have given variable results. Studies of Asian Cycas have generally shown that genetic variation within populations is low, whereas differentiation among populations is high (Yang & Meierow, 1996; Keppel, 2002; Xiao et al., 2004, 2005; Xiao & Gong, 2006; Cibrán-Jaramillo et al., 2010), although studies of New World Dioon (González-Astorga et al., 2003, 2005, 2008; Cabrera-Toledo, González-Astorga & Vovides, 2008; Cabrera-Toledo et al., 2010) and Zamia (González-Astorga et al., 2006), as well as the Australian Macrozamia riedlii (Byrne & James, 1991), have shown high variation within populations and low interpopulation differentiation. These differences may be the result of a biological trait (e.g. pollination and dispersal systems), different evolutionary histories (González-Astorga et al., 2008; Cabrera-Toledo et al., 2010) or biogeography (e.g. island versus mainland populations: Keppel, 2002) and could influence the resilience of cycads to the effects of small population size. However, these studies have examined genetic structure and levels of genetic variation without specifically testing questions relating to the resilience and augmentation of small cycad populations.

In the present study, we examined the implications of genetic structure for the augmentation of critically small cycad populations, using a case study of the Critically Endangered Albany cycad, Encephalartos latifrons. Anecdotal evidence suggests that E. latifrons was naturally rare (Pearson, 1916; Chamberlain, 1919), although habitat loss and removal of adult plants from the wild by collectors has exacerbated the species’ natural rarity (Donaldson & Bösenberg, 1999) and has increased distances between plants. Fewer than 60 plants are known in the wild and population decline has also been associated with an increasingly male-biased sex ratio (4 : 1) (Daly et al., 2006), which may be a result of the targeted collecting of female plants or differential mortality of female plants. Augmentation from artificially pollinated wild plants and from garden sources has been identified as one of the few options that remain to prevent extinction in the wild (Daly et al., 2006).

This case study on E. latifrons offered an opportunity to test one of the recommendations of the Cycad Action Plan, which is that augmentation should be based only on plants of known provenance, preferably derived from population-based collections developed for this purpose (Donaldson, 2003). There are only limited population-based collections of E. latifrons, which are not yet producing seeds, although there is a relatively large collection of 18 mature plants of wild origin (but unknown provenance) at the Kirstenbosch National Botanical Garden in South Africa. The potential value of these plants for increasing genetic diversity of E. latifrons populations could therefore be assessed.

Various DNA analyses can be applied to conservation genetics and for the monitoring and regulation of threatened species (Stelling & van der Peijl, 2001). Our study required a method that would assess genetic variation and genetic structure, assign individuals to specific subpopulations and determine the contribution of individual plants to the gene pool of the wild population in a cost-effective and time-efficient manner. Of various options available (e.g. polymerase chain reaction (PCR) short tandem repeats: Gill et al., 1994; DNA sequencing: Sanger,
Encephalartos latifrons occurs in the Bathurst district of the Eastern Cape Province, South Africa. Plants have been recorded from at least five localities scattered within the original extent of occurrence, which is estimated to be 560 km² (Fig. 1). It is not known whether the populations had a naturally scattered distribution on steep hillsides and rocky outcrops (Kemp, 1986; Norstog & Nicholls, 1997; Whitelock, 2002), or whether this was a consequence of bush clearing by farmers who settled in the area from 1820 onwards. The growth form is characterized by a few arborescent stems with numerous suckers forming at the base. This is consistent with the ‘Type 2’ category of cycad life-history classification (Donaldson, Nänni & Bösenberg, 1995), indicating species capable of persisting over long periods as a result of the longevity of individual stems and vegetative production of numerous basal suckers. As with all cycads, E. latifrons is dioecious, although cone production is infrequent and plants may produce cones at >10-year intervals. There has been no evidence of recruitment in wild populations for several decades, nor has any viable seed been observed over the past 10 years (Whitelock, 2002). Although the natural pollinators of E. latifrons are unknown and may be locally extinct, several other species of Encephalartos are pollinated by beetles (Donaldson et al., 1995; Donaldson, 1997; Suinyuy, Donaldson & Johnson, 2009).

STUDY SITES AND SAMPLING PROCEDURE
We sampled every known individual from the natural geographical range of the species and from collections in Kirstenbosch Botanical Garden in Cape Town. One leaflet was collected from each individual of E. latifrons and either dried in silica gel before extraction (wild samples) or processed within a few hours (garden samples). The use of silica gel as a preservative has been shown to have no detrimental effects on the quality and quantity of DNA extracted from dried leaf material compared to DNA extracted from fresh tissue (Chase & Hills, 1991). In the wild, 51 samples were collected from 11 private farms or nature reserves. These sites represent three different conditions: seven sites contained only plants that occurred naturally in their current location (sites 5–11); three sites (sites 1–3) contained only translocated mature plants; and one site (site 4) contained only seedlings originating from artificial pollination of wild plants. Thirty-seven samples were collected from Kirstenbosch, comprising 18 adult plants, 18 seedlings originating from the cultivated plants, and one additional seedling that was donated to the garden.

DNA EXTRACTION AND AFLP
Total genomic DNA was isolated from leaf material using a 2 × cetethylammonium bromide protocol with 2% PVP modified from Doyle & Doyle (1987).

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AFLP markers were resolved according to the AFLP plant mapping protocol from Applied Biosystems Inc. We used the ABI AFLP Regular Plant Genome Module kit optimized for a genome of 500–6000 Mb. We made a slight modification to the plant mapping protocol by replacing the restriction enzyme MseI with Tru9I (an isoschizomer of MseI) in the restriction-ligation phase of the procedure. All amplification steps were carried out in an ABI GeneAmp® PCR System 9700 thermocycler. Primer combination MseI-CTA/EcoRI-ACT (FAM-labelled EcoRI fragments) was used as the selective primer pair based on a previous primer trial carried out with 28 different primer combinations for the genus Encephalartos (Reeves et al., 2003). Selective amplification products were run on a denaturing polyacrylamide gel and analyzed on an automated DNA Sequencer (ABI Prism™ 377 DNA Sequencer). To assess reproducibility of the AFLP procedure, we re-extracted DNA from five individuals and repeated the AFLP procedure. At the end of the study, all DNA was purified using caesium chloride/ethidium bromide density gradients and deposited in the South African National Biodiversity Institute’s plant DNA bank.

**STATISTICAL ANALYSIS**

AFLP fragments were analyzed using GENESCAN, version 3.2.1 and GENOTYPER, version 2.5 from ABI. We scored AFLP profiles for the presence (1) or absence (0) of bands between 50 and 500 bp. To verify assignment, all fragment profiles were edited by visual inspection. Two wild plants were excluded from the analysis because of insufficient DNA and ambiguous amplification results. Consequently, data from 86 individuals were analyzed.

We quantified genetic diversity as: (1) the percentage of within-population polymorphic loci; (2) Shannon’s information index of diversity (Shannon & Weaver, 1949), and (3) Nei’s gene diversity (i.e. genetic identity and distance; Nei, 1973). The Shannon index measures allelic diversity within populations, taking into account the evenness in relative abundance of alleles. Nei’s index, however, calculates the average proportion of heterozygotes per locus in a population under the assumption of Hardy–Weinberg equilibrium. These genetic diversity indices were estimated separately for each locus and averaged from allele frequency data using POPGENE,
version 1.32 (Yeh et al., 1997). Percent polymorphic loci estimates were based on a 99% confidence interval that loci were not fixed for one allele.

\(F_{ST}\) values, and their corresponding significance values, were calculated using the program ARELQUIN, version 2.00 (Schneider, Roessli & Excoffier, 2000). Slatkin’s (1995) linearized \(F_{ST}\) statistic, which is based on an infinite alleles model, was obtained by analysis of molecular variance (AMOVA) to determine the partitioning of the total genetic variation. Significance levels were determined by 1023 permutations of AFLP haplotypes for each hierarchical level. Additionally, \(F_{ST}\) estimates were determined by 3024 permutations of AFLP haplotypes among subpopulations.

We used the programme STRUCTURE (Pritchard, Stephens & Donnelly, 2000) to examine genetic structure of the total population and assign individuals to inferred population clusters. STRUCTURE, version 2.2 was used to better account for the genotypic ambiguity inherent in dominant markers, such as AFLPs, in polyploids, which could not be adequately addressed in earlier versions of the software (Falush, Stephens & Pritchard, 2007). The optimal number of populations (K) is identified when the probability of giving rise to the observed genotypic distribution is maximized (Pritchard et al., 2000). In this test, values of K ranged between 1 (all plants make up a single population) and 10 (each farm/nature reserve sampled is a separate subpopulation). Batch runs were performed using: (1) only the wild plants and (2) all plants. Individuals in the sample were assigned probabilistically to populations using a burn-in period of 50 000 iterations and 10\(^6\) Markov chain Monte Carlo (MCMC) generations. Five independent MCMC realizations of this length were run for three K-values; the runs produced highly consistent results. This method assumes that within populations, the loci are at Hardy–Weinberg equilibrium and the markers are unlinked (Pritchard et al., 2000; Falush et al., 2007).

Principal coordinate analysis (PCOA), as implemented in Numerical Taxonomy System (NTSYS-pc), version 2.1 (Rohlf, 2000), was used to examine overall patterns of genetic similarity amongst all sampled individuals. Pairwise similarities among individuals were calculated using Jaccard's (1908) and simple matching coefficients (Sokal & Michener, 1958). The similarity matrices were also clustered using the unweighted pair group method with arithmetic mean (UPGMA) algorithm, as implemented in NTSYS-pc.

Genetic structure at the subpopulation level was evaluated for wild individuals by AMOVA (Excoffier, Smouse & Quattro, 1992) as performed in ARELQUIN, version 2.00 (Schneider et al., 2000). This analysis is based on hierarchical variance of gene frequencies; however, in the case of dominant markers, AMOVA partitions the genotype variation and not the variation of allele frequencies. When using AMOVA with dominant markers, it has to be assumed that there is the same mating pattern in all subpopulations (Tero et al., 2003). Two regional groups of subpopulations were considered (Fig. 1): North and South. Two sets of analyses were run to include: (1) all wild sites (North: sites 5, 6, and 7; South: sites 9, 10, and 11) and (2) only wild sites with \(N > 1\) (North: site 5; South: sites 10 & 11). We tested for significant variation at three hierarchically nested levels: (1) among populations (i.e. between northern and southern regions); (2) among subpopulations [i.e. among sites (farms) within each region]; and (3) within subpopulations. A simplified estimate of Wright’s (1951) \(F_{ST}\) statistic was obtained by AMOVA to determine the partitioning of the total genetic variation into within and between populations. \(F_{ST}\) was calculated for all three hierarchical levels, leading to estimates of \(F_{CT}\) (\(F\)-statistic among populations), \(F_{SC}\) (among subpopulations, within populations), and \(F_{ST}\) (within subpopulations). As above, significance levels were determined by 1023 permutations of AFLP haplotypes for each hierarchical level. Additionally, \(F_{ST}\) estimates were determined by 3024 permutations of AFLP haplotypes among subpopulations.

RESULTS

In total, 417 AFLP markers were scored for 86 individuals. Despite minor band differences in peak height, profiles were found to be identical (the replicated profiles possessed the same peaks, both in number and position), as observed with the separate extractions of the five tested individuals; thus illustrating the high reproducibility of these markers for this species.

GENETIC DIVERSITY

The in situ and ex situ populations exhibited similar levels of genetic variability, with 84% and 81% polymorphic loci, respectively, and similar values of both Nei’s and Shannon’s diversity indices (Table 1). For all three measures of heterozygosity, slightly lower values were observed within the Kirstenbosch plants. In both in situ and ex situ populations, mature plants possessed higher levels of diversity than their artificially propagated seedlings. The extent of this differentiation was more apparent between the seedlings and adult plants in the wild (Table 1).

NATURAL POPULATION

When examining the genetic diversity within the natural population, only sites 10 and 11 were considered because too few plants occurred at the
other sites (N = 1 for sites 6, 7 and 9; and N = 2 for site 5). Of these two, site 11 has the highest level of variation (H = 0.1684 and P = 62.11%). Site 10 has only half the proportion of polymorphic loci as the other groups considered, although it has comparable values for the other two measures of diversity. This apparent discrepancy probably results from the well-known effects of sample size on estimates of percent polymorphism (Chakraborty, 1984). Furthermore, both the other indices include a measure of evenness of allelic frequencies.

**Population Structure**

The Bayesian clustering approach used in STRUCTURE indicates that division into four and five ‘genotypic subpopulations’ for the wild and when considering all individuals, respectively, maximizes the likelihood (Wild: –lnL = –6329.5; All: –lnL = –11 193.4) of giving rise to the observed allelic frequencies among the extant individuals of *E. latifrons*. This was determined using the method outlined by Evanno, Regnaut & Goudet (2005), which calculates ‘an ad hoc quantity based on the second order rate of change of the likelihood function with respect to K (ΔK)’. Despite the implied division into four subpopulations, the vast majority of individuals reveal admixture implying weak differentiation.

Each of the four implied genotype groups found in the wild is represented in the Kirstenbosch collection. When considering all individuals in the analysis, an additional cluster was detected, which, upon further inspection, was made up solely of Kirstenbosch individuals exhibiting a full complement of the wild genotypes. This is evident in the PCOA (Fig. 2, dashed line).

The PCOA revealed similar spatial patterns as the STRUCTURE analysis with few differences in individual clustering irrespective of whether Jaccard’s or simple matching coefficients were used. The first and second principal coordinates accounted, respectively, for 9.7% and 7.2% of the variation among plants using Jaccard’s similarity coefficient. Slightly higher percentages were achieved with the simple matching coefficient, with the first and second principal coordinates explaining 14.2% and 9.1% of the variation. A genetically and spatially distinct cluster identified by the PCOAs is group D, which is comprised predominantly of individuals from site 11.

Some genotypic groups could be assigned to specific wild localities. Group C is unique in that its distribution is limited to only two individuals, both originally from site 10. Similarly, the samples assigned to group D originate from site 11. The translocation of a small number of plants from these sites means that these genotypes are now also represented at site 3 and site 2, respectively. Group B is made up of individuals from natural populations at sites 1, 3, and 10; and is also present at site 4, which comprises seedlings cross fertilized from plants from site 11 and adjacent farms. The majority of individuals in Group A are localized to the south of the species’ range, particularly sites 3, 10, and 11 (Fig. 1).

The ordination analyses were complemented by UPGMA clustering of individuals (Fig. 3: using Jaccard’s coefficient; UPGMA-simple matching not shown). The cophenetic correlations showed high levels of fit for both similarity measures to the original matrices (UPGMA–Jaccard: r = 0.86; UPGMA–simple matching: r = 0.88) suggesting that little distortion occurred (Sneath & Sokal, 1973). The two dendrograms showed only minor differences for the
four main genotype groups (A–D). In the simple matching analysis group A divided into two clusters, with one being most similar genetically to group C (similarity coefficient of approximately 0.93).

Both AMOVAs examining a North–South distribution among wild sites [(1) all wild sites and (2) wild site with \( N > 1 \)] revealed similar results, accordingly only results pertaining to (2) are reported (Table 2). No significant variation among populations (\( F_{CT} = -0.01; P = 1.00 \); proportion variance = −1.00%) and subpopulations (\( F_{SC} = 0.058; P = 0.079 \); proportion variance = 5.90%) was apparent, whereas the within subpopulation component accounted for the majority of variation (95.10%). Pairwise \( F_{ST} \) between subpopulations varied from 0.03 (sites 5 and 10) to 0.11 (sites 5 and 11). Only one pairwise comparison was considered significant at \( P < 0.05 \) (sites 5 and 10).

**Table 2.** Analysis of molecular variance for 21 individuals of *Encephalartos latifrons* from three sites (\( N > 1 \)) using 417 amplified fragment length polymorphism markers

| Source of variation                     | d. f. | Sum of squares | Variance components | Percentage of variation | \( P \)   |
|-----------------------------------------|-------|----------------|---------------------|------------------------|----------|
| Among populations (\( F_{CT} \)) North–South | 1     | 46.86          | −0.43               | −1.00                  | 0.500    |
| Among subpopulations/within populations (\( F_{SC} \)) | 1     | 65.97          | 2.56                | 5.90                   | 0.026*   |
| Within subpopulations (\( F_{ST} \)) Individual field sites | 19    | 785.58         | 41.35               | 95.10                  | <0.002*  |
| Total                                   | 21    | 898.41         | 43.48               |                        |          |

A North–South regional structure is implied. Statistics include sum of squares, variance component estimates, and the percentage of variation explained by three different hierarchical levels. \( P \)-values were calculated based on 1023 permutations. *Significant \( F_{ST} \) values based on a significance level of 0.050.

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DISCUSSION

The present study set out to use AFLP characterization to examine the genetic structuring in *E. latifrons* as a basis for answering questions relating to the survival and recovery of critically small cycad populations. The results show that AFLP characterization is feasible for *E. latifrons* and that the profiles provide unique DNA fingerprints. Additionally, the markers were effective and efficient at providing estimates of genetic similarity and diversity, as well as providing insight into the species’ population structure. We discuss some methodological caveats and the implications of our results for cycad conservation.

METHODOLOGICAL CAVEATS

A major assumption made in the analyses is that populations are in Hardy–Weinberg equilibrium. To obtain accurate estimations of null alleles from dominant markers (e.g., AFLPs or random amplified polymorphic DNA), this assumption must be made, or known departures (e.g. from codominant data) must

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**Figure 3.** Unweighted pair group method with arithmetic mean cluster analysis of Jaccard’s Similarity coefficients calculated on the basis of 417 amplified fragment length polymorphism comparisons among 86 individuals of *Encephalartos latifrons*. Clusters A–D represent the four distinct genotypes present within the population. The cluster denoted by the dashed line groups individuals from Kirstenbosch sharing a similar admixture of genotypes. Numbers denote the sites where the individual plants were collected (Fig. 1). K, Kirstenbosch Botanical Garden.
be incorporated into the estimation procedure (Lynch & Milligan, 1994; Zhivotovsky, 1999; Krauss, 2000a). Although it is uncertain whether E. latifrons is in Hardy–Weinberg equilibrium (Page & Holmes, 1998), cycads are dioecious and must represent outcrossed populations. Previous studies examining natural outcrossing plant populations with the use of dominant markers have shown no departure from Hardy–Weinberg equilibrium (Page & Holmes, 1998), and any departures are thought to be generally modest for dominant markers in outcrossing species (Krauss, 2000a). The questions posed in this study require only broad knowledge of general patterns of genetic variation and departures from Hardy–Weinberg equilibrium are not likely to greatly affect these. In addition, such departures should lead to overestimation of structure; thus, with respect to our conclusions, the analytical results should be conservative.

**GENETIC DIVERSITY AND POPULATION STRUCTURE**

The main objective of conservation genetics is to preserve species as dynamic entities, capable of evolving and adapting to environmental change (Frankham, Ballou & Briscoe, 2002). In the long term, genetic diversity helps to maintain adaptive evolutionary potential, and low levels of diversity may be associated with increased likelihood of extinction (Lande, 1988; Frankham et al., 2002). In the short term, genetic diversity helps maintain reproductive fitness; thus, low genetic diversity has been linked directly to the level of inbreeding within a population, leading to reductions in survival and reproductive success (Reed & Frankham, 2001).

In the present study, both in situ and ex situ populations of E. latifrons had very high proportions of polymorphic loci, moderate to high Nei’s and Shannon’s diversity indices, as well as moderate levels of heterozygosity (Tables 1, 2) in comparison to published values based on AFLP markers for a range of other endangered and threatened plant species, including Astragalus cremnophylax (Travis et al., 1996), Eryngium alpinum (Gaudeul, Taberlet & Till-Bottraud, 2000), Persoonia mollis (Krauss, 2000a, b), Rubus articus (Lindqvist-Kreuze, Koponen & Valkonen, 2003), and Medicago citrina (Juan et al., 2004).

No measures of genetic diversity exist for close relatives of E. latifrons; and of the studies that have been conducted on other cycad species, codominant markers were used, with the exception of three studies that used dominant inter-simple sequence repeat techniques (Xiao et al., 2004, 2005; Xiao & Gong, 2006). The ability to compare studies using markers of different dominance has been investigated in a few studies and different outcomes have been observed with dominant and codominant markers showing similar (Peakall, Smouse & Huff, 1995; Aagaard, Krutovskii & Strauss, 1998) and different levels of diversity (David et al., 2007).

A major limitation of dominant markers is their low information content, and hence accuracy (Guillot & Carpentier-Skandalis, 2011) as a result of their inability to discriminate between homozygotes and heterozygotes. Another possible limitation is low levels of polymorphism since only two alleles are available per locus, as opposed to > 10 alleles for many microsatellite loci (Bensch & Åkesson, 2005). In an attempt to overcome these limitations, some studies recommend increasing the number of dominant polymorphic markers (Bensch & Åkesson, 2005; Guillot & Carpentier-Skandalis, 2011). Guillot & Carpentier-Skandalis (2011) even found that dominant markers can achieve accuracy higher than that of codominant markers as long as the numbers of loci used satisfy a particular equation ($L_{\lambda} = \lambda L$, where $\lambda = \ln(5/8)/\ln(25/33) = 1.69$). Given that several hundred polymorphic loci were investigated in the present study and they were found to be highly polymorphic, adequate comparisons can be made to previous studies using either codominant or dominant markers.

Accordingly, studies of a few cycad species have shown that genetic variation within populations is generally low, while differentiation among populations is generally high (Ellstrand, Ornduff & Clegg, 1990; Walters & Decker-Waters, 1991; Yang & Meerow, 1996; Sharma et al., 1999; González-Astorga et al., 2003, 2005, 2006; Xiao et al., 2004, 2005; Xiao & Gong, 2006; Cibrián-Jaramillo et al., 2010), in contrast to the results reported here (Table 2). Ellstrand et al. (1990) suggested that limited seed dispersal constrains genetic variation in Macrozamia communis, whereas Yang & Meerow (1996) suggest geographical isolation as a cause of the high levels of interpopulation variation in Zamia pumila. Yet E. latifrons has both limited seed dispersal as a result of infrequent and unsynchronized coning events, and populations appear to have been historically isolated. The only cycads showing results similar to ours is Macrozamia riedlei (Byrne & James, 1991) and some New World Dioon (González-Astorga et al., 2003, 2005, 2008; Cabrera-Toledo et al., 2008, 2010) and Zamia (González-Astorga et al., 2006) species. Low population differentiation was attributed to either limited or no inbreeding or a potential heterozygote advantage. High within population differentiation could be explained by the slow rate of genetic diversity loss expected from long-lived species with long generation times (González-Astorga & Castilló-Campos, 2004; Feyissa et al., 2007; Cabrera-Toledo...
et al., 2008). The lapsed time between a habitat disturbance, which may lead to a reduction in population size, and actual time is short compared to the longevity and generation time of the species (Feyissa et al., 2007). It is difficult to explain the ‘true’ cause of the high variation within sites and low variation among sites observed for E. latifrons; however, the results do imply a sexual mode of reproduction, at least historically (Liu, Zhgang & Charlesworth, 1998; Keiper & McConchlie, 2000; Frankham et al., 2002). Additionally, the low \( F_{ST} \) values (Table 1) suggest historically high rates of gene flow among sites and indicate that all individuals belong to a single, effectively panmictic, population. The present population appears to be a relict of what was likely a much larger historical population. The present population appears to be a relict of what was likely a much larger historical population that thrived when environmental conditions were different from those of present.

The current populations comprise mostly large mature plants, and there appears to have been no recruitment within the past century. Insect pollination is common in cycads (Donaldson, 1995; Norstog et al., 1995; Oberprieler, 1995; Norstog & Nicholls, 1997), and beetle pollination occurs in at least three species of Encephalartos (Donaldson et al., 1995; Donaldson, 1997). No seed set has been recorded in any E. latifrons populations in recent years (White-lock, 2002), which suggests that either the distance between individuals of E. latifrons is now too great for pollinators or that pollinators are locally extinct. The implication is that most of the wild plants are older than 100 years and represent the genetic diversity from that time. The genetic effects of greater isolation because of habitat loss and over-collecting may not be apparent as a result of the lack of recruitment.

Despite the absence of between-population structure, some significant variation exists at the subpopulation level, although the overall proportion is tiny (Table 2). Together, the first two principle components account for approximately 17% of the overall covariation among markers, which is a relatively high proportion among loci, suggesting rather strong demographic history. Four distinct genotypic groups were detected in the wild, and two groups were confined to a single site each (i.e. group C at site 10 and group D at site 11). These groups were separated from groups A and B along the second factor in the PCOA (Fig. 2), presumably as a result of the shared absence of many fragments and the relatively large size of groups A and B. Groups C and D also form the basal groups in the cluster analysis (Fig. 3), suggesting they are the maternal genotypes and the likely source of the other in situ plants. Several other genotype groups were also present at site 11, meaning that this site has most of the genetic variation remaining in the wild (as identified in Table 1) and it should be considered an area of high conservation priority.

A goal for threatened plant conservation is to maintain genetic diversity of native plant species (Montalvo et al., 1997), and ex situ collections, such as botanical gardens, have become a key means of achieving this. Until recently, assessing the actual contribution or conservation value of such collections has proven very difficult (Schaal & Leverich, 2004). Namoff et al. (2010) devised a model for assessing the number of plants needed in a botanical garden to maintain genetic diversity of a population. Using a rare palm, Leucothrinax morrisii, as their model, they found that the natural population and ex situ collection at Montgomery Botanical Centre had similar diversity metrics. Their models revealed that as the collection size increases, so does the percent allelic diversity captured, with ten or more individuals likely to account for 80% of allelic diversity. Also, increasing the accession breadth (i.e. half-sibling groups) can lead to an increased allelic capture, albeit to a lesser extent.

As above, the ex situ collection at Kirstenbosch has similar levels of genetic diversity to wild stocks (Table 1), indicating that the number of plants in the garden should be sufficient to maintain the genetic diversity of the species. The Kirstenbosch plants were also found to represent all wild genotype groups (a key attribute of any ex situ collection) (Donaldson, 2003). Because the diversity in the wild appears to be fully represented in the Kirstenbosch collection, genotypic groups with very limited geographical ranges in the wild can be preserved.

Figure 2 highlights a group of plants from Kirstenbosch that the UPGMA analysis revealed to be most genetically similar to groups C and D (Fig. 3). These individuals include two adults (one male and one female collected from the wild, though their origins are unknown) and five seedlings cultivated at Kirstenbosch that share a mix of genotype groups, although groups C and D are more prevalent. With random sampling in the garden (acting as a form of genetic drift), this unique combination of genotype groups has been preserved. These individuals, and their offspring, can be used to supplement the wild population either through artificial fertilization or translocation. Managing breeding projects involving these plants and those in groups A and B may act to increase the accession breadth, thereby further increasing the overall genetic diversity of the progeny and the population as a whole.

Although the Kirstenbosch collection possesses similar levels of genetic variation compared to the wild, seedlings from Kirstenbosch were found to have much lower proportions of polymorphic loci and lower values for both Nei’s and Shannon’s measures of
diversity compared to their parent stock (Table 1). These statistics were shown to be more severe when seedlings and parent stock from the wild were compared. Rucińska & Puchalski (2011) experienced similar results in their study of a narrowly endemic plant now extinct in the wild, Cochlearia polonica. They attributed their results to repeated founder effects and high rates of genetic drift leading to the fixation of alleles (Lande, 1994). Given that there is a limited number of individuals in the wild and at Kirstenbosch contributing pollen and seed for artificial propagation, a similar explanation can be offered.

Despite the lower diversity metrics found in Kirstenbosch seedlings compared to their parent stock, a low $F_{ST}$ was observed, suggesting an adequate level of gene flow is still being achieved through the artificial fertilization projects currently under way. The fixation index observed for seedlings cultivated from wild plants is slightly higher, suggesting more alleles are reaching fixation. Based on the results reported in the present study, artificial fertilization projects need to be monitored to prevent the loss of genetic integrity at the same time as maintaining adequate levels of gene flow. Ideally, additional individuals should contribute seed and pollen to ensure the full complement of genotype groups is being represented. In the case of cultivation using wild plants, individuals from more diverse localities should be included, if possible.

**RE-INTRODUCTION AND TRANSLOCATION**

A primary objective of threatened species management is to increase effective population size so that stochastic environmental, demographic, and genetic threats are minimized (Given, 1994; Frankham et al., 2002). In the case of *E. latifrons*, this can be achieved by introducing seedlings propagated from plants in botanical gardens or artificially pollinated wild plants, or by relocating suckers from plants with multiple stems. Key questions that must be addressed before any of these methods can be used include whether plants and propagules from different sources can be mixed without compromising the genetic structure of the population.

Although we did not specifically investigate the effects of inbreeding or outbreeding depression on *E. latifrons*, the AFLP data reported in the present study can inform decisions regarding population management. First, the conclusion that surviving individuals represent the remnants of a single panmictic population indicates that suckers, seedlings, and pollen can be transferred between populations without reducing fitness, based on the premise that even low migration rates in panmictic plant populations would prevent divergence through genetic drift or selection (Lawrence & Marshall, 1997). In the case of *E. latifrons*, this means that all the reproductive plants can be used in a breeding programme without trying to manage subpopulations separately.

We also identified at least four genotypic groups that are not evenly spread among the surviving populations. The breeding programme therefore needs to ensure that all the genotypes are represented. Plants from Kirstenbosch are particularly important because they include one genotype group that is extinct in nature. However, breeding programmes need to focus on combinations that maintain genetic diversity in the F1 generation. Fingerprinting each seedling and conducting structure analyses, as highlighted in the Material and methods, can help distinguish which genotype groups get passed on to each seedling from the parental stock. Although dominant markers, such as AFLPs, cannot distinguish between homozygous and heterozygous individuals, which is typically key in breeding programmes, research aimed at extracting codominant information from dominant markers is being conducted (Piepho & Koch, 2000). Subsequent studies can determine whether such techniques are suitable for identifying heterozygosity in *E. latifrons*.

Our data show that reduced diversity and increases in kinship may occur when particular plants cede more frequently than others so that they are always used for breeding (as appears to occur at Kirstenbosch) effectively resulting in a few plants acting as a founder population. The results obtained in the present study suggest that *in situ* and *ex situ* pollen sources could be used to fertilize female cones and in this way balance the genetic conflict between increasing the effective population size and minimizing the loss of genetic diversity (Krauss, Dixon & Dixon, 2002).

The long-term goal of recreating self-sustaining populations of *E. latifrons* assumes that the processes of pollination and dispersal are intact or can be re-established. Studies are underway to survey pollinators and to determine the host specificity of pollinators in the Albany region. Results so far indicate that weevil pollinators (*Porthetes* spp.) have a narrow host range (Downie, Donaldson & Oberprieler, 2008) but that other pollinator beetles in the families Boganidae and Erotylidae have a wider host range that may include *E. latifrons* (J. Donaldson, unpubl. data). Seed dispersal by medium to large birds (e.g. starlings, hornbills) has been observed in the same area on *Encephalartos altensteinii*, and dispersal by animals (rodents, monkeys, baboons) that feed opportunistically on cycads has been observed in several Eastern Cape cycads (e.g. *Encephalartos cycadifolius*, *Encephalartos friderici-guilielmi*, *Encephalartos*...
longifolius, and Encephalartos villosus: J. Donaldson, unpubl. data). At least some of these dispersal agents are still present in *E. latifrons* habitat.

**CONCLUSIONS**

Our results have several implications for the conservation of critically small cycad populations. First, the present study has revealed the existence of high levels of genetic diversity within the species, as well as weak genetic structure. These findings suggest that remnant subpopulations were originally a single panmictic population, with historically high levels of gene flow. This could be important for other species with an originally small extent of occurrence (EOO). The estimated EOO for *E. latifrons* is 560 km² and at least 49 other cycads have EOoS less than 600 km² (IUCN, 2010).

Second, the results highlight the importance of *ex situ* collections, even those of unknown provenance. Many other *E. latifrons* plants exist in private and public collections that were not included in the present study as a result of the absence of locality and generation information; however, these plants should be surveyed as part of a restoration initiative of wild populations of *E. latifrons*. The mature plants at Kirstenbosch, which were mostly acquired from unknown wild localities before 1916, contain genotype groups that are either rare or extinct in nature. The garden plants are therefore an important source for population augmentation. The IUCN/SSC Cycad Action Plan (Donaldson, 2003) proposed that only *ex situ* collections based on genetic sampling of extant populations should be used for augmentation programmes, to overcome the problem of unknown provenance. Although this is appropriate for large populations, the present study indicates that this recommendation should be revised for small populations that may already have lost significant segments of the original gene pool. Where collecting is a major cause of decline, important genetic material may be found in public and private collections where the provenance is not always known. If plants are known to be of wild origin, then genetic techniques can be used to assess their potential contribution to recovery efforts.

Third, augmentation from seed stocks requires careful management of artificial pollination. Our results show that even the *F₁* generation seedlings in botanic gardens can have lower levels of heterozygosity than the parent generation as a result of over reliance on a few individuals for seed production. Stud books are commonly used in animal conservation programmes to manage reproduction and similar approaches need to be applied in cycad conservation.

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