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Genetic diversity and differentiation in *Zamia furfuracea* (Zamiaceae): an endangered, endemic and restricted Mexican Cycad

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ABSTRACT: *Zamia furfuracea* is a cycad endemic to coastal habitats of southeast Mexico and of high ecological and horticultural importance. It is threatened with extinction due to the declining population size, caused mainly by livestock raising, urban development, and poor environmental management. This work aimed to determine the genetic diversity and structure of six natural populations of *Z. furfuracea* throughout their known distribution range. To determine the genetic variation, ten molecular markers of repeated simple sequences (SSR) were used. A Bayesian assignment model, molecular variance analysis and bottleneck tests, and identification of loci with non-neutral heritage were also performed. A lower genetic diversity was identified in the southern population (Capulteolt). Four genetic groups were identified (K = 4). Ancestral polymorphism and management resulted in an increased genetic similarity between the populations of "Ciénega del Sur," "Toro Prieto," and "Playa Escondida". "Capulteolt" was the most differentiated population; a historical reduction in the population size was observed, leading to the loss of some alleles by putative directional natural selection. This study reveals the "Capulteolt" population's low genetic diversity as well as the presence of increased differentiation in the geographical limits of the species' distribution range.

KEY WORDS: Bottleneck, genetic flow, genetic structure, natural selection, SSR markers.

INTRODUCTION

Zamia furfuracea L. f. is a cycad endemic to the coastal habitats of southeastern Mexico, restricted to dunes and rocky slopes in isolated areas (Vázquez-Torres, 2001). It is the second most traded cycad in the horticultural market worldwide (Vovides *et al.,* 2002; Vovides *et al.,* 2010). Because of this, *Z. furfuracea* is threatened by the illegal extraction of seedlings and seeds (Vázquez-Torres, 2001; Osborne and Vovides, 2007). In addition to this, other anthropogenic factors such as land use change and natural phenomena have caused a decrease in its population size, with an estimated reduction of 35% of its original size in the last four decades (Chemnick and Gregory, 2010). This can cause negative impacts on density, equal sex ratio, and pollinators (Hall and Walter, 2011), decreasing gene flow and increasing genetic differentiation (Ellstrand and Elam, 1993). In fact, previous studies have reported limited gene flow of less than 30 m (Norstog *et al.,* 1986), and poor seedling survival has been reported (Octavio-Aguilar *et al.,* 2017). It is therefore listed as endangered in the IUCN Red List (IUCN, 2010) and by Mexican law (NOM-059-SEMARNAT, 2010). It was also included in CITES Appendix II (CITES, 2008).

Understanding levels of genetic diversity and structure in natural populations of threatened and endangered plants is a priority for conservation biology (Pierson *et al.,* 2016). Loss of genetic diversity in conservation programs and restoration of threatened populations can only be addressed through detailed population genetic studies (Hamrick and Godt, 1996). In addition, genetic analysis based on molecular data allows us to preserve the genetic patterns of natural species encountered (Larson *et al.,* 2014). It provides guidance for collecting plant resources from endangered plants as well as improving reintroduction success (Griffith *et al.,* 2015; Kaulfuß and Reisch, 2017). Similarly, it prevents exogamy depression and inbreeding and increases species-wide resilience (Larson *et al.,* 2014). The high-quality genome sequence for Cycads, established by Liu *et al.* (2022), opens new scientific horizons in the conservation of these valuable genetic resources because they provide us with an overview about the specific geographical location of relicts and ancestral radiation centers, as potential sources of variability, in addition to allowing us to identify historical threats to colonization of the species.

Several studies on cycads have used molecular markers to estimate population diversity and genetic structure (Xiao *et al.,* 2004; González-Astorga *et al.,* 2006; Calonje *et al.,* 2013; Feng *et al.,* 2014; Gong *et al.,* 2015). In general, genetic diversity in this group is higher than what is typically expected from a long-lived species

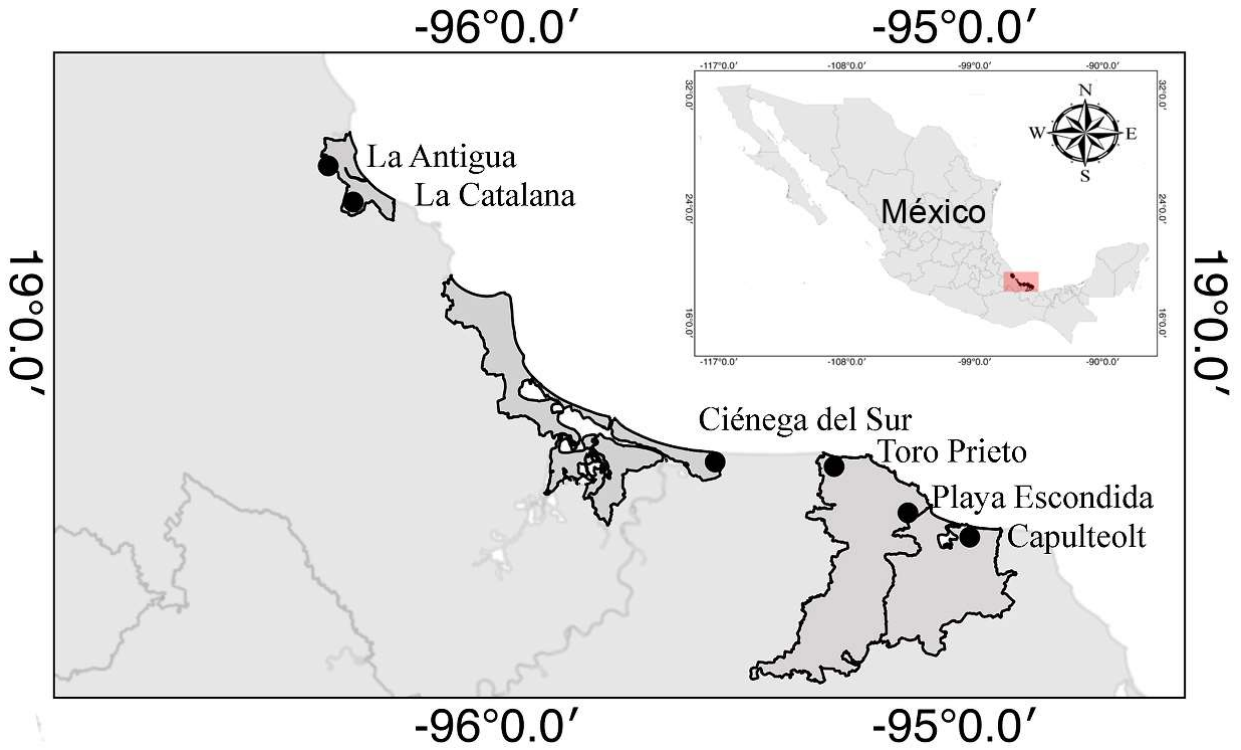

Fig. 1. Sampling sites in southeast Mexico within the natural distribution range of ***Zamia furfuracea***. Populations of "La Antigua" and "La Catalana" to the north, "Ciénega del Sur" and "Toro Prieto" in the center, and "Playa Escondida" and "Capulteolt" to the south of the natural distribution range of the species.

that is restricted in distribution and sensitive to disturbance, but high levels of differentiation have been reported (García-Montes *et al*., 2020). In five of the eight reported populations of *Z. furfuracea*, an allozyme study revealed substantial inbreeding, loss of genetic variability, and considerable differentiation (Limón-Salvador, 2009). It's important to note that this study looked at three new populations (La Antigua, and La Catalana), which were recently described (Baldo-Romero *et al*., 2013), and "Playa Escondida" was described for the first time.

Considering the above, we aim to determine the levels of genetic diversity and genetic structure in six natural populations of *Z. furfuracea*, throughout its known geographical distribution, using SSR molecular markers. It is expected that the geographical isolation and the reduction in the effective size of the populations studied have caused a decrease in diversity and greater genetic differentiation, with a particularly strong effect on the extremes of the natural distribution of the species. These findings have significant implications for the management and conservation of this important species.

MATERIALS AND METHODS

Study sites and plant material

The study was conducted in six natural populations of *Z. furfuracea* located along the coastal zone of the Gulf of Mexico, in the state of Veracruz, Mexico (Fig. 1). The study area's dominant vegetation is typical of coastal dunes and low tropical forests with thorny shrubs (Vázquez-Torres, 2001). The local climate is tropical with a warm-humid regime (Aw_2), slightly less warm and more humid towards the south of the distribution range of the species (Af) (García, 1970). The biological material of each population was collected along a 20 x 200 m (4000 m^2) transect, with 30 adult plants randomly selected per transect. The foliar tissue samples were labeled, washed with 70% ethanol, packaged in tightly sealed plastic bags, transported to the Institute of Biotechnology and Applied Ecology in the city of Xalapa, Veracruz, Mexico, and stored at -4 °C until used.

DNA extraction

Genomic DNA was extracted according to Stewart and Via (1993) using 2% CTAB (Cetylmethylammonium bromide). The integrity of the molecule was confirmed by electrophoresis in agarose gels in TAE 1X buffer for 60 minutes at 100 V. A final concentration of 50 ngμL^{-1} of genomic DNA was obtained, quantified with a benchtop Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, US).

Microsatellite amplification

The polymerase chain reaction was performed using ten SSR molecular markers developed by Meerow and Nakamura (2007). In the mix reaction, 50 ng of genomic DNA, Buffer Taq 1X, $MgCl_2$ (2.5 mM), dNTPs mix (0.6 mM), primer (25 pM), Taq DNA (Promega) polymerase

(1 U), and sterile water to a final volume of 25 μL were used in the combination reaction. The thermal cycle (Artik Thermo Scientific®) began with a 3.5-minute denaturation stage at 94 °C, followed by 35 cycles at 93 °C for 40 seconds each, 45 seconds at 52 °C (Zam 35 and 45), 54 °C (Zam 40), 59 °C (Zam 33 and 34), 60 °C (Zam 32 and 39), 61 °C (Zam 28 and 29), and a final extension stage. The amplified fragments were separated on 6% polyacrylamide gels (140 V, 90 min), stained with GelRed®, and visualized with a transilluminator (2 UVTM-UVP) under UV light. The 50-bp molecular weight marker Bioline® was employed. This method has proven to be effective and accurate for the group (García-Montes *et al*., 2020).

Data analysis

Genetic diversity

A matrix was constructed based on the size of the amplified fragments corrected by the Chybicki and Burczyk (2009) method. The genetic diversity was assessed by calculating the percent of polymorphism (*P*), average alleles per *locus* (N_A), effective allele number per *locus* (N_E), Shannon's information index (*I*), and observed and expected heterozygosity (H_O, H_E), using the software GenAlEx ver. 6.5 (Peakall and Smouse, 2006). The Hardy-Weinberg equilibrium (*HWE*) by *locus* and population was tested with a χ^2 test. An AMOVA was run to obtain the fixation index *F* (Excoffier *et al*., 1992). Nei's genetic distance was calculated, and the number of migrant individuals between pairs of populations was estimated indirectly with the formula $Nm = 1/4 \times [1/(F_{ST}-1]$ (Wright, 1969). However, it has been indicated that the estimation of the *Nm* from the F_{ST} reflects the long-term dispersion (ancestral polymorphisms) (Whitlock and Mccauley, 1999). Therefore, GENCLASS2 (Piry *et al*., 2004) was used to detect recent immigration events based on the ratio *L = L_Home/L_Max*. The resampling algorithm of Paetkau *et al*. (2004) was used, with a probability of allocation ($p < 0.01$) based on 10,000 simulated individuals. To test whether there is genetic isolation by geographic distance between population pairs, a Mantel test was carried out using two matrices, one for geographic distances (km) and another for Nei's genetic distances, using the TFPGA software (Miller, 1997).

Population differentiation

The genetic structure within and between populations was evaluated with STRUCTURE 2.3.4 (Falush *et al*., 2003). Groups of one to ten genetic clusters (*K*=1–10) were assumed. Twenty replicates of each of one million MCMC iterations (burn-in of 100,000) were run for each *K* tested using Structure Threader (Pina-Martins *et al*., 2017), on an 88-core Ubuntu Linux cluster. Our strategy for determining the optimal value of *K* (number of populations) with STRUCTURE follows recent papers (Kalinowski, 2011; Puechmaille, 2016; Pina-Martins *et al*., 2017). STRUCTURE output was evaluated and visualized using sampled populations: admixture with allele frequencies correlated, admixture with no frequency correlation, and no admixture. All other settings were maintained as default. Estimates of ΔK (Evanno *et al*., 2005), LnP (*K*) (Falush *et al*., 2003), and the four independent estimators of Puechmaille (2016), which we refer to collectively as MMK, were generated on the web server STRUCTURE SELECTOR (Li and Liu, 2018). A Q value of 0.5 was established to assign an individual to a particular group. It was used STRUCTURE SELECTOR (Li and Liu, 2018) to generate a consensus Q-matrix from replicates at optimal K and visualize the clusters in a histogram labeled by population.

Neutrality and bottleneck tests

To evaluate the evidence of genetic bottlenecks, a Wilcoxon rank assignment test (two-tailed) was performed in a gradual mutation model (SMM) using the Bottleneck v.1.2 program (Cornuet and Luikart, 1996). This analysis assumes that a severe reduction in the effective size of a population is due to excess or deficient heterozygosity relative to the expected heterozygosity by loci.

Finally, a neutrality tests were carried out at each locus using a hierarchical Bayesian method as described in Beaumont and Balding (2004) and the BayeScan v.2.1 program (Foll and Gaggiotti, 2008). This model considers the multinomial approach proposed by Dirichlet (Bernard, 2005), assuming that any differences between sites are mediated by the chromosomal link between neutral alleles containing genes under selective pressure. We used a confidence limit of 0.95 to consider non-neutral loci, an interval of 0.001 as the frequency of deviation, and a sample size of 344 individuals with the 10 loci.

RESULTS

Our results identified 52 alleles with 10 SSR primers, nine being monomorphic and 43 informative. Total genetic diversity indicators were $P = 98.33 \pm 1.67\%$, $N_A = 3.28 \pm 0.13$, $N_E = 2.42 \pm 0.08$, $H_O = 0.57 \pm 0.04$, $H_E = 0.54 \pm 0.02$, and $I = 0.93 \pm 0.04$. The highest genetic diversity (H_E) values were recorded in the "Toro Prieto" and "Playa Escondida" populations (0.61 – 0.56, respectively), and the lowest, in the "Capulteolt" and "La Catalana" (0.5 – 0.52, respectively). In "Capulteolt", the higher outbreeding level was observed (-0.201, Table 1). "La Catalana," "Toro Prieto," and "Capulteolt" were not found in *HWE* (Table 1).

Population differentiation

The AMOVA showed significant differences between the populations studied (FST (5,359) = 0.087, $p < 0.001$). The greatest variation was observed within populations (84%), far above the variation between populations (9%), with 7% of it within individuals. *The Nm* estimation at the

Table 1. Genetic diversity indicators of the six natural populations of ***Zamia furfuracea***.

Populations	*N*	*P*	N_A	N_E	*I*	H_O	H_E	*F*	*HWE*
La Antigua	31	90.0	3.10±0.34	2.40±0.24	0.89±0.12	0.54±0.11	0.53±0.06	0.008±0.18	ns
La Catalana	29	100.0	3.20±0.38	2.34±0.21	0.88±0.11	0.52±0.11	0.52±0.06	0.040±0.17	*
Ciénega del Sur	31	100.0	3.20±0.38	2.37±0.21	0.91±0.10	0.56±0.10	0.53±0.05	0.016±0.17	ns
Toro Prieto	30	100.0	3.60±0.34	2.72±0.21	1.07±0.07	0.58±0.08	0.61±0.03	0.030±0.13	*
Playa Escondida	29	100.0	3.30±0.30	2.15±0.25	0.97±0.09	0.61±0.10	0.56±0.04	-0.090±0.18	ns
Capulteolt	30	100.0	3.30±0.30	2.17±0.16	0.87±0.08	0.62±0.08	0.50±0.04	-0.201±0.11	**
Mean		98.3±1.67	3.28±0.13	2.42±0.08	0.93±0.04	0.57±0.04	0.54±0.02	-0.030±0.06	

N, Sample size; *P*, percentage of polymorphic loci; N_A, average alleles per locus; N_E, effective number of alleles per locus; *I*, Shannon´s index; H_O, observed heterozygosity; H_E, expected heterozygosity; *F*, fixation index; *HWE*, Hardy-Weinberg equilibrium; ns, no significant differences. *$p < 0.05$; **$p < 0.01$

Table 2. Pairwise gene flow between six populations of ***Zamia furfuracea***. *Nm*: values above diagonal, F_{ST}: values below diagonal.

Populations	La Antigua	La Catalana	Ciénega del Sur	Toro Prieto	Playa Escondida	Capulteolt
La Antigua	-	4.652*	1.486*	2.569*	1.982*	1.523*
La Catalana	0.051	-	2.799*	1.523*	0.993*	2.528*
Ciénega del Sur	0.144	0.082	-	1.395*	2.438*	1.924*
Toro Prieto	0.089	0.141	0.152	-	6.329	2.201*
Playa Escondida	0.112	0.067	0.093	0.038	-	2.065*
Capulteolt	0.141	0.090	0.115	0.102	0.108	-

Significant differences *$p < 0.001$

Table 3. Bottleneck analyses and immigrants of six natural populations of ***Zamia furfuracea***.

Population	Loci with *He* excess	Loci with *He* deficiency	*p* value *W*	Migrants	Origen
La Antigua	9	1	< 0.001	2	La Catalana
La Catalana	8	2	< 0.001	1	Playa Escondida
Ciénega del Sur	9	1	< 0.001	1	Playa Escondida
Toro Prieto	10	0	< 0.001	1	La Antigua
Playa Escondida	9	1	< 0.001	1	Toro Prieto
Capulteolt	7	3	< 0.001	0	-

Heterozygosity (*He)*, Wilcoxon 2-tailed test (*W)*.

total level of the species was high (2.623). The *Nm* ranged between 1.499 (La Antigua and Ciénega del Sur) and 6.328 (Toro Prieto, and Playa Escondida). “Toro Prieto” and “Playa Escondida” is not significant (Table 2), so not all pairwise comparisons are significant. The analysis of the allocation, using GENECLASS2, indicated that only six of the 180 individuals (3.33%) sampled were first-generation immigrants (Table 3). However, no genetic isolation was identified through geographic distance (Mantel test, $r = 0.283$, $p > 0.05$).

The most suitable model for the *Z. furfuracea* microsatellite data was admixture with correlated allele frequencies. This means that frequencies in the different populations are likely to be similar (probably due to migration or shared ancestry). The four MMK estimators and the ΔK method found $K = 4$ to be the most optimal structure (Fig. 2a), while the LnP (K) suggested $K = 8$ (Fig. 2b), although we reject this grouping for overestimating the separation between "La Antigua" and "La Catalana" populations that form two different clusters (purple and green colors, respectively). The third genetic cluster consists of the "Ciénega del Sur", "Toro Prieto", and "Playa Escondida" populations. Individuals from these three populations were allocated to the orange cluster, although they were partially admixture with the green cluster, and only a few individuals in each of these populations showed genotypes distinctive of the purple cluster. Individuals in the southernmost population, "Capulteolt," were assigned mainly to the fourth.

Neutrality and bottleneck tests

All populations showed recent bottlenecks of varying intensities, based on the *p* values of the two-tailed Wilcoxon test. The strongest reductions in population size occurred in the populations of "La Antigua," "Ciénega del Sur," and "Toro Prieto" (Table 3). All of them have heterozygosis excess, a related pattern to recent contractions. The neutrality analysis identified two out of 10 *loci* (Zam 33 and Zam 34) associated with a deviation from expected frequency under nonselective heritage.

DISCUSSION

Knowing the levels of genetic diversity in *Z. furfuracea* and understanding how it is distributed across populations are highly relevant aspects for developing management and conservation strategies. The results obtained in this work revealed high genetic diversity values at the species level.

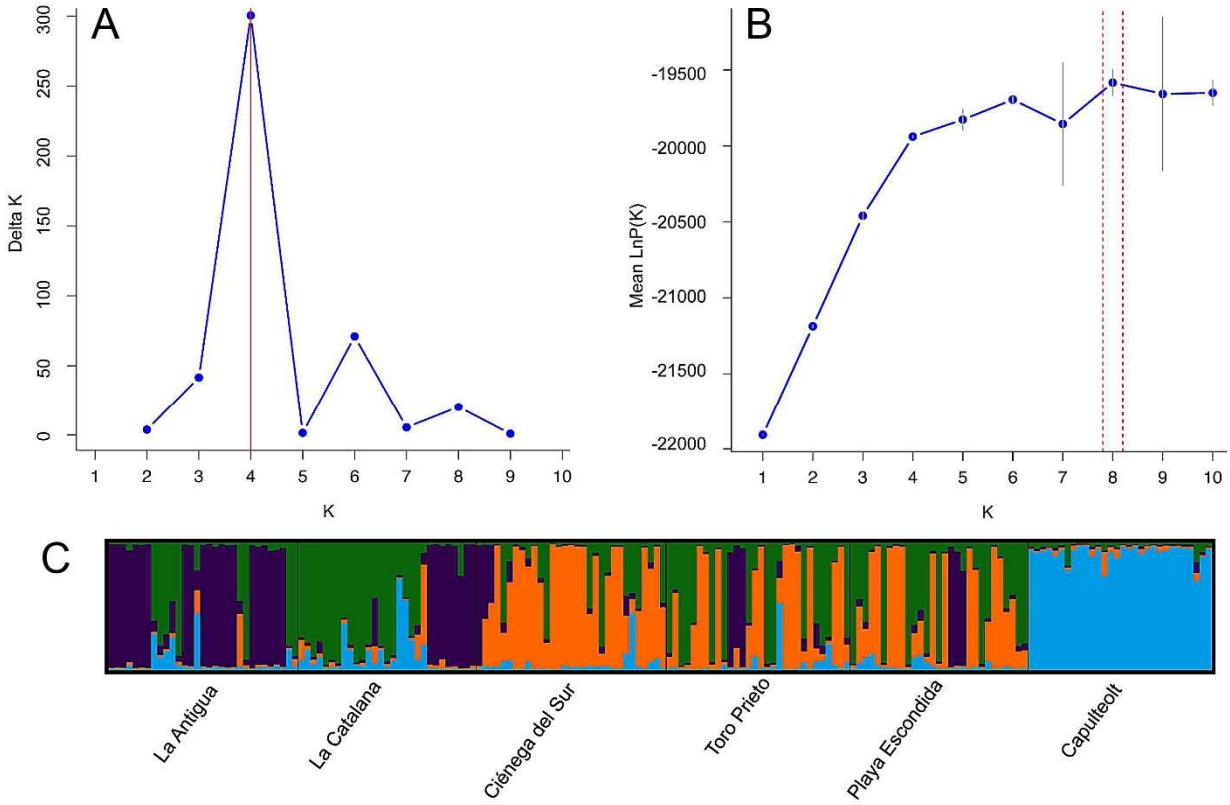

Fig. 2. Bayesian assignment of ***Zamia furfuracea*** individuals from six populations to genetic groups. Sites are arranged according to their geographic location (Fig. 1). Δ*K* values (**A**) and the estimated mean logarithmic likelihood of *K* values (**B**) for each K. The *y*-axis quantifies subgroup membership. **C**. The *x*-axis shows different populations. Each solid bar represents a single individual, while colored areas (dark blue, orange, light blue, and green) correspond to different genetic clusters (*K* = 4).

A lower genetic diversity was identified in the northern population (La Catalana) (Table 1). Two different genetic structures were identified (*K* = 4, Fig. 2a; and *K* = 9, Fig. 2b). We see a reduction in the number of genetic groups at the boundaries of the distribution range of the species (Fig. 2c). The "Ciénega del Sur," "Toro Prieto," and "Playa Escondida" populations were genetically similar and showed the highest number of genetic groups (Fig. 2c).

Relatively high levels of genetic diversity were found in the populations of *Z. furfuracea* (Table 1) compared to other species of the old world (Xiao *et al*., 2004; Feng *et al*., 2014; Gong *et al*., 2015). The genetic variation of a species is a product of its long-term evolution and represents its evolutionary potential for survival and development (Gitzendanner and Soltis, 2000). In general, cycads have long life cycles and overlapping generations, so it is expected that high levels of genetic variation would have accumulated during a long evolutionary history (Feng *et al*., 2014). The high levels of *Nm* estimated from the F_{ST} (Table 2) suggest that genetic polymorphism has accumulated throughout its evolutionary history, although these levels are based on biological assumptions that assume an infinite number of populations, absence of selection or mutation, equilibrium between migration and drift, and the nonexistence of a relationship between geographic distance and genetic flow (Whitlock and Mccauley, 1999). On the other hand, the genetic diversity indexes analyzed are like those previously reported for other species of the family *Zamiaceae* (González-Astorga *et al*., 2006; Meerow and Nakamura, 2007; Meerow *et al*., 2012; Calonje *et al*., 2013), particularly for the species that grow in the coastal areas of the Gulf of Mexico and the Caribbean islands. A previous study using allozymes showed low diversity and high genetic differentiation across all populations of *Z. furfuracea* (Limón-Salvador, 2009). Allozyme markers failed to identify the genetic similarity between "Ciénega del Sur," "Toro Prieto," and "Playa Escondida". These results probably differ because allozymes are less polymorphic (Leinonen *et al*., 2008). *Zamia furfuracea* populations are geographically isolated (Fig. 1), but do not meet the principle of genetic isolation by geographic distance (Mantel test, r = 0.283, p >0.05). Management has increased the genetic flow (Table 2) (Vázquez-Torres, 2001), thus decreasing the genetic structure in the populations at the center of the distribution. On the other hand, the reduction of the population size, founder effects, and evidence of deviation from neutrality, together with the presence of natural barriers (Antigua River in the north and

Sontecomapan Lagoon in the south), increases the differentiation at the boundaries of the populations (Fig. 2). This genetic structure corresponds to the Δ*K* method and the four MMK estimators (*K* = 4). The LnP (*K*) method differs (*K* = 9). The LnP (*K*) method allows the identification of subtle subdivisions within populations by using allele frequencies within each population (Falush *et al*., 2003). Similarly, the performance and accuracy of the program are affected by the sampling scheme (Schwartz and McKelvey, 2009) or when close relatives are included in the data set (Anderson and Dunham, 2008; Rodríguez-Ramilo *et al*., 2014). *Z. furfuracea* has a small population size (100 adult individuals per population) and an aggregated population structure (Vázquez-Torres, 2001; Octavio-Aguilar *et al.,* 2017). Therefore, it is possible that the LnP (*K*) model can indicate a substructure within the populations caused by neighbor mating. A previous fine-scale study in "La Catalana" identified genetic neighborhoods less than 10 m from well defined (Octavio-Aguilar *et al*., 2017).

"La Antigua" and "La Catalana" form two different genetic clusters (Fig. 2C). Although those are two km away, they imagine a high genetic flow (Table 2) and the largest number of first-generation migrants (Table 3). Various factors may have contributed to this genetic separation, including the following: i) They are separated physically by the La Antigua River sub-basin; and ii) In 2010, Hurricane Karl overflowed this river, causing significant loss of settlements and flooding a large area of land between these two populations for more than five days (Larios-Tlali *et al.,* 2015). Hundreds of plants died, reducing the population size (Baldo-Romero *et al.,* 2013); iii) the completion of an urban project begun in 2008, which cleared a large portion of the local vegetation, exacerbating the population size reduction. In addition, evidence of historical changes in population sizes, such as founder effects (presence of loci with heterozygosity excess) and bottlenecks (presence of loci with heterozygosity deficiency), increases the divergences among populations. The factors described above increased genetic differentiation and reduced genetic diversity.

The populations of "Ciénega del Sur," "Toro Prieto," and "Playa Escondida," geographically isolated, form the third genetic cluster (Fig. 2). Taking into consideration that cycads show a limited dispersal of pollen and seeds (Norstog and Nicholls, 1997; Gong *et al*., 2015), and in *Z. furfuracea*, the area of study involved has been estimated at less than 30 m (Norstog *et al*., 1986). This may be due to several anthropogenic and historical factors. The development of rural nurseries, the reintroduction and transplantation of individuals, and the illegal extraction of seedlings and seeds are all common practices in the "Ciénega del Sur", "Toro Prieto", and "Playa Escondida" populations (Vázquez-Torres, 2001). Management plans have further increased heterozygosity due to the exchange of seeds, functioning as a source of external pollen. Recent events mentioned above may explain the genetic similarity observed in these three populations (Fig. 2). A similar event was reported in *C. multipinnata* (Gong *et al*., 2015). "Capulteolt" forms the fourth genetic group, comprising an isolated and relatively well-preserved population on the opposite edge of the "Sontecomapan Lagoon" (Fig. 2). Our results suggest an old bottleneck (Table 3) derived from a founding effect. A colonization event may have occurred from floating seeds. In this area, some individuals of *Z. furfuracea* grow on isolated rocky islands located several meters away from the shore. The likelihood of island colonization by cycads through floating seeds has been suggested previously (Eckenwalder, 1980; Hamada *et al*., 2015). On the other hand, "Capulteolt" is a population widely managed by local people. Management decreases genetic diversity (Dobson *et al*., 1992). As a result, we believe that historical divergence and management may have increased this population's genetic homogeneity.

Each population of *Z. furfuracea* is subjected to unique microevolutionary forces related to anthropogenic factors, natural phenomena, and historical processes. Some authors (Dobson *et al*., 1992; Caro and Laurenson, 1994) have argued that microevolutionary forces play a minor role in extinctions. However, the combination of several factors increases genetic isolation and reduces population size, hence increasing the risk of extinction of a given species. This is important under a genetic isolation scenario like the one described in this work, where the loss of genetic diversity and the differentiation of the populations of "La Antigua" and "La Catalana" in the far north and "Capulteolt" in the south increase their risk of extinction. "Ciénega del Sur" and "Toro Prieto" are the populations with the largest number of genetic groups. These populations could be subjected to increased management through seed collection and cultivation practices, thereby maintaining genetic diversity and benefiting local producers. "Playa Escondida" is also highly important, as it includes many genetic groups and one exclusive allele; therefore, an *in-situ* conservation program should be established. On the other hand, ex-situ conservation, by collecting seeds or seedlings, may be an element of a viable supplementary conservation approach aimed at protecting the genetic variability detected in all populations.

It is concluded that although the populations of *Z. furfuracea* examined have gradually decreased in size due to changes in land use and natural phenomena, which have caused bottleneck effects, they have not affected their levels of genetic diversity or the specie. It has been observed that populations at the extremes of their natural distribution have increased their genetic differentiation, which is why it is necessary to develop strategies to protect their genetic diversity. The results obtained provide the basis to establish an effective strategy for the management of germplasm in this valuable species.

AUTHOR CONTRIBUTION

LGIA designed the work. EFV performed the field work and laboratory and data analyses. All authors read and approved the manuscript. AWM and POA contributed to the processing and interpretation of the results.

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