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SPECIAL FEATURE REVIEW

Current topics on cycad biology: Deciphering the Rosetta Stone of plant evolution

Progress in the study of dioecy in Mexican cycad species and its implications for conservation

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Abstract

Dioecy is a system of sexual reproduction in plants, characterized by the spatial separation of reproductive structures into different individuals. This system is thought to have evolved to reduce the adverse effects of inbreeding that can occur in hermaphroditic ancestors. Although significant progress has been made recently in understanding this phenomenon, the mechanisms of sex determination in plants remain largely unexplored. These mechanisms may have a genetic, physiological, or epigenetic basis, which has been a focus of interest in cycads, a plant group with a strong association with dioecy. Cycads are threatened plants that exhibit a long period of sexual maturity, making it difficult to determine their sex at an early age, which hinders conservation efforts. In this review, we outline the research history on finding effective methods for early sex identification in cycads, with an emphasis on Mexican cycads. Additionally, we include a discussion by presenting genetic data for the endemic Mexican species *Ceratozamia mexicana* Brongn., *Zamia furfuracea* L. f., and *Zamia inermis* Vovides, J.D. Rees & Vázquez-Torres. We also discuss how sex identification could aid in developing effective conservation plans for these critical species, many of which are recognized by the IUCN (International Union for Conservation of Nature) and federal norms in Mexico.

KEYWORDS

cycads, dioecy, molecular markers, sex determination, sexual dimorphism

1 | INTRODUCTION

1.1 | Cycads and their reproductive system

Among extant plants, cycads belong to the most primitive group of seminiferous species, with a fossil history dating back nearly 300 million years (Coiro et al., 2023). They hold significant evolutionary and ecological importance (Gutiérrez-Ortega et al., 2024), as well as rich symbolic and utilitarian value, given their roles in religious practices and therapeutic applications (Du et al., 2024; Pérez-Farrera & Vovides, 2006). Worldwide, cycads are represented by 394 species (Calonje et al., 2025), which

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are grouped into two globally distributed families: Cycadaceae and Zamiaceae (Christenhusz et al., 2011; Yang et al., 2022). Most cycad species are found in the intertropical zone, encompassing a range of habitats from continuous canopy forests to open savannas and semi-arid scrublands, as well as on nutrient-poor soils, limestone or serpentine outcrops, dunes, and cliffs.

To date, all known fossil cycad specimens exhibit unisexual reproductive structures (Gorelick & Osborne, 2007), a condition known as dioecy. Dioecy is a reproductive system characterized by distinct male and female individuals, meaning that reproductive structures are divided among different plants. Available data suggest that dioecy has evolved from hermaphroditism more than 100 times throughout plant history (Bentz et al., 2024; Charlesworth & Guttman, 1999), resulting in dioecy being present in 170 angiosperm families and 1303 genera (Charlesworth, 1991). Notably, 75% of angiosperm families include at least one dioecious species; however, only about 5% of all plants are dioecious overall (Ainsworth, 2000). Dioecy is more common among gymnosperms, with around 65% of taxa being dioecious, and includes families such as Ephedraceae, Ginkgoaceae, Gnetaceae, Welwitschiaceae, and all cycads in the Cycadaceae and Zamiaceae families (Walas et al., 2018).

Two hypotheses have been proposed to explain how this system might have evolved from hermaphroditism. The first hypothesis suggests that dioecy evolved as a solution to mitigate the negative consequences of inbreeding by promoting outcrossing, which increases genetic variation and gene flow (Parasnis et al., 2000). The second hypothesis, known as the "sexual specialization hypothesis," posits that it emerged due to selection pressures that encouraged individuals to specialize in one of the two sexes (Kohn, 1988). Pannell and Santos del-Blanco (2014) further explained that bisexuality evolved because it is beneficial for individuals to specialize as either male or female, which also helps prevent inbreeding depression. In both cases, dioecy is considered an adaptive strategy that is theoretically effective for species consisting of small populations living in resource-limited environments (Thomson & Barrett, 1981), such as cycads, for which it is worthwhile to briefly discuss the demographic consequences of dioecy.

1.2 | Demographic consequences of dioecy

In cycads, producing offspring with high genetic diversity may be a strategy to ensure the recruitment of at least a few individuals into the next generation. High levels of genetic diversity may enhance the probability of seedling survival, which is significant given that seedlings are the most vulnerable to stress, exhibiting higher mortality rates (Pérez-Farrera et al., 2000; 2006; Pérez-Farrera & Vovides, 2004; Vovides, 1990). However, maintaining a balance between the number of males and females in natural populations is necessary to ensure mating among fertile adults and to ensure effective connectivity with neighboring populations through pollen or seed dispersal (Clark & Clark, 1988). A recent study published in this Special Feature (Yang et al., 2025) demonstrated that the species *Cycas panzhihuaensis* has a well-balanced male to female ratio of 1:1 in a wild population in China. Similarly, Octavio-Aguilar, Iglesias-Andreu, Vovides, and Rivera-Fernandez (2017) reported that the population of *Zamia inermis* in the town of La Catalana, Municipality of La Antigua, Veracruz, Mexico, has an equal sex ratio. Another example is in *Zamia furfuracea* (Vázquez-Torres et al., 2001), in which there is a slightly higher number of males than females in the Los Tuxtla Region, Veracruz, Mexico, with a male-to-female ratio of 1:0.9. On the other hand, as reported by Segalla et al. (2021), a male predominance has been observed in other species, such as *Zamia pumila* (Ornduff, 1987), *Zamia boliviana* (Segalla et al., 2021), *Ceratozamia norstogii* (Pérez-Farrera & Tejeda-Cruz, 1995), *Ceratozamia matudae* (Pérez-Farrera & Vovides, 2004), and *Dioon purpusii* (Yáñez-Espinosa & Sosa-Sosa, 2007).

Research indicates that dioecious populations require a minimum number of males to maintain a viable genetic structure (Wilson & Harder, 2003), and that male reproductive maturity must be synchronized with that of females. Because only females produce seeds, this reproductive system may be more vulnerable to extinction due to demographic constraints, as successful reproduction and seed dispersal rely on only half of the population (assuming a balanced sex ratio). Therefore, finding effective ways to identify the sex of cycad individuals has been both challenging and crucial, as it is essential for informing conservation actions. Below, we outline the implications of sex determination in Mexican cycads and provide novel information that may be useful for sex identification in four species from three genera: *Ceratozamia mexicana* Brongn., *Z. inermis* Vovides, Rees & Vázquez Torres, and *Z. furfuracea* L. f. (Figure 1a–d), and *Dioon merolae* De Luca, Sabato & Vázquez Torres.

1.3 | Mexican cycads and their conservation status

Mexico has the second greatest diversity of cycad species (after Australia), with 77 recognized species across three genera: *Ceratozamia*, *Dioon*, and *Zamia*. Notably, over



FIGURE 1 Adult individuals of four cycad species. (a) *Ceratozamia mexicana*, (b) *Zamia inermis*, (c) *Zamia furfuracea*, and (d) Dion merolae.

95% of these species are endemic to Mexico (Calonje et al., 2025), which underscores the importance of conservation efforts and the implementation of protective regulations. Under the federal norm NOM-ECOL-059-94, the Mexican cycad species are classified into various conservation categories according to the demographic and ecological characteristics of their populations (SEMARNAT, 2010). This classification is based on understanding their survival and reproduction dynamics in the face of human-induced threats. Similarly, the IUCN Red List of Threatened Species (2022) reports that, in Mexico, 12 species are classified as Critically Endangered, 35 as Endangered, 10 as Vulnerable, three as Near Threatened, and one as Least Concern, with several species yet to be determined. Many cycad species are additionally subjected to regulations under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2021), which also contributes to their conservation.

The main threat to Mexican cycad species is habitats deforestation caused by agriculture, livestock grazing, mining, and urban development (Favián-Vega et al., 2019; Nadarajan et al., 2018; Octavio-Aguilar, Iglesias-Andreu, Núñez de Cáceres-González, et al., 2017), which occurs at an alarming rate of 500 000 hectares per year (Vovides, 2000). In addition to habitat loss, other factors include their use for medicinal and religious purposes, the spread of invasive exotic plants, damage by invasive species, and climate change (Carey et al., 2014). As a result, many Mexican cycad populations have experienced severe declines in recent years, becoming fragmented, isolated, and composed of a limited number of individuals (Cousins & Witkowski, 2017).

1.4 | Dimorphism as a consequence of dioecy

As mentioned above, accurate sex identification of plants in a population using observational or analytical methods is essential for conservation actions involving threatened species. In cycads, the reproductive structures differ clearly different between male and female plants, making them the most easily observable trait for sex identification (Figure 2). In the three genera that occur in Mexico (*Dioon*, *Ceratozamia*, and *Zamia*), the female strobilus (megastrobilus) is round-shaped, larger, and heavier than the male strobilus (microstrobilus), which is thinner and smaller. When mature, the microstrobilus releases pollen that, in Mexican cycads, is carried by beetle pollinators of the families Curculionidae or Languridae (Norstog, 1987; Norstog et al., 1986) to the female plant when the megastrobilus is receptive. In *Ceratozamia* and *Zamia*, the megastrobilus remains for over a year, during which seed



FIGURE 2 Plants of *Zamia furfuracea* from the population of Cienega del Sur, Veracruz, Mexico. (a) Female strobilus: female plant; (b) male strobilus: male plant.

fertilization and maturation occur (Norstog & Fawcett, 1989). In some species, seeds may undergo a post-maturation dormancy period of up to 1 year after being released from the strobilus (Sánchez-Tinoco et al., 2000; Sánchez-Tinoco et al., 2018). This reproductive stage represents the easiest period for sex identification. Otherwise, identifying the sex of juveniles' plants or those are not in their reproductive stage is challenging. In some species, such as *C. mexicana*, plants take up to 15–20 years to develop their first strobilus. Although sex is not determined by conspicuous chromosomal differences (Liu et al., 2022), finding alternative methods has become an ongoing area of research.

2 | HISTORICAL REVIEW OF THE METHODS FOR SEX IDENTIFICATION IN CYCADS

2.1 | Chromosome sex-based identification

Dioecy associated with heteromorphic sex chromosomes is relatively uncommon among plants. In fact, it is believed that sex chromosomes have evolved in only a limited number of dioecious angiosperms (Charlesworth, 2002). As a result, the presence of sex chromosomes in dioecious plants is rare. When sex chromosomes do occur, they typically follow an XY system, in which males are heterogametic (XY) and females are homogametic (XX) (Ainsworth, 2000). In some instances, these sex chromosomes can be homomorphic, meaning they are morphologically indistinguishable from the autosomes. Many dioecious plants, such as papaya and kiwi, do not show chromosomal heteromorphism. In these species, the genes responsible for sex determination appear to be in small chromosomal regions that closely resemble autosomes, specifically the non-sex chromosomes (Harvey et al., 1997). In other dioecious species, sexual differentiation has been linked to nuclear genes on the sex chromosomes, although these genes are often difficult to distinguish (Baránková et al., 2020).

Chromosome studies have helped identify the sexes in various angiosperm species, including *Phoenix dactylifera* L. (Siljak-Yakovlev et al., 1996). However, research on the cytology of sex determination in cycads remains limited, revealing a significant gap in our understanding of this important topic. Nonetheless, early evidence has shown the presence of sex-associated chromosomes in various dioecious plants across both lower and higher orders. Abraham and Mathew (1962) studied the karyotype and sex chromosomes of *Cycas pectinata* Buch.-Ham. They found that both male and female plants of this species share the same chromosome number ($2n = 22$) and exhibit nearly identical morphologies. However, female plants have satellites on two chromosomes in the somatic complement (pair III).

In contrast, male plants display heteromorphism for these chromosomes, with only one of the pairs carrying a satellite chromosome. Cytological studies conducted on *Cycas revoluta* Thunb. by Segawa et al. (1971) were the

first to suggest an XY system of sex chromosomes in *Cycas* species, indicating that females are the homogametic sex (XX) and males are the heterogametic sex (XY). Furthermore, XY-type sex chromosomes have been identified in five other *Cycas* species (Cycadaceae) and seven *Zamia* species (Zamiaceae), as reported by Sangduen et al. (2009).

The authors noted that satellite chromosomes can be used to differentiate between male and female *Cycas* plants. In contrast, the seven species of the genus *Zamia* exhibit considerable variation in chromosome numbers, ranging from $2n = 16$ to $2n = 24$. This variability presents a considerable challenge in distinguishing male from female *Zamia* plants through karyotyping, as both sexes lack defining satellite chromosomes. Other cycad species, including the Mexican species mentioned earlier, possess homomorphic sex chromosomes, further complicating sex identification using conventional cytogenetic techniques.

The fluorescence in situ hybridization (FISH) technique has been used in molecular cytotaxonomic studies by Tagashira and Kondo (2001), who studied the chromosomal phylogeny of *Zamia* and *Ceratozamia*. As noted by Kokubugata and Forster (2006), FISH could be particularly useful for sex identification, which cannot be determined with traditional cytogenetic methods. This emphasizes the need for alternative approaches to accurately identify sex within this genus, which can help maintain an appropriate sex ratio in their populations.

2.2 | Morphological traits-based sex identification

As Norstog and Nicholls (1997) pointed out, juvenile cycads do not exhibit distinguishable morphological features that allow sex identification. The ability to identify sex at the juvenile stage is highly desirable, as most dioecious species reveal their sex only at maturity, when they produce reproductive structures known as strobili (Chamberlain 1935). However, the sex identification process in cycads can be time-consuming, due to their long-life cycles. Early, accurate, non-destructive, rapid, and inexpensive methods for identifying sex are essential, particularly for long-lived species. However, clear identification of sex is possible only at the adult stage when the plants present their reproductive structures or remnants from the previous year (Sánchez-Tinoco et al., 1990).

Overall, cycads are expected to display vegetative traits associated with sexual expression. In most dioecious species, females tend to have higher reproductive costs than males (Obeso, 2002; Calonje et al., 2011). Typically, females begin their reproductive stage at a later age and larger size, while males tend to flower more frequently, exhibit faster vegetative growth, and often live longer (Delph, 1999). However, when fruiting is considered, females experience a significantly greater reproductive cost than males (Agren, 1988; Delph, 1990; Korpelaine, 1992; Pannell, 2025). Likewise, regarding nutrient requirements, females may demand more than males, particularly for nitrogen and phosphorus content (Agren, 1988); however, some research indicates that there may not be significant differentiation (Hemborg & Karlsson, 1999). It is important to note that it has recently been proposed that disparities in resource distribution cannot be used ad hoc to explain differences between the sexes in vegetative or other traits, since these distinctions may be more closely linked to variations in sexual function, as is the case in animals, rather than to differences in net allocation to reproduction (Midgley, 2022).

Over the past century, several studies have been conducted to identify the sex of cycad plants in natural populations based on vegetative traits. Notable works include those by Britton and Wilson (1926) on *Zamia latifoliata* Prenl (syn. *Z. pumila* L.), and by Liogier and Martorell (1982), and by Newell (1985) on *Zamia debilis* L. f. (syn. *Z. pumila* L.) and *Z. pumila* L. These studies focused on the vegetative morphology of cycads, which exhibits intrapopulation variation, particularly in leaves and petioles, some of which are also used as diagnostic characters in taxonomy (Griffith et al., 2014; Rubio-Méndez et al., 2019; Stevenson, 1981). In Mexican species, morphometric analyses conducted by Sánchez-Tinoco et al. (1990) examined a natural population at Coacotzintla Hill, municipality of Coacoatzintla, Veracruz, Mexico, where *C. mexicana* dominates the lower understory of a tropical montane cloud forest (Figure 1a). Alejandre-Rosas et al. (1990) quantified 1722 adult individuals within one hectare, reporting a sex ratio of 328 female to 449 male plants, with 945 individuals labeled as "undetermined" due to insufficient information regarding their sex. Sánchez-Tinoco et al. (1990) also highlighted that an important distinguishing feature of mature fronds in adult specimens of *C. mexicana* is the density of spines on the petioles. In male individuals, these spines are longer and denser than those found on female plants (Figure 3a,b).

The hypothesis of sex differentiation by spines was tested during the 1990s in *Ceratozamia* (*C. hildae* Landry & Wilson, *C. robusta* Miquel, *C. kuesteriana* Regel and *C. euryphyllidia* Vázq. Torres, Sabato & D.W. Stev). The hypothesis was also tested in the genus *Zamia* (*Z. furfuracea* L. and *Z. spartea* De Candolle) and *Dioon* (*Dioon edule* Lindley, *D. caputoi* De Luca, Sabato & Vázq. Torres, and *D. califanoi* De Luca & Sabato), but no results supported the hypothesis (Sánchez-Tinoco et al., 1990).



FIGURE 3 *Ceratozamia mexicana* plant. (a) Petioles of the mature fronds of the male plant with details of the spines (arrow). (b) Details of the spines on the petiole of a mature frond of a female plant (arrow).

Another study (Sánchez-Tinoco et al., 1993) was conducted on a population of *Z. inermis* (Figure 1b), located on the Chalagüites Hill, Sierra de Manuel Díaz, Veracruz, Mexico, where this species was present as a component of the low deciduous forest. The population included 218 plants, and 18 females and 21 males. In Sánchez-Tinoco et al. (1993), it was reported that *Z. inermis* whose specific name refers to the absence of spines on the petioles, exhibits a combination of traits useful for sex segregation. These traits include the length of the main leaf, the apical width of the upper surface, and the diameter of the petiole, among others. Similar findings were reported in a natural population of *Z. inermis* from Mozomboa, municipality of Actopan, Veracruz, Mexico (Iglesias et al., unpublished data). Among the eight morphometric variables analyzed (number of leaves, leaf length, number of leaflets per leaf, leaflet length, leaflet width, stem height, stem diameter, and number of stems per plant), only two were significantly greater in females: the number of leaflets per leaf and leaflet width.

In a population of *Z. furfuracea* (Figure 1c), Baldo-Romero et al. (2013) observed similar patterns. The number of leaflet pairs, leaf width, average leaflet width, and petiole diameter differed between sexes. According to Baldo-Romero et al. (2013) among the nine morphological variables analyzed, only the number of leaflet pairs, leaf width, average leaflet width, and leaflet diameter were significantly greater in females than in males ($p = 0.43$, $p = 0.32$, $p = 0.041$, and $p = 0.002$, respectively). Based on their results, the authors concluded that these characters may serve as useful morphometric markers for determining sex in this population. This distinction may be attributed to the fact that female cycads require more resources to produce larger cones and mature seeds. As a result, they may have a greater number of reserve cells not only in the trunk but also at the base of the petiole.

In *Dioon*, Santos-Cruz (2016) found sexual dimorphism in *D. merolae* De Luca, Sabato & Vázq. Torres (Figure 1d), within a population in the municipality of Jiquipilas, Chiapas, Mexico. Her study identified significant differences between several variables, including rachis length and leaf number. The results of the principal component analysis (PCA) conducted using Past 4.15 software for Mac (Hammer et al., 2001), indicated that the two principal axes explained 84.74% of the population variation between males and females. Rachis length and stem length were the variables that contributed most to this variation. The PCA successfully separated the studied population into two distinct groups (males and females). Overall, females exhibited a longer rachis and a greater number of leaves per crown compared to males. Additionally, Acero-Cruz (2016) evaluated 31 vegetative traits to analyze the sexual dimorphism in *D. merolae* under disturbed and undisturbed conditions in the municipality of Jiquipilas, Chiapas. Nine and six variables (for disturbed and undisturbed populations, respectively) were identified, allowing discrimination between males and females. The findings indicate that all these variables contribute to females being larger than their male counterparts. In the undisturbed population, clear sex segregation was observed; however, this segregation was less pronounced in the anthropogenically disturbed population. This suggests that anthropogenic disturbances may negatively affect the expression of sexual dimorphism in this species. The number of leaves and the length of the middle leaflet were the traits that most significantly explained the variation in sexual dimorphism (Figure 4).

In *Cycas*, Kaviani et al. (2014) compared morphological traits between 2-year-old male and female seedlings of *C. revoluta* Thunb., with the sex of the plants determined from those that had already reached sexual maturity. They found that the distance between leaflets 29 and 30 on the leaf rachis differed significantly between the sexes. De Luca et al. (2024) further demonstrated that it is possible to distinguish the sexes at a very early stage of plant growth in this species. Their results indicated that the average distance between leaflets on the leaf rachis is a sexually dimorphic morphological trait in *Cycas* seedlings. According to these authors, incorporating morphological characteristics for sex differentiation in

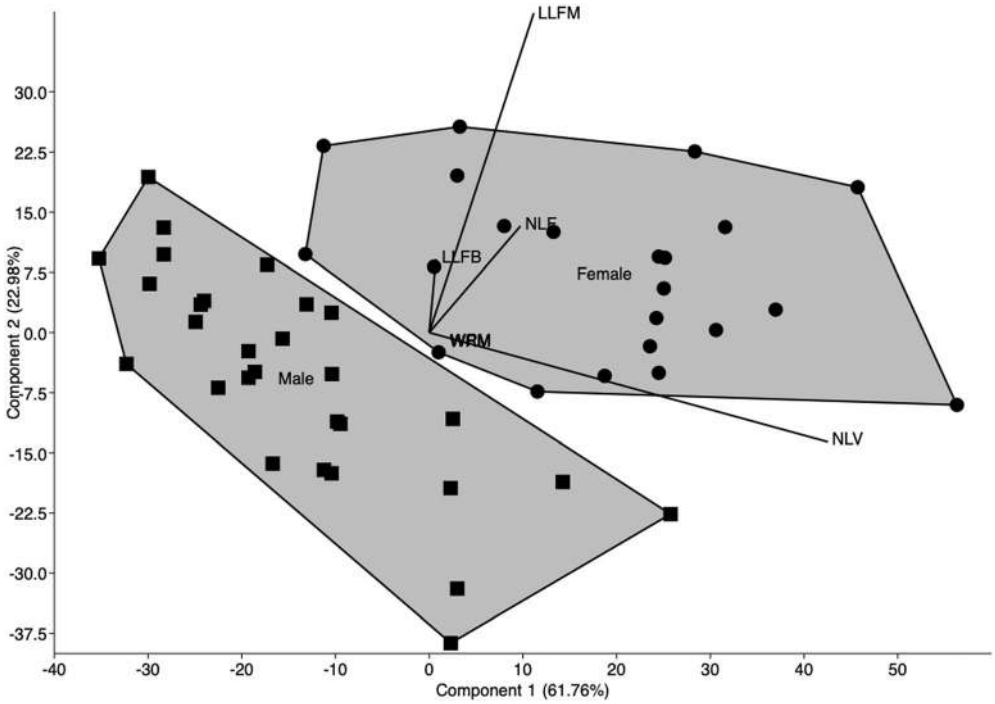


FIGURE 4 Principal component analysis (PCA) of six morphological traits used to differentiate sex in *Dioon merolae* from an undisturbed population in the Municipality of Jiquipilas, Chiapas. The biplots (LLFB) = length of the leaflet of the basal part; (LLFM) = length of the middle leaflet; (NLF) = number of leaflets; (WRM) = width of the rachis in the middle part; (WPM) = width of the petiole in the middle part; (NLV) = number of leaves. The length and direction of each biplots indicate relative importance of each trait in representing the variation between sexes (unpublished data, reanalyzed from Acero-Cruz, 2016). The circles represent the female individuals and the squares the male individuals measured.

greenhouses and nurseries is both quick and cost-effective, especially when combined with automated image recognition systems.

2.3 | Biochemical markers-based sex identification

Isoenzymatic techniques have been employed to identify biochemical markers that may correlate with plant sexual types. In particular, the variation in peroxidase isoenzymes (EC 1.11.1.7) has proven to be quite effective for this purpose, as these enzymes are suggested to play a role in the hormonal control of sex in many dioecious species such as *Populus deltoides* L. and *Populus ciliata* W. Bartram (Shirkot et al., 2006), *Hippophae salicifolia* D. Don (Shirkot et al., 2009), and *P. ciliata* Wall. ex-Royle (Kumari et al., 2014). Using cation peroxidase banding patterns in papaya, it was possible to differentiate males from females; however, females could not be distinguished from hermaphroditic plants on this basis (Sriprasertsak et al., 1988). Begum et al. (2010) studied the effect of quantitative and qualitative changes in foliar peroxidase on sexual expression in the Pant Papaya-1 variety and found that female plants had higher peroxidase levels than male plants. However, the peroxidase pattern showed no correlation with the sex type. In *Cannabis sativa* L., Truta et al. (2002) found significant differences in peroxidase and esterase patterns in the leaf tissue of male and female plants when studying the variation in these isoenzymes. They also detected differences between the sexes in catalase activity and soluble protein levels. Similar variation was detected by Garcia-Olvera & Iglesias-Andreu (unpublished data) when analyzing the activity of several enzyme systems in the leaf tissue of male and female plants from the *C. mexicana* population in Coacoatzintla, Veracruz (Figure 5).

The differences observed in esterase activity between male and female individuals from this population of *C. mexicana* were of particular interest. The functional characterization of plant esterases has generally been little explored. However, it has been suggested that cholinesterase may have proteolytic activity in addition to their cholinergic activity, acting as proteases to regulate cell growth and development (Small, 1990). It remains unclear how esterase activity in plants influences sexual expression, especially in female reproductive structures and various differentiation processes. Future studies are needed to clarify their roles in these areas.

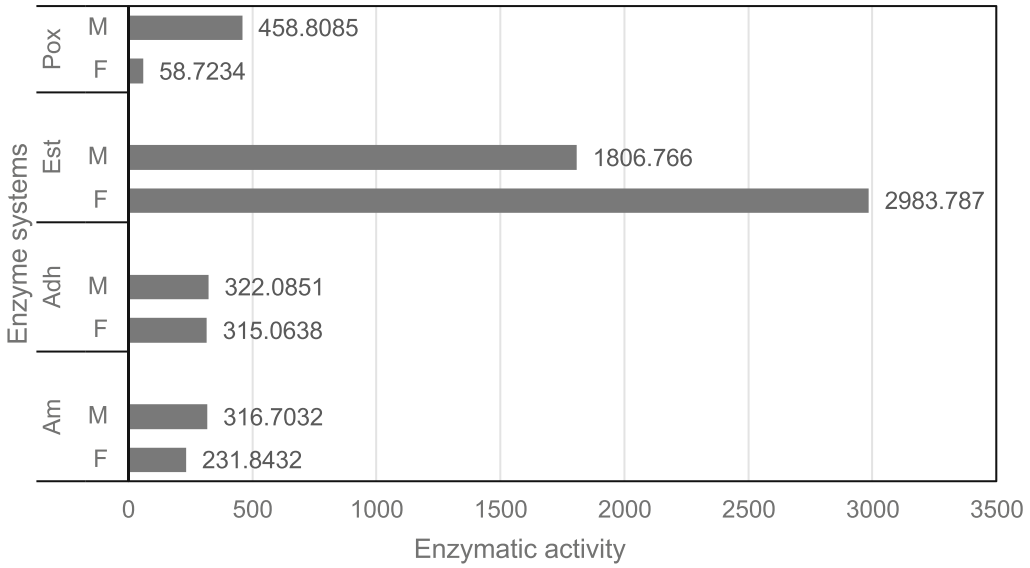


FIGURE 5 Variation in the activity of four enzyme systems (Am: amylase; Adh: alcohol dehydrogenase; Est: esterase; Pox: polyphenoloxidase) in the leaf tissue of male (M) and female (F) *Ceratozamia mexicana* plants.

Roy et al. (2012) aimed to identify a distinctive peroxidase isoenzyme marker in *Zamia fischeri* Miq. However, they found that male and female genotypes could not be differentiated using vegetative tissue. Consequently, this eliminated the peroxidase isoenzyme as a viable candidate for sex determination at the pre-flowering stage. However, significant alterations in the peroxidase profile were observed when reproductive tissue samples (microspores and ovules isolated from plants of the respective sexes) were used. The same authors also used the technique of two-dimensional gel electrophoretic analysis of male and female plants of *Z. fischeri*, using microspores and ovules, and detected fifteen unique points of relatively high molecular mass in the microspore-derived protein sample (male). Twenty-five unique points, with a molecular weight between 20 and 94 kDa, were detected in the ovule-derived protein sample (female).

2.4 | Molecular markers-based sex identification

Molecular markers associated with sex chromosomes, along with bulked segregant analysis (BSA; Michelmore et al., 1991), have been highly effective for early sex identification in various plant species. In cycads, early attempts to distinguish sexes based on DNA included random amplified polymorphic DNA (RAPD) markers, as studied in *Encephalartos natalensis* Dyer & Verdoorn (Prakash & Van Staden, 2006) and *Cycas circinalis* L. (Gangopadhyay et al., 2007). Earlier molecular analyses used primers OPK_{10} (5′ GTGCAACGTG 3′) and OPE_{17} (5′ CTACTGCCGT 3′) to demonstrate the presence of sex-specific bands in *C. mexicana*. Two of the three male DNA pools showed a 500 bp band with primer OPK_{10}, and two of the three pools showed bands of 1400 bp and 1100 bp with primer OPE_{17} (Iglesias-Andreu et al., 2010). For this review, we include new results from five Operon RAPD primers evaluated in *C. mexicana*. No distinct sex-specific bands were detected in the DNA pools tested using primers OPB_{08} and OPB_{17} (Figure 6).

In *Z. furfuracea*, no sex-associated RAPD markers were detected using different RAPD primers (OPM_{02}, OPM_{04}, OPM_{06}, OPM_{10}) (Iglesias, unpublished data). According to Iglesias-Andreu et al. (2010), sex-associated bands tend to appear inconsistently at low frequency (Figure 7).

Regarding other molecular markers, studies conducted on *C. mexicana* have detected one Inter Simple Sequence Repeat (ISSR) band that appears to be associated with males (Sánchez-Coello, 2012). According to the cited authors, the 124 bp sequence associated with males resembles the NEEDLY gene, which is a functional orthologue of the LEAFY gene and a floral transcription factor. This band subsequently became a Sequence Characterized Amplified Region (SCAR) marker to identify male individuals within the species (Sánchez-Coello et al., 2018). Conversion from a random primer to a longer, specific primer ensures a high level of repeatability and reliability. In this case, the use of sex-linked SCAR markers has proved particularly valuable for sex differentiation, as they can indicate the presence or absence of the sex-specific band. Similarly, in *Cycas tanqingii* DY Wang the female sex-linked RAPD marker was

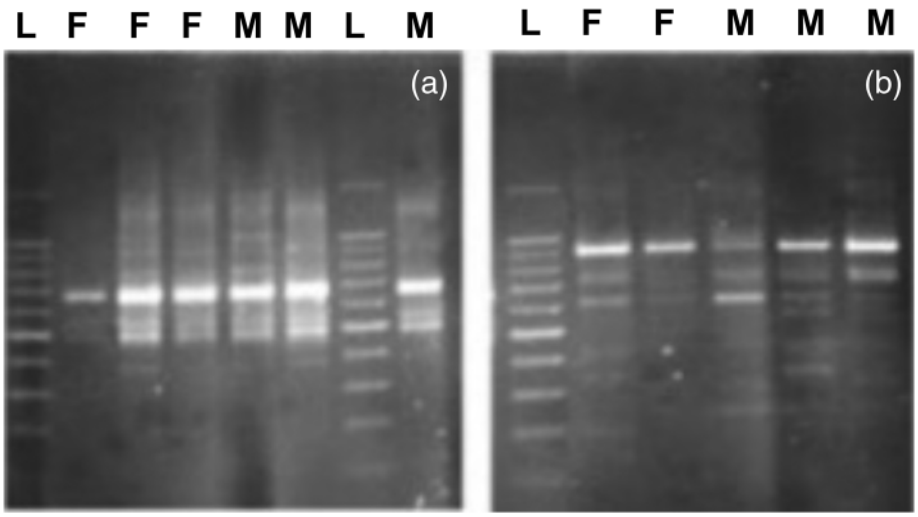


FIGURE 6 RAPD profiles of male and female DNA mixtures in *Ceratozamia mexicana*. (a) RAPD profiles obtained with OPB_{08} primer; (b) RAPD profiles obtained with the OPB_{17} primer; L: ladder 100 bp; M: male; F: female. RAPD, random amplified polymorphic DNA.

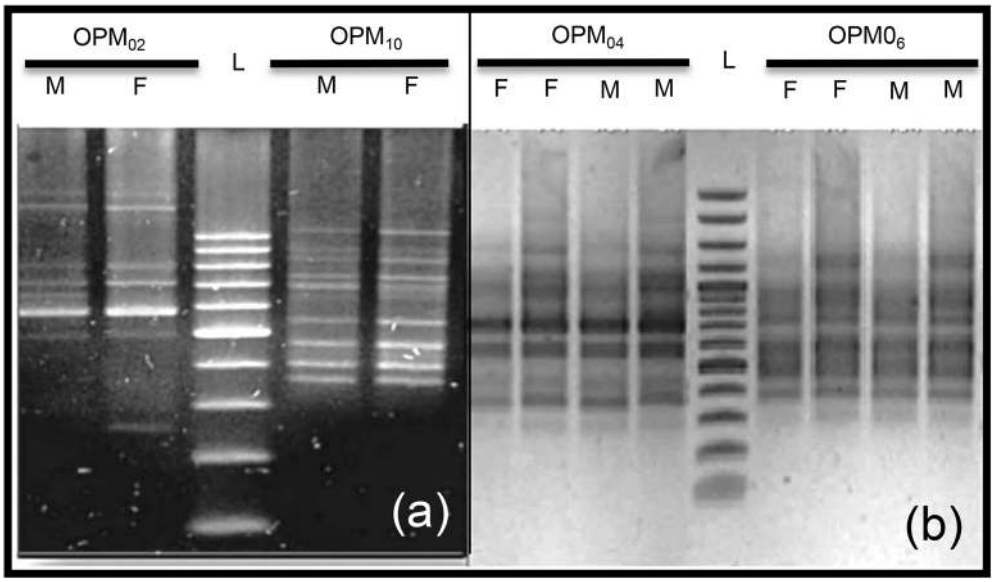


FIGURE 7 RAPD profiles of male and female pools in *Zamia furfuracea*. (a) RAPD profiles obtained with OPM_{02}, and OPM_{10} primers; (b) RAPD profiles obtained with OPM_{04} and OPM_{06} primers; L: ladder 100 bp; M: male; F: female. RAPD, random amplified polymorphic DNA.

effectively transformed into a stable SCAR marker, providing a robust and reliable conservation tool (Milewicz & Sawicki, 2013).

In a study by Iglesias-Andreu and Favian-Vega (2021) using six ISSR primers on the *Z. inermis* population of Mozomboa, Veracruz, differences were observed in the number of bands in the DNA pools of male (59 bands) and female (56 bands) individuals. Using the IS-18 primer (5′ ACTGACTGACTG3 ′), a band of 867 bp was detected in four of the six DNA pools tested and appears to be associated with the female sex. This band was detected in more than 60% of the female DNA pools tested.

Based on the results obtained by Iglesias-Andreu and Favian-Vega (2021) from the Principal Coordinates Analysis (PCoA), the first two principal coordinates explained 72.17% of the total variance, allowing an adequately detection of the spatial distribution of sexually differentiated individuals into two groups. Due to the heterogeneity observed in the groups, resulting from the presence of two DNA pools of male (3M) and female (6F) individuals, which displayed an atypical spatial distribution, a clear grouping by sex among the individuals under study could not be observed.

From the examples shown above, detecting sex-associated RAPD and ISSR markers appears to be challenging, as they typically occur at very low frequencies and may not be consistently present. Some researchers, such as Mandolino et al. (1999), have used a larger number of primers—up to 20—to identify male sex markers

in *C. sativa* L. Similarly, Polley et al. (1997) reported using between 400 and 900 RAPD primers for *Pistachio vera* L. and *Humulus lupulus* L., with mixed results. These findings highlight the complexity involved in identifying such markers.

Later, Fernández et al. (2024) used PCR markers to identify the sex of male ($n = 17$) and female ($n = 13$) individuals of *Cycas micronesica* K.D. Hill. Using a set of male-specific primers, CYCmic_MADSY (F/R), they demonstrated greater precision and reliability for correct sex identification for this species. More recently, the findings of Liu et al. (2021) have generated significant progress in this topic. They published the first cycad genome, enabling the identification of sex-specific loci was finally accomplished. Consequently, Yang et al. (2015), in an article published in this Special Feature, identified the sex of all individuals in a population of *C. panzhihuaensis*. Clugston and Kenicer (2022) also identified two genes involved in the development of female (CYCAS_010388 on chromosome 2) and male (CYCAS_034085 on chromosome 8) strobili. In assessing the utility of the CYCAS_034085 gene, Clugston et al. (2024) confirmed that it is present exclusively in microsporangial plants. They used a simple qualitative PCR assay to identify the sex of *Encephalartos* Lehm. Individuals. The application of these findings shows strong promise for sex identification in cycads, which will contribute to their conservation.

2.5 | Exploring eco-physiological basis for sex-determination in cycads

As an exploratory avenue for research, it has been suggested that physiological differences between male and female plants may lead them to occupy distinct microsites (Dawson & Bliss, 1989). One mechanism to enhance resource acquisition from the environment is through the differential selection of microsites based on sex (Dawson & Geber, 1999). If females experience higher reproductive costs, selection pressure may favor individuals with higher photosynthetic rates, even at the expense of greater water use, prompting females to grow in more humid environments. In contrast, males, with lower reproductive costs, can thrive in more challenging habitats due to their ability to tolerate or avoid water deficits (Dawson & Bliss, 1989; Dawson & Ehleringer, 1993; Kohorn, 1994; Freeman et al., 1976). Females may be favored in microsites with limited fertility if they increase biomass allocation to roots and enhance nutrient uptake. In radiation-limited environments, females can develop a self-shading canopy with biochemical characteristics that maximize light capture and carbon acquisition (Dawson & Geber, 1999). Conversely, males may allocate more resources to vegetative growth, particularly to the root system, which may confer tolerance to extreme environments (Hoffmann & Alliende, 1984).

Reproductive costs can influence the ratio of females to males in a population (Nicotra, 1998). In dioecious plants, the sex ratio often skews toward a higher number of males, particularly in habitats impacted by abiotic factors such as poor soil or low humidity (Barrett et al., 2010). When reproductive costs are similar for both sexes, the expected male-to-female ratio is 1:1 (Cipollini & Stiles, 1991). However, males are typically found to be more abundant than females (Delph, 1999). This male predominance may stem from their greater competitive abilities and lower reproductive costs compared with females (Agren, 1962). It could also arise from higher mortality rates among females (Obeso et al., 1998). In environments where individuals lack sex-specific adaptations and females incur higher reproductive costs, males are likely to perform better overall, as their energy requirements are lower than those of females. Consequently, males may be more abundant and exhibit greater vigor than females (Dawson & Ehleringer, 1993). These ideas have not been explored in cycads but seem promising as new lines of inquiry.

2.6 | Discussion on the epigenetic basis for sex-determination in cycads

It has been suggested that epigenetic factors may be involved in the mechanisms of sex determination in plants (Tanurdzic & Banks, 2004). It is well established that the epigenome is more susceptible to environmental influences than that of animals. However, unlike genetic inheritance, not all epigenetic changes are heritable. Nonetheless, epigenetic regulation can be heritable for at least a few generations and may act together with genetic variation to shape evolutionary trajectories (Webster & Phillips, 2024). Some heritable epigenetic marks generate signals that is transmitted from the mother plant to the progeny and direct restoration in the early zygote or in meristematic cells of the next generation. This information may represent a form of molecular memory considered part of a "soft inheritance system" (Richards, 2006). In sex identification systems, epigenetic changes may be mediated by chromatin modifications in sex-determining regions, involving DNA methylation and nucleosome histone acetylation (Kanchanaketu et al., 2007; Leite-Montalvâo et al., 2021).

In early explorations on cycads, Gorelick (2003, 2005) and Gorelick and Osborne (2007) suggested that sex expression in cycads might have an epigenetic basis.

According to the authors, this phenomenon could be influenced by processes such as cytokine methylation and chromatin formation, both of which regulate the genes responsible for gamete or sex hormone production. Gorelick (2003) proposed that dioecy and sex chromosomes evolved from the diploid ancestors of hermaphrodites, originating as a pair of autosomes. In this scenario, one of the chromosomes possessed more methyl groups near a region controlling sex than its counterpart. This methylation could have suppressed transcription, including the genes involved in gamete production, allowing hermaphrodites to evolve into distinct male or female forms. Additionally, differential methylation processes may have contributed to reduced recombination and accelerating the Muller ratchet effect. Specifically, the authors suggested that males should exhibit a higher number of methylated regulatory loci in genes related to female functions. As a result, both sexes may display distinct patterns of methyl group distribution in their nucleotide sequences. To test this hypothesis, the methylation-sensitive amplified fragment length polymorphism (MS AFLP) technique, as described by Cervera et al. (2002), has proven effective in identifying the methylation patterns in plants. This technique is very useful for studying plant methylation (Bolukbasi & Aras, 2016) and differentiating between the methylation patterns of the two sexes. However, the results reported by Baldo-Romero (2015) in *Z. furfuracea* did not establish a clear correlation between methylation patterns and the sex of the sexually differentiated individuals studied. They observed variation in total methylation frequency, with males showing 37.5% and females 31.5%. Similarly, Sultana et al. (2016), when comparing the sexual expression of cycads using various molecular methods, concluded that sex identification in cycads might be result from DNA methylation rather than from sex-specific markers.

However, contrasting results have been published before. Kanchanaketu et al. (2007) identified differences in methylation patterns between the sexes in species of the genera *Cycas* and *Zamia*. Related to this, Osborne and Gorelick (2007) documented 33 instances of sex reversal in six genera and 17 cycad species, often linked to physical damage or major environmental disturbances. They found that changes from male to female were typically associated with low-light conditions or exposure to severe cold, whereas changes from female to male occurred in response to extreme drought conditions. However, to date, whether temperature-based treatments can alter sex in cycads has not been evaluated under controlled conditions. In general, the idea of epigenetic influence on sex determination in cycads has been explored with mixed results, and the genetic basis of sex determination reported by Liu et al. (2022) may represent the strongest evidence for the underlying mechanisms.

2.7 | Further research on the sexual dimorphism of cycads

It should be noted that next-generation sequencing (NGS) methods are facilitating advances in cycad sex determination. These methods, which include reduced representation sequencing and whole genome sequencing (WGS), allow the identification of sex-specific genetic markers even in species with morphologically identical chromosomes, such as cycads (De Luca et al., 2025). These authors validated the efficacy of double digestion restriction site-associated DNA sequencing (ddRAD-seq) technique, which is especially useful for species with large genomes, such as cycads, as this approach is more advantageous over WGS.

Finally, the development of proteomics has opened new avenues to find out early on the sex of dioecious plants (Yang et al., 2015). Although only a few studies that have used proteomics to identify sex, ginkgo (*Ginkgo biloba* L.) represents a noteworthy example (Yang & Fu, 2012). This approach can reveal sex-specific protein expression patterns, which could facilitate in the development of tools for early sex identification in cycads. Mass spectrometry allows the analysis of protein content in biological samples, enabling researchers to identify proteins unique to individuals of a specific sex. The basis for sex determination and the resulting demographic and evolutionary effects in cycads may also be better understood with the help of these methods.

3 | CONCLUSIONS

Understanding sexual differentiation in dioecious species is crucial for studying their reproductive mechanisms and strategies. This is particularly important for cycads, a group that includes many threatened and endangered species. Early sex identification is of great interest for elucidating the genetic and epigenetic mechanisms involved in sex determination. It also plays a crucial role in maintaining the viability of natural populations of threatened species. From a conservation perspective, these findings emphasize the importance of establishing management programs that ensure adequate numbers of both male and female plants are spatially distributed to maximize reproductive success and sustain successful reproduction within these populations. This review aims to promote further research in this field, as it will help clarify the mechanisms of sex determination and inform the

management and conservation of these vital, long-lived species, whose natural populations have persisted to the present day.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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