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A PCR-Based Method for Sex Determination of *Cycas micronesica* (Cycadaceae)*

MICHAEL ANGELO P. FERNANDEZ[1], BLAIZE SAN NICOLAS[2], JAMES MCCONNELL[3] & MARI MARUTANI[4,†]

[1]*Pacific Biosciences Research Center, University of Hawaiʻi at Mānoa, 1993 East-West Road, Honolulu, HI, 96822*

[2]*Proferre, Inc./ Pacific Federal Management. 202 Hilton Road Unit #10 Tumon Bay, Guam 96931*

[3]*Guam Plant Extinction Prevention Program, College of Natural & Applied Sciences, University of Guam, UOG Station, Mangilao, Guam 96923*

[4]*Agriculture and Life Science Division, College of Natural & Applied Sciences, University of Guam, UOG Station, Mangilao, Guam 96923*

Abstract— *Cycas micronesica* is an arborescent, dioecious cycad native to Micronesia. Once the most abundant tree species in Guam, the native *C. micronesica* population on the island has sharply declined due to invasive insect predation. While conservation efforts have focused on restoring *in situ* populations with *ex situ* propagated plants, sexing at the time of outplanting remains challenging in the absence of sexually dimorphic traits. Despite these limitations, a reliable method of sexing remains critical in ensuring reproductive function within a population for the conservation of the species. Recently, a PCR-based method was reported for sexing *Cycas panzhihuaensis* using primers that amplify a male-specific *MADSY/CYCAS_034085* gene and its nearest autosomal homolog *CYCAS_010388* as an internal control. However, the compatibility of this sexing primer set with *C. micronesica* is not well understood given the lack of genetic information for *C. micronesica*. The present study evaluated these PCR-based methods in sexing male (n = 17) and female (n = 13) *C. micronesica* individuals, finding amplification inconsistencies indicative of primer incompatibility with *C. micronesica*. From our findings, we developed an improved primer set with redesigned male-specific primers *CYCmic_MADSY* (F/R) demonstrating its improved accuracy and reliability in correctly sexing all *C. micronesica* individuals. Our revised PCR-based method provides a rapid, reliable protocol for sexing *C. micronesica*. The information generated from this method has potential for guiding conservation studies and efforts aimed at re-establishing viable, seed-producing populations of this native species.

Introduction

Cycas micronesica (Cycadaceae) K.D.Hill, commonly known as *fadang*, *fadan, federiko,* or *federico* in Guam (Falanruw *et al.* 1990), is an arborescent, dioecious cycad species native to Micronesia (Fig. 1) (Hill 1994, Hill *et al.* 2004). In 2002, *C. micronesica* was recorded as the most abundant tree species in Guam (Donnegan *et al.* 2004). However, the native cycad population rapidly

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† Corresponding author: marutanim@triton.uog.edu

declined following the introduction of the cycad scale, *Aulacaspis yasumatsui* Takagi, to the island in 2003 (Marler & Muniappan 2006). According to the most recent forest inventory in 2013, over 93% of plants showed signs of scale damage (Lazaro *et al.* 2020). Currently, *C. micronesica* is listed as a threatened species under the United States Endangered Species Act (U.S. Fish & Wildlife Service 2015). Most recently, it has been assessed as endangered by the International Union for Conservation of Nature (IUCN) Red List (Bösenberg 2022). Numerous recovery efforts have since been undertaken in pest management and the outplanting of nursery propagated plants to restore *C. micronesica* populations *in situ*.

Although the dioecious *C. micronesica* displays reproductive organs at maturity, namely microstrobili in males (Fig. 1b) and megastrobili in females (Fig.1c), sexing in *Cycas* is morphologically indistinguishable during the juvenile stage and non-reproductive periods at maturity

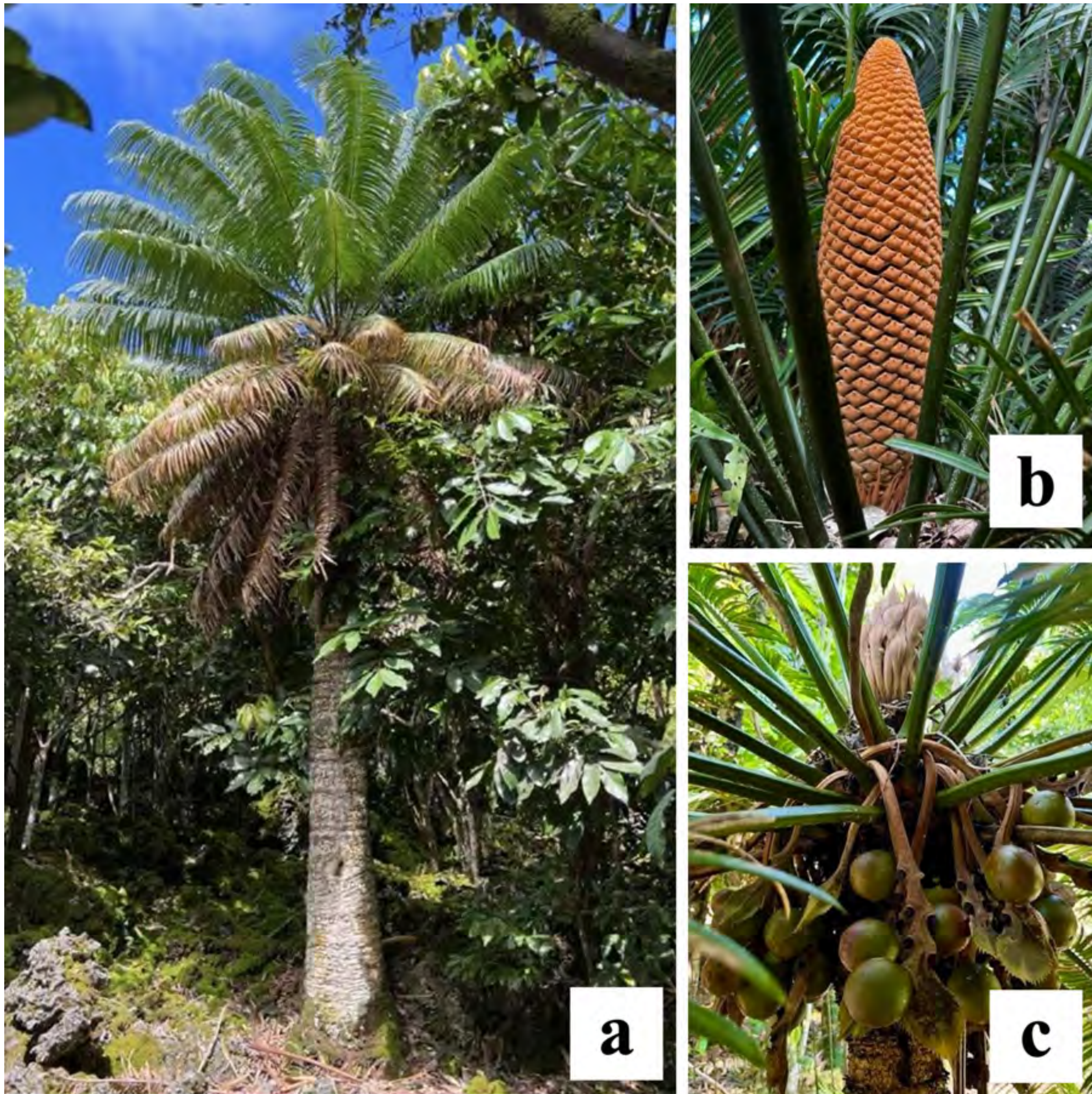

Fig. 1. (a) Mature Cycas micronesica growing in limestone forest in northern Guam. (b) Close-up of microstrobili (male). (c) Close-up of megastrobili (female).

(Norstog & Nichols 1997). Some studies have suggested using other sexually dimorphic traits such as stem branching patterns for sexing *C. micronesica* (Marler & Calonje 2020, Niklas & Marler 2008) and other cycad species (Ornduff 1996, Norstog & Nichols 1997). However, these traits may not be reliable, especially considering the time required to develop such traits or the possibility of mechanical damage from storms or pest predation (Hirsh & Marler 2002, Lindstrom *et al.* 2023, Marler *et al.* 2020).

Previous cytological studies in *C. pectinata* Buch.-Ham. (Abraham & Mathew 1962) and *C. revoluta* Thunb. (Segawa *et al.* 1971) using karyomorphological comparisons were the first to suggest an X.Y. system of sex chromosomes in *Cycas* with females being the homogametic sex (X.X.) and males being heterogametic (X.Y.). Subsequent research in *C. circinalis* L. using microsatellite markers identified potential male- and female-specific randomly amplified polymorphic DNA (RAPD) markers suitable for polymerase chain reaction (PCR)-based analysis, although their relationship to the sex chromosomes in *Cycas* remained unclear (Gangopadhyay *et al.* 2007). Among all these efforts, developing a reliable sexing method presents a major challenge for conservation and restoration efforts as knowing the sex of outplanted juveniles or existing individuals *in situ* is necessary in ensuring effective sexual reproduction, especially for *C. micronesica* in Guam.

In 2022, the first *Cycas* reference genome of any species was assembled for the endangered *Cycas panzhihuaensis* L.Zhou & S.Y.Yang native to China. Genome-wide association studies revealed a differentially regulated gene *CYCAS_034085*, or *MADS-Y*, on the Y chromosome which encodes a GGM13-like MADS-box transcription factor involved in floral development that is highly expressed in males (Liu *et al.* 2022). Its closest autosomal homolog, *CYCAS_010388*, is expressed in both males and females. Based on these findings, Liu *et al.* (2022) developed a multiplexed PCR-based method for sexing *C. panzhihuaensis* and potentially other cycad species with a primer set amplifying the male-specific *MADSY/CYCAS_034085* gene for sexing and the autosomal *CYCAS_010388* gene as an internal control for ensuring the presence of viable DNA. To date, genetic research on the sex chromosomes of *C. micronesica* remains unstudied and it remains unclear whether the primer set developed by Liu *et al.* (2022) is compatible with *C. micronesica*.

In our study, we evaluated the PCR-based methods developed by Liu *et al.* (2022) by testing the compatibility and reliability of their primer set in sexing *C. micronesica* in Guam. From our findings, we developed an improved PCR-based method for sexing *C. micronesica* using a redesigned primer set with the male-specific primers *CYCmic_MADSY* (F/R) and demonstrated its increased accuracy. These findings provide a valuable tool for guiding conservation studies and restoration approaches aimed at establishing viable, seed-producing populations of *C. micronesica* *in situ* which is important given the critically compromised state of the species in Guam.

Materials and Methods

STUDY SITES & INDIVIDUALS

Two study sites, Site A (Lat. 13.55°N, Long. 144.93°E) and Site B (Lat. 13.60°N, Long. 144.90°E), in northern Guam, were selected. These sites are located near the type locality originally described for *Cycas micronesica* (Hill 1994). Both sites predominantly consist of limestone forest habitat, with Site A featuring Ritidian series soil type (clayey-skeletal, gibbsitic, nonacid, isohyperthermic Lithic Ustorthents) and Site B featuring Guam series soil type (clayey, gibbsitic, nonacid, isohyperthermic, Lithic Ustorthents) (Young 1988). We sampled mature individuals of *Cycas micronesica* of known sex from Sites A and B, based on reproductive phenology data collected from 2020 to 2023 monitoring microstrobili and megastrobili development to identify male and female individuals, respectively. In total, we sampled five females and eight males from Site A and eight females and nine males from Site B.

LEAF SAMPLE COLLECTION & DNA EXTRACTION

Young, tender leaflets were collected from known male and female individuals from each study site. Leaf samples were separately washed under running distilled water to remove any surface contaminants and stored dry at -20°C with silica beads until total genomic DNA could be extracted. About 10mg of dried leaf tissue was disrupted using a Qiagen TissueLyser III (Qiagen, Germany) for 4 min at 25Hz. Total genomic DNA was extracted using sucrose extraction solution described by Berendzen *et al.* (2005). DNA extracts were diluted with nuclease-free water to a final concentration of no more than 50ng/µL, as quantified by a NanoDrop spectrophotometer (Thermo Scientific, USA). DNA extracts were centrifuged at 11,000 rpm for 2 min and the resulting supernatant was used as a DNA template for PCR.

EVALUATION OF EXISTING SEXING PRIMER SET FOR *CYCAS*

PCR conditions for the sexing primer set by Liu *et al.* (2022) (Table 1) were initially optimized using one representative male and female sample of *C. micronesica* from Site A. A gradient PCR was performed for both samples in a multiplexed design with the male-specific *MADSY/CYCAS_034085* (F/R) and autosomal *CYCAS_010388* (F/R) primers pooled at equimolar concentration. By this multiplexed design, the primer set was expected to produce one amplicon in female samples and two amplicons in male samples. PCR amplification was carried out in a total reaction volume of 16 µL containing 2 µL of DNA template, 8.5 µL of HotStarTaq DNA Polymerase (Qiagen, Germany), 4.5 µL of sterile water, and 0.5 µL of each forward and reverse primer (10 µM). The gradient PCR protocol consisted of an initial denaturation at 94°C for 3 min; denaturation at 94°C for 45 s, annealing from 53°C–63°C for 45 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR was carried out for 35 serial cycles of amplification in a MultiGene Gradient Thermal Cycler (Labnet International Inc., USA). PCR products and a 100 bp molecular ladder were separated by gel electrophoresis on a 1.0% agarose gel stained with GelGreen (Biotium, USA) at 100V for 30 min and visualized on a U.V. transilluminator. An optimal annealing temperature of 60°C was identified based on clear separation of a 681 bp amplicon corresponding to the male-specific *MADSY/CYCAS_034085* and an approximately 275 bp amplicon corresponding to its autosomal homolog *CYCAS_010388*.

Table 1. Name, gene type, nucleotide sequence, orientation, and base pair size of primers examined in the study.

Primer Set	Primer Name	Gene Type	Nucleotide Sequence (5’-3’)	F/R	Size (bp)
This Study	*CYCmic_MADSY*	Male-Specific	AATTTTGGGTCGTGGGGGTT	F	196 bp
	CYCmic_MADSY		TCCCCCGTCATATGCCTACA	R	
	CYCAS_010388	Autosomal	TCTGCGAGATGACAAGAATCA	F	~275 bp
	CYCAS_010388		CAGCCACTTCTAGCTGCTGT	R	
Liu *et al.* (2022)	*MADSY/CYCAS_ 034085*	Male-Specific	AAGTGACATTCTCCAAGCGTAG	F	681 bp
	MADSY/CYCAS_ 034085		TTCGAACACGAGTTGCTGAAAT	R	
	CYCAS_010388	Autosomal	TCTGCGAGATGACAAGAATCA	F	~275 bp
	CYCAS_010388		CAGCCACTTCTAGCTGCTGT	R	

In a preliminary study using these optimized PCR conditions, five females and six males from Site A were sexed with the primer set by Liu *et al.* (2022). The autosomal *CYCAS_010388* was consistently amplified across all male and female samples of *C. micronesica* of approximately 275 bp, validating the presence of viable DNA for PCR. However, the male-specific *MADSY*/*CYCAS_034085* by Liu *et al.* (2022) failed to amplify in two male samples, lacking an amplicon at around 681 bp, and incorrectly amplified in two female samples, showing a non-specific or "false positive" amplicon also of 681 bp.

DESIGN AND EVALUATION OF MALE-SPECIFIC PRIMERS FOR *C. MICRONESICA*

To improve the reliability of the male-specific primers *MADSY*/*CYCAS_034085* (F/R) by Liu *et al.* (2022), the four correctly identified male amplicons and two non-specific female amplicons from our preliminary study were sequenced by Sanger sequencing (Azenta, USA). Sequences were aligned to produce one male and female consensus sequence with 100% identity using the MUSCLE algorithm (Edgar 2004). Each consensus sequence was then subjected to a BLAST search on NCBI. The results indicated that the male consensus sequence of *MADSY/CYCAS_034085* from *C. micronesica* was homologous to a male-specific MADS-box transcription factor, *GbMADS9*, in *Gingko biloba* with 80% sequence identity (KP260627), validating its association with the sex chromosomes. A representative sequence for the *MADSY/CYCAS_034085* gene for *C. micronesica* using primers *MADSY*/*CYCAS_034085* (F/R) was deposited into NCBI GenBank under accession number PQ335231 and is also included in the Appendix. In contrast, the consensus sequence of the non-specific female amplicons from *C. micronesica* did not closely resemble any known sequence.

Using the male consensus sequence, we designed a new forward (F) and reverse (R) primer, *CYCmic_MADSY* (F/R), to amplify a shorter 250 bp region nested within the *MADSY/CYCAS_034085* gene of *C. micronesica* using Geneious Prime (ver. 2023.2.1) (Biomatters, New Zealand) (Table 1). We confirmed the identity of the nested amplicon by Sanger sequencing showing 100% identity to the target *MADSY/CYCAS_034085* gene spanning from base pair 417 to 612. A representative sequence for the *MADSY/CYCAS_034085* gene for *C. micronesica* using primers *CYCmic_MADSY* (F/R) is included in Appendix 1. We evaluated the sexing accuracy of our redesigned male-specific primers paired with the autosomal *CYCAS_010388* (F/R) and compared it with the original sexing primer set by Liu *et al.* (2022). Both primer sets were used to sex 30 known male and female *C. micronesica* samples collected from Site A and Site B. DNA extraction and PCR conditions were standardized and maintained consistent across both primer sets. Amplified PCR products were analyzed by electrophoresis and visualized under U.V. light. Both primer sets were assessed on the correct amplification or presence of the male-specific and autosomal amplicons in electrophoresis.

Results

In the sexing primer set of our study and by Liu *et al.* (2022), primers *CYCAS_010388* (F/R) consistently amplified the autosomal *CYCAS_010388* gene of approximately 275 bp in all male and female samples of *C. micronesica*. The presence of these amplicons as an internal control confirmed that each sample contained sufficient viable cycad DNA for PCR, eliminating the possibility of "false negatives" due to the absence of viable cycad DNA. In contrast, sexing primers *MADS-Y/CYCAS_034085* (F/R) from Liu *et al.* (2022) yielded inconsistent results wherein amplicons for the male-specific *MADS-Y/CYCAS_034085* gene were absent in four male samples at the expected 681 bp. Additionally, these primers produced unpredictable, weaker signals in other samples during gel electrophoresis despite optimized PCR conditions (Fig. 2b and Fig. 3b). In comparison, our newly developed primers *CYCmic_MADSY* (F/R) provided more consistent results, producing clear amplicons of about 196 bp exclusively in male samples (Fig. 2a and Fig. 3a), demonstrating improved accuracy in sexing *C. micronesica.* Our primer set with redesigned primers,

CYCmic_MADSY (F/R), correctly confirmed the sex of all 30 male and female samples of *C. micronesica* from Sites A and B, whereas the primer set by Liu *et al.* could not confidently identify sex and showed incorrect identification in five individuals. Table 2 summarizes the results of the PCR-based tests of our primer set and the primer set reported by Liu *et al*. (2022). We further illustrate the specificity of each primer set as the proportion of correctly sexed samples.

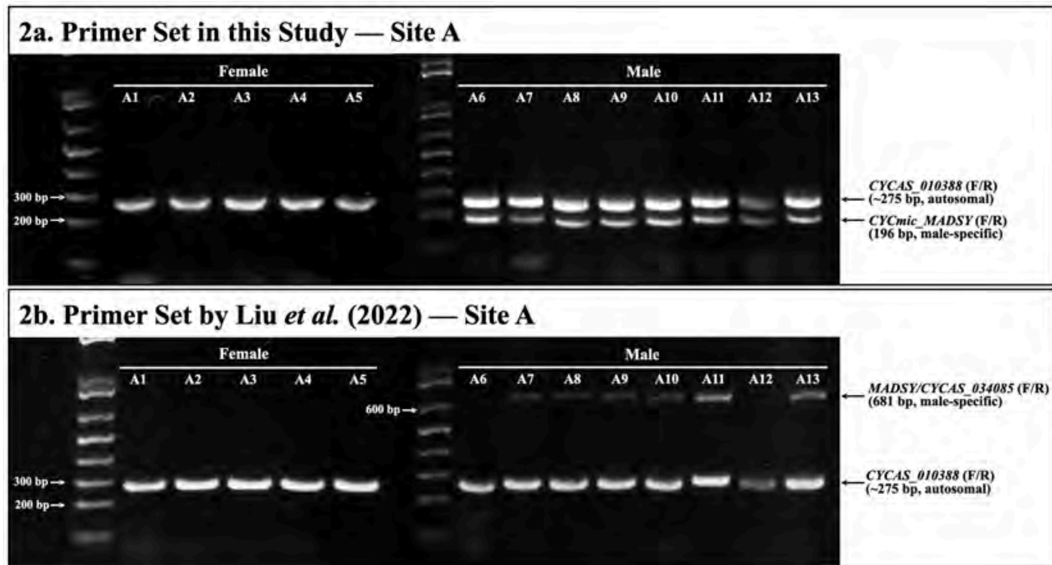

Fig. 2. Comparison of electrophoresed PCR products using (a) primer set developed in this study and (b) primer set by Liu et al. (2022) for sexing *Cycas micronesica*. Samples were collected from male (n = 5) and female (n = 8) *C. micronesica* individuals from Site A. PCR products of both primer sets were separated on 1.0% agarose gel.

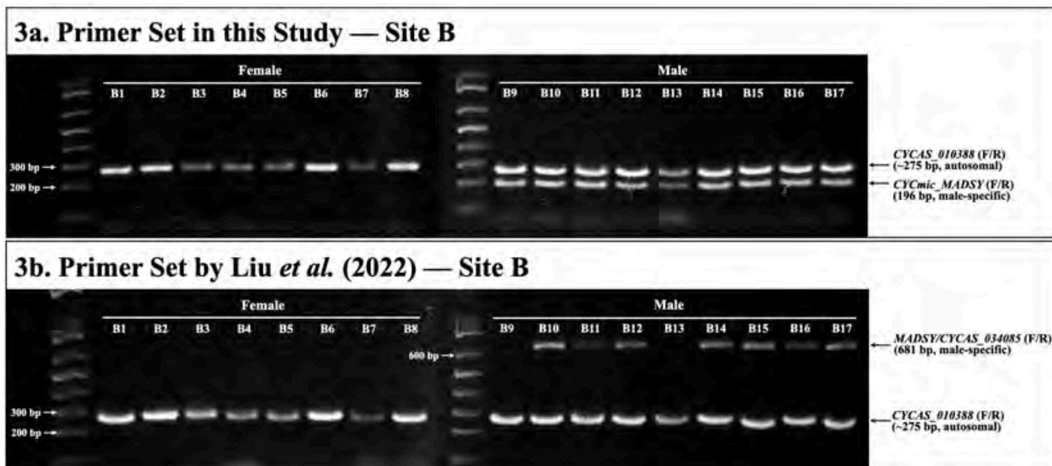

Fig. 3. Comparison of electrophoresed PCR products using (a) primer set developed in this study and (b) primer set by Liu *et al.* (2022) for sexing Cycas *micronesica.* Samples were collected from male (n = 5) and female (n = 8) *C. micronesica* individuals from Site B. PCR products of both primer sets were separated on 1.0% agarose gel.

Table 2. Summary of presence (+) or absence (-) of electrophoresed PCR products using sex-determination primer set developed in this study and by Liu *et al.* (2022). Leaf DNA samples were collected from known male (M) and female (F) *Cycas micronesica* from Site A and Site B.

Site	**Sex**	**Plant ID**	**Primer Set in this Study**		**Primer Set by Liu *et al.* (2022)**	
			***CYCAS_010388* (*Autosomal*)**	***CYCmic_MADSY* (*Male-Specific*)**	***CYCAS_010388* (*Autosomal*)**	***MADSY/CYCAS_034085* (*Male-Specific*)**
Site A	**F**	A1	+	-	+	-
		A2	+	-	+	-
		A3	+	-	+	-
		A4	+	-	+	-
		A5	+	-	+	-
	M	A6	+	+	+	-
		A7	+	+	+	-
		A8	+	+	+	-
		A9	+	+	+	-
		A10	+	+	+	-
		A11	+	+	+	+
		A12	+	+	+	-
		A13	+	+	+	+
Site B	**F**	B1	+	-	+	-
		B2	+	-	+	-
		B3	+	-	+	-
		B4	+	-	+	-
		B5	+	-	+	-
		B6	+	-	+	-
		B7	+	-	+	-
		B8	+	-	+	-
	M	B9	+	+	+	-
		B10	+	+	+	+
		B11	+	+	+	-
		B12	+	+	+	+
		B13	+	+	+	-
		B14	+	+	+	+
		B15	+	+	+	+
		B16	+	+	+	-
		B17	+	+	+	+
Total Correct Sex ID			**30**		**26**	
Specificity (%)			**100% (30/30)**		**86.67% (26/30)**	

Discussion

In the dioecious *Cycas micronesica* native to Guam, sexing by morphological methods remains challenging due to the time required for plants to reach maturity and exhibit sexually dimorphic traits, which can be altered or damaged by environmental factors. Although PCR-based sexing methods have been proposed as a novel, reliable method for determining sex (Liu *et al.* 2022), their compatibility with *C. micronesica* is not well understood due to the limited genetic information available for the species. In this study, we developed and validated a new primer set with male-specific primers *CYCmic_MADSY* (F/R) for reliably sexing *C. micronesica* in Guam.

When comparing our sexing primer set and the primer set by Liu *et al.* (2022), our primers *CYCmic_MADSY* (F/R) demonstrated increased specificity with consistent amplification of the male-specific MADS-Y/CYCAS_034085 gene exclusively in male samples. Unlike our primers which were directly designed from the sequenced MADS-Y/CYCAS_034085 gene of *C. micronesica*, the primers by Liu *et al.* (2022) were developed from the distantly related *C. panzhihuaensis*. When designing cross-species primers, it is common to use a reference genome from the target species and empirically test primer compatibility in other species. In developing their sexing primer set for *C. panzhihuaensis*, Liu *et al.* (2022) also tested their sexing methods on other cycad species including *C. debaoensis* Y.C.Zhong & C.J.Chen, *C. sexseminifera* F.N.Wei, *C. segmentifida* D.Y.Wang & C.Y.Deng, and *C. fairylakea* D.Yue Wang and observed similar results as with *C. panzhihuaensis.* However, predicting the compatibility of primers in other, untested species is difficult when the evolutionary history of the primer binding region is unknown, as is the case for *C. micronesica* (Primmer *et al.* 1996, Housley *et al.* 2006, Merritt *et al.* 2015).

Previous phylogenetic studies based on plastid and nuclear genome data identified *C. panzhihuaensis* within the basal sect. Panzhihuaensis, distributed across south and southwest China (Liu *et al.* 2018). The other cycad species tested by Liu *et al.* (2022), *C. debaoensis*, *C. sexseminifera*, *C. segmentifida*, and *C. fairylakea*, were all classified to sect. Stangerioides, which is closely related to sect. Panzhihuaensis and shares similar distribution in south and southwest China, and north Indochina (Liu *et al.* 2018). The observation of similar sexing results across all these cycad species may be indicative that specificity of the sexing primers by Liu *et al.* (2022) may have been maintained among these closely related species.

In contrast, *C. micronesica* represents a more recently evolved species within sect. Cycas, subsect. Rumphiae, with a distinct distribution across the Indo-Pacific (Hill 1994, Hill *et al.* 2004, Liu *et al.* 2018). By virtue of their evolutionary distance, the male-specific primers *MADS-Y/CYCAS_034085* (F/R) by Liu *et al.* (2022) may have performed poorly in sexing C. micronesica by several factors including base pair mismatches in the primer binding region of the MADS-Y/CYCAS_034085 gene between *C. micronesica* and *C. panzhihuaensis*. This is evidenced by the inconsistent and sometimes weak amplification of the primer across all male samples of *C. micronesica*, suggesting primer incompatibility with this species. Given the limited genomic information on *C. micronesica* particularly for the sex chromosomes, we adopted a *de novo* approach for a redesign of the primers *MADS-Y/CYCAS_034085* (F/R), establishing more reliable sexing primers *CYCmic_MADSY* (F/R) specific to *C. micronesica*.

This improved PCR-based approach to sexing *C. micronesica* presents numerous benefits in the conservation and restoration of this species in its native habitat. Accurate and reliable sexing is necessary in optimizing population structure and reproductive success in sessile, dioecious plant species such as cycads. In our initial implementation of the sexing primer set of our study, we tested 136 *ex situ* juvenile *C. micronesica* propagated from seeds originating from Sites A and B. We identified 69 females and 67 males, revealing a near 1:1 sex ratio (unpublished). Although little is known about the optimal population structure for sexual reproduction in *C. micronesica*, this information can be used to support futures that involve determining optimal sex ratios, spatial distribution by sex, and appropriate population sizes to ensure adequate access to mates (Percy &

Cronk 1997, Heilbuth *et al.* 2001, Stehlik *et al.* 2008, Lazcano-Lara & Ackerman 2018). These insights can guide management strategies and outplanting efforts aimed at augmenting existing populations or establishing new populations *in situ* with optimal population structure for recruitment. The findings of our study have great potential to strengthen current conservation efforts for *C. micronesica* in Guam.

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Appendix

Primers, base pair size, GenBank accession number, and DNA sequence of male-specific amplicons in this study.

Primers (F/R)	Size (bp)	GenBank Accession No.	Sequence (5'-3')
MADSY/CYCAS_034085 (F/R)	681 bp	PQ335231	GCACGACTCTCCGTGTTATGTGACGCAGAGGTC GCTCTGATAATATTTTCAAGCACTGGAAAGCTCT TCGAGTATGCAAGCTCGAGTAGCAGGTTAGTAA CGGTTTGGATCAGCCTATAATGTGAGACTGTTTC TACTTTCTGGCCCTCATATCGTGTTTTTCTTTTTC TTCTCCCTCCGAGGACACGCACATATATTTTACAT GGCTTCTATTCATTAAACTGAAATTCAAGCCTATC TGTTATGTCGAAAAGCATAAAGAGAATTCTCGAA AGATATCAGAGGGTCTCCGGAGTACGACTTTGG GATTATGAGCATCAAGTAAGACAAATCTTGTGCA TTTCGCCTTTTATTGTCGATGATTTACCGTAGTCA TTGTTTAGGTGGGTACCGTAATGCGCAAACTAAT TTTGGGTCGTGGGGGTTGCTATTTGGCCAAACAG CAGTTGTTTTGTGAGATGACAAGATTGAAGAATG AGAATGAAAAACTTCAAAATGCTTTGAGGTATTA TAACTGTATTATTTTTCAAAAATTTCGATGATCCT ATTGTTGGTGTATTTGATAAAAATAAAAAGAGCA ATCTACCATCGAATATATTGTAGGCATATGACGGG GGAGGACCTCAATTCGCTGTCCACGAACGAGCT GCATCATCTAGAGCAAAATCTTGAAATTTCAGC
CYCmic_MADSY (F/R)	196 bp	None	CGTGGGGGTTGCTATTTGGCCAAACAGCAGTTG TTTTGTGAGATGACAAGATTGAAGAATGAGAAT GAAAAACTTCAAAATGCTTTGAGGTATTATAACT GTATTATTTTTCAAAAATTTCGATGATCCTATTGTT GGTGTATTTGATAAAAATAAAAAGAGCAATCTAC CATCGAATATATTGTAGGCATATGAC