

IDENTIFICATION OF SEX IN *ZAMIA INERMIS* USING ISSR MARKERS

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Abstract: *Zamia inermis* is a dioecious cycad endemic to the State of Veracruz that is on the risk extinction. Sex-specific markers are important in understanding the mechanism of sex determination. However, since little is known about the mechanism of sex determination in *Z. inermis*, we proposed looking for Inter-simple Sequence Repeats (ISSR) markers that could be linked to sexual expression in this species. Using DNA bulk samples of male and female genotypes and 6 ISSR primers, a female marker (~867 bp) was identified with primer ISSR-18, which was present in 66% of the DNA mixtures of the female genotypes analyzed. The results of the Principal Coordinate Analysis performed revealed a tendency for clustering of genotypes of the same sex.

Keywords: conservation, cycad, dioecious, molecular markers, sex.

Introduction

Cycads are a type of gymnosperm that can be found in tropical and subtropical areas of the world. There are 54 species of cycads in Mexico, divided into three genera (*Ceratozamia* Brongn., 26 species, *Zamia* L., 15 species and *Dioon* Lindl., 13 species). MARTÍNEZ-DOMÍNGUEZ & al. (2018) found that 89 percent of them are endemic and provide 17% of the diversity.

Zamia inermis Vovides, J. D. Rees & Vázq. Torres (Zamiaceae, Cycadales), is an endemic species of Veracruz, Mexico, which is stated as a critically endangered species on the International Union for Conservation of Nature's Red List (IUCN, 2011) and as an endangered species on SEMARNAT's NOM-059-SEMARNAT-2010 [SEMARNAT, 2010]. This is mainly due to the absence of their natural pollinator to low recruitment rates, illegal trade, and land use change [OCTAVIO-AGUILAR & al. 2017; IGLESIAS-ANDREU & al. 2017]. Like all cycad species, *Zamia inermis* is dioecious and it is difficult to determine the sex of seedlings during the early stages of development; additionally, reproductive events are rare, with low and sporadic seed production, from which only a small proportion of seedlings are obtained [OCTAVIO-AGUILAR & al. 2017]. The morphological evaluation of cycad reproductive structures has been used to determine sex [SÁNCHEZ-TINOCO & al. 1990; SÁNCHEZ-TINOCO & al. 1993; BALDO-ROMERO & al. 2013]. Other molecular markers, such as Random Amplified Polymorphic DNA (RAPD marker) [WILLIAMS & al. 1990] and Amplified Fragment Length Polymorphism (AFLP marker) [VOS & al. 1995], have lately gained popularity.

Although some molecular markers have been successfully employed for sex identification in cycad species such as *Encephalartos natalensis* R. A. Dyer & Verdoorn. [PRAKASH & VAN STADEN, 2006], and *Ceratozamia mexicana* Brongn. [IGLESIAS-

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ANDREU & al. 2010], it should be highlighted that markers based on Simple Sequence Repeats (ISSR) [ZIETKIEWICZ & al. 1994] have been highly efficient for distinguishing the sexes of some cycad species, like *Cycas circinalis* L. [GANGOPADHYAY & al. 2007] and *Cycas tanqingii* D. Y. Wang [JING & al. 2007], and *Ceratozamia mexicana* Brongn. [SÁNCHEZ-COELLO & al. 2018] due to their simplicity, abundance, reproducibility, low cost, and speed of analysis. However, no information about its use for this purpose has been found in *Z. inermis* to date. To contribute to the identification of sex in this species, we propose to develop a genetic sex marker that allows us to gender as well as sex ratio determination at any stage in the life cycle and, eventually, the understanding of sex ratio dynamics in this unique natural population.

Material and methods

Study site and plant material. The only natural population of *Zamia inermis* is observed in the Sierra de Manuel Diaz, close to the town of Mozomboa (municipality of Actopan), in the central part of Mexico's Veracruz State [VOVIDES, 1983], between 19°32'54.90" north latitude and 96°27'29.80" west longitude [VOVIDES, 1983]. This population, which is distributed across about 2.5 km² of seasonally dry and scattered grassland, is extremely small. This population has an approximate altitude ranging from 200 to 300 m.a.s.l. This locality is characterized by an AW₁ climate with two climatic subtypes according to the Koppen climate classification system modified by GARCÍA (1988). This type of climate is characterized by being hot and sub-humid, with an intermediate level of humidity, between AW₀ and AW₂, with rainfall in summer, and a mean annual temperature of the coldest month higher than 18 °C and the hottest month higher than 22 °C.

Sample collection was conducted in the 9.7 km² area located on two hills in central Veracruz [OCTAVIO-AGUILAR & al. 2017] from adult individuals sexually identified based on their reproductive structures. To conduct this study, 2-4 leaflets were collected from each of the 36 individuals (18 males and 18 females) in four 20 m × 20 m plots. With the aid of a GPS (Garmin eTrex Legend, Olathe, Kansas, USA), all the individuals were georeferenced. Each leaf sample was individually labeled and transported in a cooler to the "Instituto de Biotecnología y Ecología Aplicada" (INBIOTECA), from "Universidad Veracruzana", for molecular studies.

Genomic DNA isolation. The genomic DNA from the collected individuals of *Z. inermis* was extracted using the STEWART & VIA (1993) procedure, which depends on the use of CTAB (Cetyltrimethylammonium bromide, Sigma Aldrich). A fluorometer (Qubit 2.0, Invitrogen, USA) has been used to assess the purity of the DNA once it has been correctly obtained. Each sample's DNA stock solution was diluted to a concentration of 50 ngL⁻¹ for PCR amplification. The Bulk Segregant Analysis (BSA) method was used to establish six DNA bulk samples. The bulk DNA samples came from three individuals (one for each sex) [MICHELMORE & al. 1991]. This method was used to screen the individuals of known sex to rapidly detect markers linked to any genomic region that may be sex-linked. A potential sex-linked marker was one that was present in the sex bulks analyzed but not in the alternate sex bulk.

ISSR markers. Twenty-five ISSR primers were selected (UBC, University of British Columbia, Vancouver, Canada) (Table 1).

Table 1. List of ISSR primers, their sequences and annealing temperature

No.	ISSR primer	*Sequence of nucleotides (5'-3')	Annealing temperature (°C)
1	UBC-809	AGAGAGAGAGAGAGAGG	52
2	UBC-818	CACACACACACACAG	52
3	UBC-827	ACACACACACACACG	52
4	UBC-829	TGTGTGTGTGTGTGTC	52
5	UBC-848	CACACACACACACARG	55
6	ISSR-01	GTAGTAGATAGATA	40
7	ISSR-02	GTAGTAGATAGATARG	45
8	ISSR-03	GTAGTAGATAGATARY	45
9	ISSR-04	GACAGACAGACAGACA	48
10	ISSR-05	GACAGACAGACAGACARG	55
11	ISSR-06	GACAGACAGACAGACARY	55
12	ISSR-07	GACACGACACGACACGACAC	55
13	ISSR-08	ACTGACTGACTGACTG	48
14	ISSR-09	ACTGACTGACTGACTGRG	55
15	ISSR-10	ACTGACTGACTGACTGRY	55
16	ISSR-11	GATAGATAGATA	35
17	ISSR-12	GTAGTAGATAGATARG	37
18	ISSR-13	YRGATAGATAGATA	37
19	ISSR-14	GACAGACAGACAGACA	40
20	ISSR-15	GACAGACAGACARG	45
21	ISSR-16	YRGACAGACAGACA	40
22	ISSR-17	GACACGACAC	35
23	ISSR-18	ACTGACTGACTG	40
24	ISSR-19	ACTGACTGACTG	45
25	ISSR-20	YRACTGACTGACTG	40

*R, purine (A or G), Y, pyrimidine (C or T). The primers selected are indicated in bold. UBC: University of British Columbia, Canada.

PCR reactions were performed in duplicate, using a reaction mix in a 25 µL volume containing: 5X PCR buffer, 25 mM MgCl₂, 5 mM dNTPs, 25 pM primers, 1.5U of Taq DNA polymerase, and 50 ng of DNA. Amplifications were carried out in a thermal cycler (AXIGEN®, Foster City, California, USA) under the following thermal conditions: a 7-minute denaturation step at 94 °C, 35 cycles of 30 seconds at 94 °C and 45 seconds at various alignment temperatures (37-55 °C), depending on the primer, and a 10-minute final extension at 72 °C. Positive and negative controls were included in all cases. Electrophoretic separations were performed at 100 V for 80 min on 2% (w/v) agarose gels in 1X TAE buffer. A 100 bp molecular weight marker (Bioline brand, Memphis, Tennessee, USA) was used to evaluate 5 µL of each

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of the amplified products. Afterwards, the gels were stained with ethidium bromide (10 mgmL⁻¹) and photographed using a photo-documenter (UVP, Upland, California, USA).

Analysis of data. Based on the type of primer and their size in base pairs (bp), bands that exhibited appropriate resolution and reproducibility in the obtained band profiles were recorded in a primary data matrix (values of 1: presence and 0: absence of the band). The latter was determined using a 100-bp DNA molecular size marker (Bioline brand) as a reference. Using the Paleontological Statistics (PAST) software, the data matrix was analyzed using Principal Coordinate Analysis (PCA) to identify the grouping patterns among the genotypes evaluated according to their sex [HAMMER & al. 2001].

Results and discussions

According to the results, only 6 of the 25 ISSR primers examined allowed for the detection of repeatable and reproducible band profiles. The ISSR protocol was correctly developed on this basis, enabling the detection of 56 and 59 bands, respectively, for the DNA bulk male and female samples evaluated. ISSR-18 was one of the primers in this analysis that had the best resolution and repeatability. In this study, the number of bands detected ranged from 7 to 14 in DNA bulk male samples and from 6 to 15 in DNA bulk female samples, with an average of nine ISSR bands in both sex expressions (Table 2).

Table 2. Sequence of ISSR primers and number of bands detected by sex

ISSR Primers	*Sequence of nucleotides (5'-3')	No. of bands / sex	
		Male	Females
UBC-848	CACACACACACACARG	7	7
ISSR-04	GACAGACAGACAGACA	14	13
ISSR-05	GACAGACAGACAGACARG	8	6
ISSR-06	GACAGACAGACAGACARY	10	15
ISSR-18	ACTGACTGACTG	9	10
ISSR-20	YR ACTGACTGACTG	7	8
Total		56	59

*R, purine (A or G), Y, pyrimidine (C or T). Y: C/T; R: A/G. UBC: University of British Columbia, Canada.

The presence of an 867 bp band was detected with the ISSR-18 primer in four of the six DNA bulk samples of males and females examined; that is, it was present in 66% of the DNA samples of female individuals (Figure 1) evaluated.

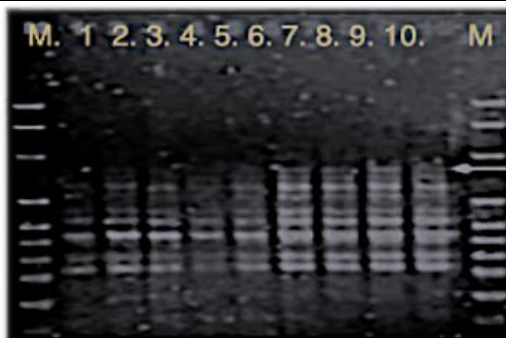


Figure 1. ISSR band profiles. The arrow indicates the 867 bp band detected with the ISSR-18 primer in DNA bulk female samples. Lanes 3-8: DNA bulk male samples (1-5); lanes 6-10: DNA bulk female samples (from left to right); M: 100-bp ladder.

According to the results of principal coordinate analysis (PCA), the first two principal coordinates explained 72.17% of the total accumulated variance (Figure 2). DNA samples from sexually differentiated individuals were spatially distributed within the first two coordinates (Figure 2). Group I, located in the lower left part of the graph, explained 51.4% of the total variance. This group consisted of five bulk DNA samples, of which four corresponded to female bulk DNA samples. Group II, located at the top of the graph, explained 20.87% of the total accumulated variance (Figure 2). This group was composed of five DNA bulk samples, corresponding to one female DNA bulk sample and four male DNA bulk samples (Figure 2). It was not possible to observe in this study a clear grouping of individuals by sex, using Principal Coordinates Analysis (PCoA) due to the atypical spatial distribution presented by two of the bulk DNA samples evaluated (3M: bulk male DNA sample, and 6H: bulk female DNA sample) (Figure 2).

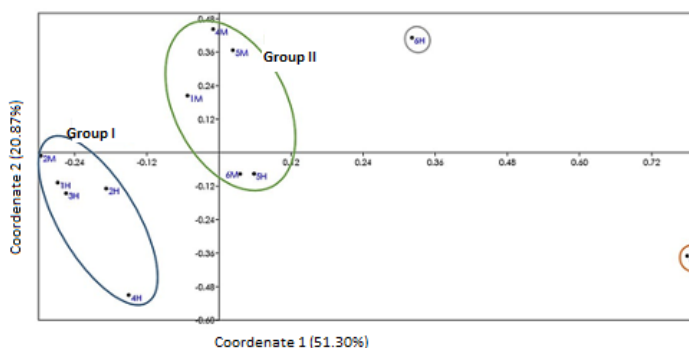


Figure 2. Principal Coordinates Analysis (PCoA) of DNA bulk samples of sexually differentiated individuals in *Z. inermis*. Group I (4 DNA bulk female samples), Group II (4 DNA bulk male samples). H = female, M = male.

Even though dioecy is a common trait among gymnosperms, accounting for roughly 65 percent of all currently identified species [WALAS & al. 2018], the search for sex-segregating molecular markers in this group of plants has been limited [ROY & al. 2012]. Sex-

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linked bands, as is widely known, occur rarely and not always in the same way. As is known, sex-linked bands usually occur very infrequently and not always consistently. As a result, very little is known regarding cycads, although they will presumably be the first dioecious seed-bearing lineage and occupy a major evolutionary position among land plants.

In *Zamia fischeri*, two RAPD markers were reported to be linked with each sex, as well as a male gender-specific band which showed some homology with a microsatellite sequence from *Araucaria angustifolia* (Bertol.) Kuntze [ROY & al. 2012], potentially useful for early male sex identification. Sex-specific RAPD markers have been identified in *Cycas circinalis* [GANGOPADHYAY & al. 2007] and *C. mexicana* [IGLESIAS-ANDREU & al. 2017].

It was interesting to find that the ISSR marker detected was associated with the female genotype even though, according to AINSWORTH (2000), most molecular markers are associated with the male sex. Bands are linked with the female sex in other cycad species, like *Encephalitis natalensis*. PRAKASH & VAN STADEN (2006) found RAPD markers specific to the female method analysis, and a RAPD marker specific to the female gender in *Cycas tanqingii*, which was later converted to a “Sequence Characterized Amplified Regions” (SCAR marker) for use in male and female identification prior to flowering [JING & al. 2007]. SCAR markers have the advantage of becoming more reliable and enabling the identification of a single locus [JIANG & SINK, 1997].

The ISSR marker found to be associated with female sex in this study is only a starting point to contribute to sex identification in *Z. inermis*. However, it is necessary to validate its usefulness, for which future studies will be developed to detect a SCAR marker like those found in other gymnosperms [JING & al. 2007; LIAO & al. 2009; SÁNCHEZ-COELLO & al. 2018].

Conclusions

The results demonstrated the ISSR marker linked with female sex found in *Z. inermis* is a suitable starting point to start looking for other genetic and epigenetic molecular markers to improve early sex identification in this significant genetic resource. We can't rule out the possibility that sex expression in this species is influenced by epigenetic factors (gene expression changes without changes in the nucleotide sequence), so it'd be advisable to evaluate the usefulness of markers such as Methylation-Sensitive Amplification Length Polymorphism (ms-AFLP) in elucidating the role of possible epigenetic factors in sex determination.

Notes on contributors

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Enrique FAVIAN-VEGA – he has a PhD in Ecology and Biotechnology, and his work has focused on the conservation biology of Mexican cycads.

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