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Sex determination of South Africa's *Encephalartos* - A conservation perspective

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ABSTRACT

Species within *Encephalartos* Lehm., the largest cycad genus in Africa, face considerable threats from habitat loss and illegal collection. Living collections in botanical gardens play a crucial role in conserving these slow-growing plants, necessitating the presence of both male (microsporangiate) and female (megasporangiate) individuals for successful sexual reproduction. Recent genomic research has identified sex-specific genes in cycads, offering an opportunity for sex determination in *ex situ* collections. This study tested the utility of the *CYCAS_034085* gene, found exclusively in microsporangiate plants, for identifying the sex of *Encephalartos* individuals using a simple qualitative PCR assay. A total of 88 accessions representing six *Encephalartos* species were collected from Kirstenbosch National Botanical Garden. The results demonstrated that the developed primer combination effectively differentiated between microsporangiate and megasporangiate plants, achieving an accuracy of 86.3 %. This PCR-based sex determination method represents a valuable tool for cycad conservation management and the establishment of effective *ex situ* collections.

1. Introduction

Encephalartos (Zamiaceae) is the largest cycad genus endemic to Africa, with around 65 species currently recognized (Stewart et al., 2023). Nine species are represented in northern Africa, 11 in central Africa, and 45 south of the Zambezi River, of which 37 are found in South Africa (Mankga et al., 2020) – accounting for more than half of the number of described species of *Encephalartos* (Williamson et al., 2016). Species occur in three main habitat types: woodland, grassland, and savannah (Bamigboye and Tshisikhawe, 2022). *Encephalartos* species are all long-lived; the typical adult life span varies from around 150 years for species with a subterranean stem (e.g., *Encephalartos villosus* Lem.) to ≥ 1000 years for multi-stemmed species (e.g., *Encephalartos cycadifolius* (Jacq.) Lehm.) (Raimondo and Donaldson, 2003).

More than 60 % of the world's cycad species are currently considered endangered (IUCN, 2022) – a consequence of their slow growth and reproductive cycles, susceptibility to rapid change in climatic conditions, habitat loss, and their appeal among plant collectors (Donaldson, 2003). These factors have contributed to cycad populations being highly fragmented and often isolated (Cousins and Witkowski, 2017). The horticultural value of many *Encephalartos* species has garnered interest from all over the world, and collectors have been known to pay exorbitant prices to obtain species (Cousins et al., 2011). This has led to significant poaching of plants from wild populations – making the horticultural trade one of the greatest threats to *Encephalartos* populations (Donaldson, 2003). Additionally, there are threats from the traditional medicinal markets in South Africa, where *Encephalartos* bark strips, leaf bases connected to an underlying layer of cortical tissue, as well as parts of complete stems and roots are sold for medicine (Cousins and Witkowski, 2017). Due to these threats posed to *Encephalartos* and other cycad genera, it has been crucial to prioritize conservation efforts in conjunction with research to help understand and safeguard these vulnerable species.

The *ex situ* conservation of plants has become critical to the long-term survival of many plant species, including cycads. Methods for *ex situ* conservation of genetic resources include plant cultivation and

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propagation in botanical gardens, as well as utilizing germplasm collections in the form of seeds (Griffith et al., 2015). Although cycad pollen can be frozen and stored, cycad seeds appear to be recalcitrant i.e. desiccation-sensitive (Woodenberg et al. 2007) and are only viable for up to two years post-harvest (Nadarajan et al., 2018). Therefore, the only effective way to conserve the genetic diversity of cycads and their populations is through a combination of in situ and *ex situ* conservation (curated botanic garden collections; Calonje et al., 2011). In *ex situ* conservation collections of botanic gardens, optimal population structure with an even sex ratio is imperative to maximally utilize the often-limited resources that are available while still being effective at conserving genetic diversity. Achieving this for cycads is further complicated by the fact that all species are dioecious, and thus, balanced sex ratios are needed to maintain a viable, seed-producing population in both in situ and *ex situ* populations. Prior to a cycad's first strobilus production, there are no diagnostic sex-specific morphological characters that can be easily used to differentiate between microsporangiate and megasporangiate plants (Clugston and Kenicer, 2022). Additionally, the age at which cycads mature varies greatly between species, with some species in the genus *Zamia* L. taking around two to three years to mature from seed, while growers have been known to wait 20 years or more for some species of *Encephalartos* (Raimondo and Donaldson, 2003). This poses a particular issue for the living collections of botanic gardens, as storing large numbers of cycads in an attempt to obtain a good balance of sex ratios is not easy from a space perspective (Griffith et al., 2015).

Sex determination of cycads was originally assumed to be based on the sex-linked chromosomes (Segawa et al., 1971). Early attempts to sex cycads without the need for cone morphology included observing chromosome variances to differentiate between male and female plants, but this was largely unsuccessful (Abraham and Mathew, 1962). These findings, together with several observations of sex changes occurring in cycads that were most often preceded by major environmental changes (drought and cold) or substantial physical damage (see Osborne and Gorelick, 2007), lead to the hypothesis that sexual differentiation in cycads was controlled by differential DNA methylation between females and males, i.e., sex was epigenetically determined. Later, Sultana et al. (2016) compared sex expression in cycads from various published molecular methods and concluded that sex determination in cycads is likely due to DNA methylation and not sex-specific markers. Nevertheless, to date, attempts to alter the sex of cycads purposefully have largely been unsuccessful (Gorelick and Osborne, 2002).

Recently, Liu et al. (2022) published the first cycad genome of the Chinese *Cycas panzhihuaensis* L. Zhou & S. Y. Yang (the first cycad full genome), which finally solved the puzzle of cycad sex determination (Clugston and Kenicer, 2022). The authors used sex as a binary phenotype in a genome-wide association analysis (GWAS) and discovered two genes related to XY sex determination found on chromosomes 2 and 8. These two genes are involved in the initiation of female (the *CYCAS_010388* gene on chromosome 2) and male (the *CYCAS_034085* gene on chromosome 8) strobili, respectively. Liu et al. (2022) determined that megasporangiate plants exclusively possessed the *CYCAS_010388* gene, while microsporangiate plants had both the *CYCAS_034085* and the *CYCAS_010388* gene. Microsporangiate plants, therefore, have the necessary genetic makeup to generate both microsporangiate and megasporangiate strobili (Clugston and Kenicer, 2022). A set of primers was subsequently developed for amplifying homologues to these markers, and they were tested across multiple genera (*Cycas* L., *Macrozamia* Miq., and *Zamia*) representing both cycad families (Cycadaceae and Zamiaceae; Liu et al., 2022). All tested male cycad samples yielded a male-specific polymerase chain reaction (PCR) product of *CYCAS_034085*; however, this product was not detected in female samples. Conversely, both male and female cycad samples yielded a PCR product of *CYCAS_010388*. Because *CYCAS_034085* is exclusively expressed in males and due to its presence in the male-specific region of chromosome 8, Lui et al. (2022) designated it as *MADS-Y* – a putative gene that can be used for sex determination in cycads by employing a simple qualitative PCR assay. Unfortunately, its utility was not demonstrated across all cycad genera, but the presence of *GbMADS4* (a homologue of the *Cycas MADS-Y*) in *Ginkgo* L. male-specific contigs suggests that the same mechanism for sex determination might have originated before the split of Cycadales and Ginkgoales. This, in turn, implies that *MADS-Y* would likely be useful for PCR-based sexing across the order. Further testing is, however, required for confirmation. This study aims to determine i) if the *CYCAS_034085* gene can be used to accurately sex species in the genus *Encephalartos* (Zamiaceae) using a simple PCR-based test, and ii) if PCR-based sex designations correspond with strobilus morphology-based observations.

2. Methods

2.1. Sample collection

Fresh silica gel dried leaf material was collected from the living collection at Kirstenbosch National Botanical Garden (NBG), South Africa. A total of 88 samples from the genus *Encephalartos* (*Encephalartos altensteinii* Lehm., *Encephalartos latifrons* Lehm., *Encephalartos lebomboensis* I.Verd., *Encephalartos princeps* R.A.Dyer, *Encephalartos trispinosus* (Hook.) R.A.Dyer, and *Encephalartos woodii* Sander; Zamiaceae), representing 43 microsporangiate plants (♂), 30 megasporangiate plants (♀), and 15 seedling specimens of unknown sex were collected (Supplementary Table 1). Additionally, a positive control was collected from a single microsporangiate *Cycas revoluta* Thunb. (Cycadaceae) plant within the living collection of the University of Johannesburg (UJ).

2.2. DNA extraction

Total genomic DNA was extracted using a 10 × CTAB protocol for plant DNA extraction (Doyle and Doyle, 1991). In short, leaf tissue samples were ground at room temperature using a mortar and pestle with liquid nitrogen or sterile grinding sand until homogenized. The homogenate was then transferred to a 50 mL falcon tube containing 10 mL of 10 × CTAB isolation buffer and 80 μL of β-mercaptoethanol preheated to 65 °C. The falcon tube was then incubated at 65 °C for 15–20 min with occasional inversion. Following incubation, 10 mL of SEVAG (chloroform/isoamyl alcohol in a ratio of 24:1) containing 2 % polyvinyl pyrrolidone (PVP) was added and agitated for 30 min using an orbital shaker set to 100 rpm. Thereafter, samples were centrifuged at 4000 rpm for 20 min, after which 500 μL of the aqueous layer was carefully transferred to a 1.5 mL Eppendorf tube containing 150 μL of 70 % isopropanol. After centrifuging at 14,000 rpm for 10 min, the supernatant was decanted off, and the resulting DNA pellet was washed by adding 70 % ethanol to the Eppendorf tube, gently inverting the tube 10 times, centrifuging it at 14,000 rpm for 10 min, and carefully decanting off the supernatant. This wash step was repeated two more times, after which the DNA pellet was dried overnight in a fume hood to eliminate any remaining traces of ethanol. Lastly, the extracted DNA pellet was re-suspended in 50 μL nuclease-free H_2O. Purified genomic DNA was then visualized on a 1 % agarose gel to which ethidium bromide (EtBr; 0.5 μg/mL) was added (Supplementary Fig. 1).

2.3. PCR amplification

PCR amplification was performed from purified genomic DNA with primers designed by Liu et al. (2022) to amplify the *CYCAS_034085* and *CYCAS_010388* genes. Since the *CYCAS_010388* gene is present in both megasporangiate and microsporangiate plants

(results will always show a PCR band), our work was focused on amplification of the *CYCAS_034085* gene.

Although various primer combinations were tested, most success was achieved with the 6265F–Zamia (forward, TCC ACG AAC GAR CTG CAT CA) and 6745R–Zamia (reverse, GTG GGC AAA ACA YGC TGT AG) primer set (Liu et al., 2022) for *Encephalartos*. Each reaction contained 12.5 μL Taq DNA polymerase Red Master Mix (Ampliqon, Denmark), 0.3 μL 6265F–Zamia, 0.3 μL 6745R–Zamia, 0.8 μL BSA (3.2 %), 0.5 μL dimethyl sulfoxide (4.5 %; DMSO) (Kramer and Coen, 2001), and 0.5 μL $MgCl_2$ to help enhance the activity of the DNA polymerase (Schmidt et al., 2014). Between 1 μL and 2 μL of the DNA template was added to the PCR reactions. The volume of template DNA added was adjusted based on the relative intensity of DNA bands in the 1 % agarose gel used to visualize the purified genomic DNA, i.e. the brighter the bands, the higher the DNA concentration and the lower the amount of template DNA used. PCR reactions were completed on the Applied Biosystems™ ProFlex™ PCR System (Thermo Fisher Scientific, USA). PCR amplification settings for the 6265F–Zamia and 6745–Zamia primers were an initial pre-denaturation for 5 min at 94 °C, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 90 s followed by a final elongation step for 5 min at 72 °C. PCR products were then visualized, together with a 1 kb DNA ladder (size range: 500 bp to 10 kb) (New England BioLabs), on a 1 % agarose gel to which EtBr was added (0.5 μg/mL).

2.4. Cycle sequencing

Four of the microsporangiate accessions of *Encephalartos* representing two species (*Encephalartos altensteinii* and *Encephalartos latifrons*) were purified using the ExoSAP-IT™ protocol - incubation at 37 °C for 30 min then 80 °C for 15 min. The purified samples were then sequenced to determine sequence variations between species of the *CYCAS_034085* gene. Cycle sequencing was performed using the Applied Biosystems™ ProFlex™ PCR System (Thermo Fisher Scientific, USA) and the BigDye Terminator Cycle Sequencing Kit v3.1 (Thermo Fisher Scientific, USA). The cycle sequencing products were precipitated in 25 μL 100 % ethanol and 1 μL sodium acetate to remove any unincorporated dye terminators. Sequencing products were then suspended in 10 μL HiDi formamide (Thermo Fisher Scientific, USA) before being sequenced using a 3500 Series Genetic Analyzer (Thermo Fisher Scientific, USA). Generated sequence reads were then aligned using Geneious® v8.1.9 (https://www.geneious.com, Kearse et al., 2012), using the MAFFT v 7.017 plugin (Katoh and Standley, 2013) default settings. The algorithm used was FFT-NS-1, and the scoring matrix was set to be 200PAM/k = 2, gap penalty=1.53 and the offset value=0.123. Geneious was then used to compare the sequence variations between the *CYCAS_034085* gene sequences.

2.5. Statistical and data analysis

A 2 × 2 confusion matrix was made using the package caret 6.0–94 (Kuhn, 2008) in R version 4.3.1 (R Core Team, 2023). This was done to assess the performance of the qualitative PCR assay. It does this by presenting the relationship between the sexual states identified by the PCR-based test and the 73 samples with known sexes identified through traditional morphology-based methods. The matrix comprised four values: correctly identified microsporangiate plants – true positive (TP), correctly identified megasporangiate plants – true negative (TN), megasporangiate plants incorrectly identified as microsporangiate – false positive (FP), and microsporangiate plants incorrectly identified as megasporangiate – false negative. The resulting confusion matrix was, in turn, used to calculate the sex identification accuracy of the PCR method by determining the sum of the correct sex identifications (TP + TN identifications) divided by the total number of identifications returned. The precision, specificity, and sensitivity of the PCR-based sexing was also calculated using the following formulas: precision = TP/(TP+FP); specificity = TN/(FP+TN); sensitivity = TP/(TP+FN) (Hajian-Tilaki, 2013).

3. Results

3.1. DNA extraction and PCR amplification

Total purified genomic DNA was successfully extracted for 89 accessions (88 *Encephalartos* and 1 *Cycas*). Although the genomic DNA for all accessions was not quantified, it was visualized on 1 % agarose gel to confirm the presence and quality of the genomic DNA for the accessions prior to PCR. All accessions showed bands on the gel, confirming the presence of genomic DNA. The *CYCAS_034085* gene was successfully amplified for the 43 microsporangiate *Encephalartos* samples using the primer pair 6265F–Zamia + 6745R–Zamia (Supplementary Fig. 1). We were able to identify ten accessions as different sexual states than what were reported in the living collection records of Kirstenbosch NBG. Among the 30 accessions reported to be megasporangiate by Kirstenbosch NBG, five samples also tested positive for the microsporangiate-specific *CYCAS_034085* gene (AL38, AL46, LF18, TP39 and TP41). Similarly, of the 43 microsporangiate plants, five samples lacked the presence of PCR bands (AL15, LF11, LF14.2, LF30 and PR15) – indicating either that these accessions were potentially megasporangiate plants (Table 1).

Table 1
Sex determination of accessions before and after PCR amplification of the CYCAS_034085 gene using the 6265F–Zamia and 6745R–Zamia primer pair. Species No. = Our defined sample collection number for each sample collected. Accession No. = The defined accession number of each plant from Kirstenbosch National Botanical Garden, Sex – KNBG records = the sex of each plant defined based on the Kirstenbosch National Botanical Garden living collecting database, Sex determined post PCR = the final sex determination following PCR amplification of the CYCAS_034085 gene.

Species No.	Accession No.	Species	Sex - KNBG records	Sex determined post PCR
9	AL15	*Encephalartos altensteinii*	♂	♀
23	AL38	*Encephalartos altensteinii*	♀	♂
30	AL46	*Encephalartos altensteinii*	♀	♂
42	LF11	*Encephalartos latifrons*	♂	♀
46	LF14.2	*Encephalartos latifrons*	♂	♀
49	LF18	*Encephalartos latifrons*	♀	♂
52	LF30	*Encephalartos latifrons*	♂	♀
71	PR15	*Encephalartos princeps*	♂	♀
81	TP39	*Encephalartos trispinosus*	♀	♂
82	TP41	*Encephalartos trispinosus*	♀	♂

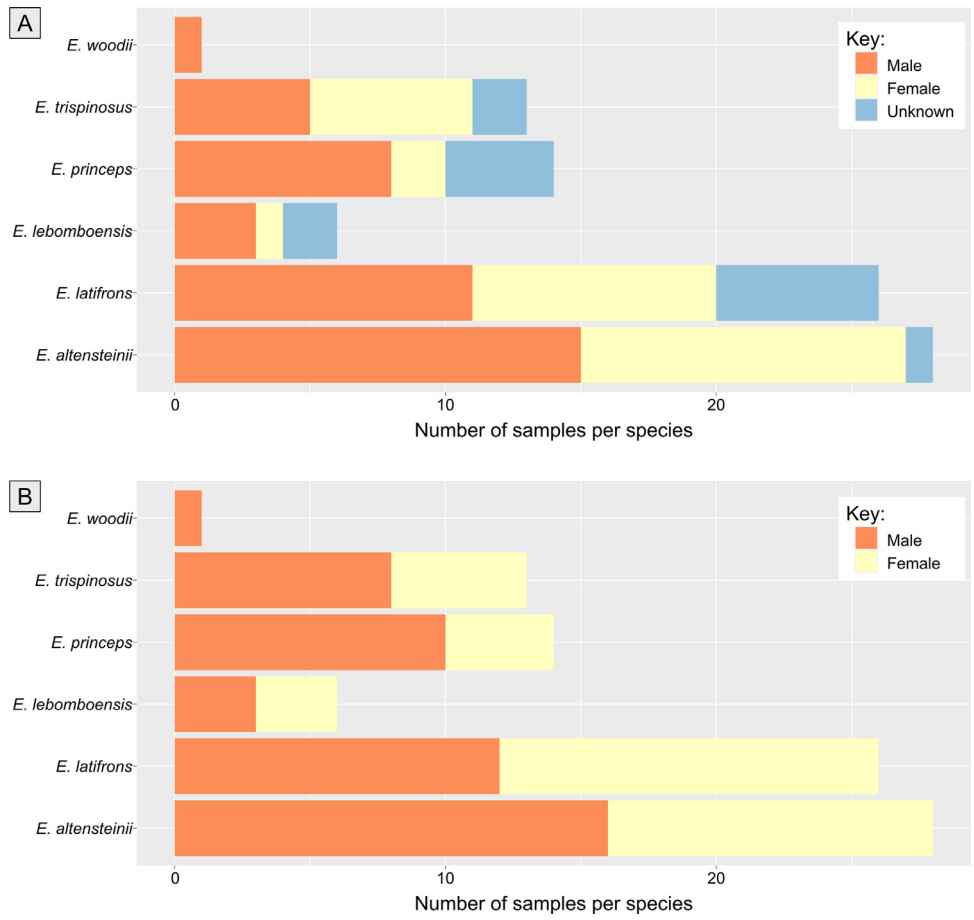

Fig. 1. Total number of accessions collected and identified per species of *Encephalartos*. A) Representation of the total number of accessions that were collected of either sex based on records of the Kirstenbosch National Botanical Garden and the number of unknown sex per species. B) Total number of accessions that were determined by PCR and grouped as either microsporangiate or megasporangiate plants per species.

The stacked bar chart (Fig. 1) compares the number of specimens per species that were collected. *Encephalartos altensteinii* was sampled the most (n = 28). *Encephalartos latifrons* (n = 6) had the highest number of unknown plants that have yet to produce strobili. The total number of microsporangiate accessions to megasporangiate accessions was found to be 43:30 prior to PCR and 57:44 post PCR. Most species had more microsporangiate accessions sampled (with the exception of *E. latifrons*). (Table 2)

3.2. DNA sequencing

The results from sequencing of the CYCAS_034085 gene for four microsporangiate accessions of *Encephalartos* showed a sequence length of f ≤ 520 bp (max=522, min=520, Table 2). The base call accuracy (HQ%) of the sequences is between 81.4 % and 91.3 %; GC content (GC%) ranges from 37.60 % to 38.50 %. The alignment of these sequences shows three polymorphic sites, two of which were potential transversional substitutions (sites 75 and 200) and an insertion/deletion at 203 bp position.

Table 2
The results from cycle sequencing of the CYCAS_034085 gene sequence for four sequences of two different *Encehalartos* species (*E. latifrons* and *E. altensteinii*). Species ID = the accession number of each sample. HQ% = The high-quality base score percentage, Sequence Length = the length of the resulting sequences, GC% = the percentage GC components in each sequence. NCBI Acc. = NCBI accession number (to be uploaded after review).

Species ID	Species	HQ%	Sequence Length	GC%	NCBI Acc.
LF7	*Encephalartos latifrons*	91,30	522	37,60	
AL214	*Encephalartos altensteinii*	88,30	521	38,50	
AL56	*Encephalartos altensteinii*	84,30	520	38,10	
LF3	*Encephalartos latifrons*	81,40	521	37,60	

3.3. Statistical analysis

Performance metrics were calculated using the confusion matrix. From the total known sample (n = 73), 63 were correctly predicted by the PCR test (TP = 38; TN = 25), and 10 samples were incorrectly predicted by the PCR test (FP = 5; FN = 5). The accuracy, precision, specificity and sensitivity performance metrics for the PCR test are given in Fig. 2.

4. Discussion

Cycads are dioecious gymnosperms with long generation times. Because of this, botanic gardens, growers, and collectors need to wait many years for plants to mature and produce their first strobilus before sex can be determined (Abraham and Mathew, 1962). However, with the recent discovery of sex-specific genes in cycads, we can now use PCR and locus-specific primers to amplify them – offering a new approach to sexing cycads at any life stage. In this study, we have demonstrated that a PCR-based qualitative assay can be used to determine the sex of microsporangiate and megasporangiate plants in the genus *Encephalartos* using the *CYCAS_034085* gene. Furthermore, the approach developed here has the potential to identify plants that may have changed sex.

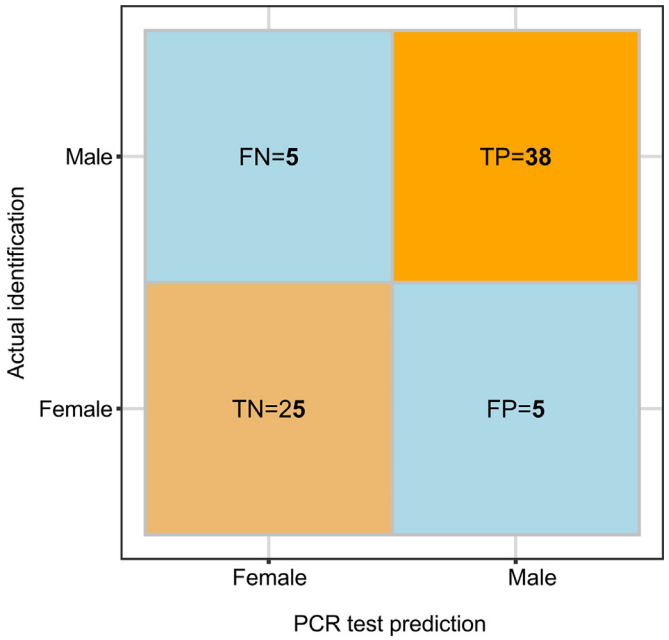

Fig. 2. A confusion matrix showing the overlap between the morphological sexual determination of the plants and what the PCR test predicted. TP = True Positive; TN = True Negative; FN = False Negative; FP = False Positive. Accuracy =86.3 %; positive precision = 88.3 %; negative prediction = 83.3 %, specificity (true positive rate) =88.3 %; sensitivity (true negative rate) = 83.3 %.

4.1. *Sex determination in* Encephalartos

The 6265F–Zamia and 6745R–Zamia primer pair was able to produce positive PCR bands in the range of ~500 bp in length in microsporangiate plants. Additionally, as expected, there were mostly no PCR bands recovered in megasporangiate plants due to the lack of the *CYCAS_034085* gene. Based on living collection records of Kirstenbosch NBG, we found inconsistencies in the sex state recording in ten accessions (Table 1) when compared to the results of the PCR tests (even after multiple PCR attempts, the results remained consistent) – with five apparent megasporangiate plants identified as microsporangiate and five apparent microsporangiate plants identified as megasporangiate.

Two of these accessions (LF11 and TP41) were growing in proximity with the accessions of the opposite sex, and it is possible this is a records error from Kirstenbosch NBG. However, two other accessions (AL46 and PR15) were recently damaged during maintenance. Accession AL46 had been completely cut down (~ six years ago) and has produced basal offsets without any recorded strobili since the damage occurred. Accession PR15 was recorded as producing microsporangiate strobili before 2019, but the plant was disturbed to remove numerous basal offsets. This disturbance may have played a role in a potential sex change. This might indicate that certain abiotic and biotic factors such as severe cold, drought, disruptions to the root system (transplanting) and physical stress could play a role in affecting the regulation and expression of the *CYCAS_034085* and *CYCAS_010388* genes in microsporangiate plants.

4.2. *Sex change in cycads and* Encephalartos

Sex expression in cycads is biased towards microsporangiate plants as they have the ability to express both the *CYCAS_034085* and the *CYCAS_010388* genes (Liu et al., 2022). Megasporangiate cycad plants may lack the *CYCAS_034085* gene involved in the initiation of the microsporangiate strobilus. Thus when testing for the presence or absence of the *CYCAS_034085* gene in megasporangiate plants, PCR amplification is 100 % accurate for plants that have developed as megasporangiate plant (has a megasporangiate genotype and can only ever produce megasporangiate strobili) (Clugston and Kenicer, 2022). When the sex systems of cycads are compared to other gymnosperms, 64.6 % of gymnosperm species are dioecious, although only a few representatives are known to possess heteromorphic sex chromosomes. Because for many gymnosperm species there are no observed differences in their karyotypes, and as such, sex differentiation between species was mostly epigenetically controlled (Ohri and Rastogi, 2020). However, when gymnosperms are compared to angiosperms where dioecy is relatively uncommon, as Leite Montalvão et al. (2021) found that dioecy in angiosperms is often linked to sex-determining genomic regions. This is also likely the case for many gymnosperms species, but further research would be needed to determine the specific sex-determining genomic regions. It was also once assumed in cycads that the sex of plants was epigenetically controlled (Osborne and Gorelick, 2007) prior to the discovery of sex determining regions (SDRs) by Liu et al. (2022). Nevertheless, unidirectional sex changes from male-to-female as well as from female-to-male have been recorded in both *Cycas* and *Encephalartos* (Osborne and Gorelick, 2007). Based on what is currently known, we hypothesize that it is biologically possible for a cycad with a microsporangiate genotype to produce megasporangiate strobili through the up regulation of the *CYCAS_010388* gene and down regulation of the *CYCAS_034085* gene – which could result in a decrease in the transcription of the of the *CYCAS_034085* gene and a megasporangiate phenotype. Reversal of the latter phenomenon could, in turn, explain apparent female-to-male or male-to-female sex changes.

If the regulation of the genes changes during the strobilus development, a transition cone could be produced that is both microsporangiate and megasporangiate. Fig. 3 shows the strobilus of *Encephalartos longifolius* (Jacq.) Lehm. that is male on the terminal part of the central strobilus axis and female below. This figure helps to demonstrate the sex change occurring in *Encephalartos* and likely shows a transition in the up regulation of the *CYCAS_010388* gene and down regulation *CYCAS_034085* gene likely occurring within the meristematic tissue. Zemp et al. (2016) found that sex biased gene expression in dioecious plants favours male genes due to less resource allocation required in the development of microspores, when compared to the greater resources required for the development of female reproductive organs. In cycads, Krieg et al. (2017) found that megasporangiate cycad plants have greater water usage efficiency and that their uptake of nitrogen from cyanobacteria in coralloid roots was greater when compared to microsporangiate plants. This helps to demonstrate that in cycads, the production of megasporangiate strobili requires higher levels of nitrogen and more careful use of water. This could explain why microsporangiate plants are more likely to develop microsporangiate strobili more often when compared to megasporangiate strobili and why our sample representation is male-biased (Fig. 1).

So why could the sexual representation be changing in megasporangiate plants and how could this aid in the long-term population survival of cycads? Correspondingly, Liu et al. (2021) found that when dioecious plants are growing in high biotic stress environments (e.g. drought and high temperature), the microsporangiate genes tend to be favoured over the megasporangiate genes in plants because of the lower resources required in the development of the microsporangiate strobilus (Marler, 2010). This provides insights into the adaptive processes that may occur when cycads are exposed to biotic or abiotic stress, as cycads may be less likely to ignite a sex change event in a stressful environment, which could lead to a microsporangiate biased population. This is possibly an adaptation for cycads ensuring population resilience because a higher ratio of microsporangiate plants in a population could allow more flexibility in adapting to adverse biotic factors (Munné-Bosch, 2015).

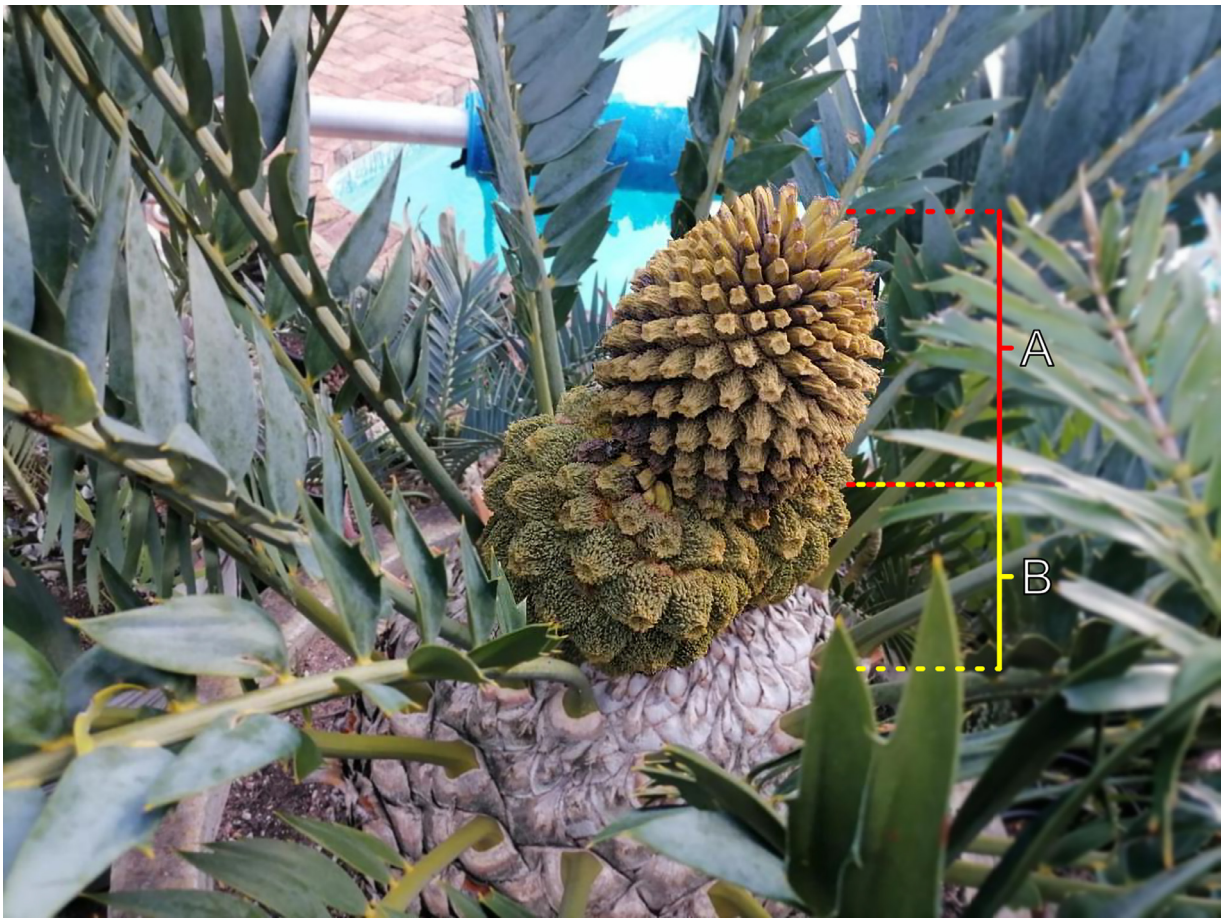

Fig. 3. A photo of a rare bisexual cone from *Encephalartos longifolius* growing in cultivation. A: shows the microsporangiate potation of the strobilus and B: shows the megasporangiate potation of the strobilus. The cone demonstrates potential for an intermediate between both sexes and the down regulation of the CYCAS_034085 gene and the up regulation of the CYCAS_010388 gene. Photo provided by and credited Wilfred Cloete, Sedgefield, South Africa.

4.3. PCR-based sex determination accuracy

The accuracy of the PCR-based sex determination is important when determining the state of any *Encephalartos* species, and our results have shown an accuracy of 86 % (TP=52 % and TN=34 %). Comparatively, Guo et al. (2023) tested a PCR-based sexing approach on *Actinidia arguta* (Siebold & Zucc.) Planch. ex Miq. and managed to achieve a higher sex identification accuracy of 96.09 %. We, however, found that determining the sex of cycads is more challenging as microsporangiate plants have the potential to change sexual phenotype (Sultana et al., 2016). Nevertheless, our results demonstrate the effectiveness of PCR in identifying and determining the sexual state of the tested accessions. However, the presence of the FN highlights that there is room for improvement in the PCR method, and reducing the FN errors would enhance the overall accuracy of this method. Making sure the concentration and quality of the DNA is suitable to amplify the gene of interest by utilizing additional quality control assays prior to PCR (e.g., spectrophotometry and/or fluorometry) would assist with reducing the number of FN. Furthermore, by using the *CYCAS_010388* gene as a positive control for each query sample, it would be possible to infer if the PCR reaction failed or if one is dealing with a true negative result. A precision of 88 % was calculated from all the microsporangiate accessions (n = 43). This metric (also known as the positive predictive rate) indicates the precision of microsporangiate determinations, i.e., of all the microsporangiate assignments made by the PCR test, how many are actually microsporangiate accessions. Based on the results, there is more than 85 % probability that the plants will be accurately sexed using this method, and the methodology presented can help minimize the errors when collecting.

4.4. The utility of cycad sex determination for conservation

The identification of the sexual state of cycads has important implications for conservation, as it has the capacity to directly lead to better and more effective *ex situ* and in situ population management of species which are at risk of extinction (Clugston and Kenicer, 2022). From a conservation perspective, determining the sex of cycads at any life stage is important to aid in effective pollination and seed production from plants growing in both cultivation and the wild. It has been demonstrated that the living collection of botanic gardens have the ability to capture the genetic diversity found in wild populations for cycads (Griffith et al., 2015). The knowledge to create a balanced sex ratio in mega collections of plants is important for maintaining genetic diversity and in the development of stable living assurance colonies (Salgotra and Chauhan, 2023). Due do the conservation concern of cycad genera like *Encephalartos* (sensu Stewart et al., 2023), determining the sexual state in Multiple species can allow for the assessment of the sexual viability of any populations/collections. This methodology will allow the ability to build living collections of cycads with balanced sex ratios and help monitor the sex ratios of wild populations (Wood et al., 2020).

For *ex situ* conservation, the knowledge of the sex of plants will help in curation of assurance colonies that not only represent the genetic diversity found in wild, but also have a balanced sex ratio to optimize breeding. As well curated assurance colonies are most

optimal way to preserve plant species like *Encephalartos* – especially those that are extinct in the wild, critically endangered or endangered (Chen and Sun, 2018). This was demonstrated by Jian et al. (2020) carried out reintroduction programs for four species of *Cycas* in China by growing plants in *ex situ* collections and through a process of planning and post mentoring, had great success. Nevertheless, being able to ensure a balanced sex ratio would have provided better knowledge of population dynamics and increase long-term survivability. Thus, sex determination of cycads at any life stage will have major benefits for conservation practices and help in the process of making informed decisions regarding the implementation of best practices in the population management. As many species of cycads (including *Encephalartos* plants) need to occur in close proximity to allow for successful pollination to aid re-establishment of wild populations using the genetic resources from *ex situ* collections (Swart et al., 2018).

5. Conclusion

Cycads are one of the most conservation-significant groups of organisms, and due to their dioecious nature and short seed viability, *ex situ* conservation in the living collections of botanic gardens is important for their long-term survivability (Donaldson, 2003). To establish effective in situ assurance cycad colonies, not only do these colonies need to represent the genetic diversity found in wild populations, but also balanced sex ratios to ensure optimum pollination and subsequent seed production. With the recent finding that *CYCAS_034085* and *CYCAS_010388* genes are involved in the initiation of the microsporangiate and megasporangiate strobilus (Liu et al. 2022), a PCR-based method has been determined to be reliable for determining the sex in *Encephalartos* species at any life stage. The precision of PCR-based sex identification was 88 % for plants that had developed as microsporangiate and 83 % for megasporangiate plants. Microsporangiate plants have copies of both the *CYCAS_034085* and the *CYCAS_010388* gene, allowing for potential sex changes. Cycads populations are likely naturally male biased as it allows for more adaptation in less amiable environments and enables sex changes to occur in optimum environmental conditions or a stress related incident through upregulation of one gene and the down regulation of another, allowing a shift between sexes. The use of a PCR-based sex determination method for *Encephalartos* and other cycads will have implications for their *ex situ* conservation, allowing for better curation of living collections. These living collections would be able to both represent the genetic diversity found in the wild and have a balanced sex ratio – aiding in the creation of genetically diverse assurance colonies, which will help to ensure the future survival of cycads. However, this implies that for a plant like *Encephalartos woodii*, where only microsporangiate plants have ever been found, all the genes necessary to produce megasporangiate strobili are present and if the appropriate conditions are provided/gene silencing technologies are applied, feminization is feasible.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

James A.R. Clugston: Writing – review & editing. **Nathasha R. Mahunye:** Visualization, Writing – original draft. **Ross D. Stewart:** Data curation, Formal analysis, Methodology, Software. **Hendrik Niemann:** Writing – review & editing. **Michelle van der Bank:** Funding acquisition, Project administration, Resources, Supervision.

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Supplementary materials

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References

Abraham, A., Mathew, P.M., 1962. Cytological studies in the cycads: sex chromosomes in *Cycas*. Ann. Bot. 26 (2), 261–266.
Bamigboye, S.O., Tshisikhawe, P.M., 2022. Climatic changes may not affect the distribution range of South African endemic *Encephalartos* species (Cycadales). Contributii Botanice(57).
Calonje, M., Kay, J., Griffith, M.P., 2011. Propagation of cycad collections from seed: applied reproductive biology for conservation. Sibbaldia: Int. J. Bot. Gard. Horticult. (9), 79–96.
Chen, G., Sun, W., 2018. The role of botanical gardens in scientific research, conservation, and citizen science. Plant Diver. 40 (4), 181–188.
Clugston, J.A., Kenicer, G.J., 2022. Sexing cycads—a potential saviour. Nat. Plant. 8 (4), 326–327.
Cousins, S.R., Witkowski, E.T.F., 2017. African cycad ecology, ethnobotany and conservation: a synthesis. Bot. Rev. 83, 152–194.
Cousins, S.R., Williams, V.L., Witkowski, E.T., 2011. Quantifying the trade in cycads (*Encephalartos* species) in the traditional medicine markets of Johannesburg and Durban, South Africa. Econ. Bot. 65, 356–370.
Donaldson, J.S., 2003. Cycads: Status Survey and Conservation Action Plan. IUCN—the World Conservation Union.
Doyle, J., Doyle, J.L., 1991. CTAB total DNA isolation. Molecular Techniques in Taxonomy, pp. 283–293.
Gorelick, R., Osborne, R, 2002. Inducing sex change and organogenesis from tissue culture in the endangered African cycad *Encephalartos woodii* (Cycadales, Zamiaceae). S. Afr. J. Sci. 98 (3), 114–117.
Griffith, M.P., Calonje, M., Meerow, A.W., Tut, F., Kramer, A.T., Hird, A., Magellan, T.M., Husby, C.E., 2015. Can a botanic garden cycad collection capture the genetic diversity in a wild population? Int. J. Plant Sci. 176 (1), 1–10.
Guo, D., Wang, R., Fang, J., Zhong, Y., Qi, X., 2023. Development of sex-linked markers for gender identification of Actinidia arguta. Sci. Rep. 13, 1–8. https://doi.org/10.1038/s41598-023-39561-0.
Hajian-Tilaki, K., 2013. Receiver operating characteristic (ROC) curve analysis for medical diagnostic test evaluation. Casp. J. Intern. Med. 4 (2), 627.
IUCN, 2022. The IUCN Red List of Threatened Species. Version 2022-1. .. https://www.iucnredlist.org/ (accessed 5.06.23).
Jian, S., Gong, X., Liu, N., 2020. Reintroduction of Wild Cycas Species in China. Conserv. Reintroduct. Rare Endanger. Plant. China 225–233.
Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30 (4), 772–780.
Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28 (12), 1647–1649.
Kramer, M.F., Coen, D.M., 2001. Enzymatic amplification of DNA by PCR: standard procedures and optimization. Curr. Protocol. Molecul. Biol. 56 (1) 15-1.

Krieg, C., Watkins, Jr, J.E., Chambers, S., Husby, C.E, 2017. Sex-specific differences in functional traits and resource acquisition in five cycad species. AoB Plant. 9 (2), plx013.
Kuhn, M., 2008. Building predictive models in R using the caret package. J. Stat. Softw. 28 (5), 1–28. https://doi.org/10.18637/jss.v028.i05.
Leite Montalvão, A.P., Kersten, B., Fladung, M., Müller, N.A, 2021. The diversity and dynamics of sex determination in dioecious plants. Front. Plant. Sci. 11, 580488.
Liu, Y., Wang, S., Li, L., Yang, T., Dong, S., Wei, T., Shengdan, W., et al., 2022. The Cycas genome and the early evolution of seed plants. Nat. Plant. 8 (4), 389–401.
Liu, M., Korpelainen, H., Li, C., 2021. Sexual differences and sex ratios of dioecious plants under stressful environments. J. Plant Ecol. 14 (5), 920–933.
Mankga, L.T., Yessoufou, K., Chitakira, M., 2020. On the origin and diversification history of the African genus *Encephalartos*. S. Afr. J. Bot. 130, 231–239. https://doi.org/10.1016/j.sajb.2019.12.007.
Marler, T.E., 2010. Cycad mutualist offers more than pollen transport. Am. J. Bot. 97 (5), 841–845.
Munné-Bosch, S., 2015. Sex ratios in dioecious plants in the framework of global change. Environ. Exp. Bot. 109, 99–102.
Nadarajan, J., Benson, E.E., Xaba, P., Harding, K., Lindstrom, A., Donaldson, J., Seal, C.E., Kamoga, D., Agoo, E.M.G., Li, N., King, E., Pritchard, H.W., 2018. Comparative biology of cycad pollen, seed and tissue - a plant conservation perspective. Bot. Rev. 84 (3), 295–314.
Ohri, D., Rastogi, S., 2020. Sex determination in gymnosperms. Nucleu. 63, 75–80.
Osborne, R., Gorelick, R., 2007. Sex change in cycads: cases, causes, and chemistry. Mem. N. Y. Bot. Gard. 97, 335–345.
R Core Team (2023) R: a Language and Environment For Statistical computing, Version 4.3.1. R Foundation For Statistical Computing, Vienna.
Raimondo, D.C., Donaldson, J.S., 2003. Responses of cycads with different life histories to the impact of plant collecting: simulation models to determine important life history stages and population recovery times. Biol. Conserv. 111 (3), 345–358.
Salgotra, R.K., Chauhan, B.S., 2023. Genetic diversity, conservation, and utilization of plant genetic resources. Gene. (Basel) 14 (1), 174.
Schmidt, H.F., Sakowski, E.G., Williamson, S.J., Polson, S.W., Wommack, K.E., 2014. Shotgun metagenomics indicates novel family A DNA polymerases predominate within marine virioplankton. ISME J. 8 (1), 103–114.
Segawa, M., Kishi, S., Tatuno, S., 1971. Sex chromosomes of *Cycas revoluta*. Jpnese. J. Genet. 46 (1), 33–39.
Sultana, M., Mukherjee, K.K., Gangopadhyay, G., 2016. Determination of sex expression in cycads. Reproduct. Biol. Plant. 118.
Stewart, R.D., Clugston, J.A.R., Williamson, J., Niemann, H.J., Little, D.P., Bank, M.Van Der, 2023. Species relationships and phylogenetic diversity of the African genus *Encephalartos* Lehm. (Zamiaceae). S. Afr. J. Bot. 152, 165–173. https://doi.org/10.1016/j.sajb.2022.12.001.
Swart, C., Donaldson, J., Barker, N., 2018. Predicting the distribution of Encephalartos latifrons, a critically endangered cycad in South Africa. Biodivers. Conserv. 27, 1961–1980.
Williamson, J., Maurin, O., Shiba, S.N.S., Van der Bank, H., Pfab, M., Pilusa, M., Kabobgo, R.M., Van der Bank, M, 2016. Exposing the illegal trade in cycad species (Cycadophyta: *Encephalartos*) at two traditional medicine markets in South Africa using DNA barcoding. Genome 59 (9), 771–781.
Wood, J., Ballou, J.D., Callicrate, T., Fant, J.B., Griffith, M.P., Kramer, A.T., Havens, K., 2020. Applying the zoo model to conservation of threatened exceptional plant species. Conserv. Biol. 34 (6), 1416–1425.
Woodenberg, W., Erdey, D., Pammenter, N.W., Berjak, P., 2007. Post-shedding seed behaviour of selected Encephalartos species. S. Afr. J. Bot. 73 (3), 496.
Zemp, N., Tavares, R., Muyle, A., Charlesworth, D., Marais, G.A.B., Widmer, A., 2016. Evolution of sex-biased gene expression in a dioecious plant. Nat Plant. 2 (11), 1–7.