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Heterozygote excess in ancient populations of the critically endangered *Dioon caputoi* (Zamiaceae, Cycadales) from central Mexico

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Dioon caputoi is a long-lived cycad known from only four populations that range in size from 50 to 120, mostly adult individuals. *Dioon caputoi* has the most narrow geographical range of all *Dioon* spp. (less than 10 km), existing completely within the boundaries of the Tehuacán–Cuicatlán Biosphere Reserve, Mexico. Negative inbreeding values were found in all four populations ($F_{IT} = -0.242$) and within subpopulations ($F_{IS} = -0.379$). Only *c*. 10% of the total genetic variation was partitioned among populations ($F_{ST} = 0.099$). We also found that most mean values of genetic variation ($A = 1.91 \pm 0.12$; $P = 78.9 \pm 10.2$; $H_E = 0.35 \pm 0.01$) are within the range reported for other *Dioon* species with larger populations and with wider geographical ranges. These results support recent findings that rare plant species maintain high levels of genetic diversity. The heterozygote excess found at all loci is discussed in detail from a neutral evolutionary perspective, leaving arguments as working hypotheses for further research.

ADDITIONAL KEYWORDS: allozyme – bottleneck – conservation genetics – endemic – genetic diversity – population genetics – rare species – small populations.

INTRODUCTION

Cycads are dioecious, long-lived and entomophilous gymnosperms that, together with *Ginkgo*, are the most primitive living seed plants as evidenced by their fossil history which dates back to the Permian and perhaps the Carboniferous. As a group, they are considered to be in global decline as a result of illegal trade and habitat loss (Norstog & Nicholls, 1997) and they are threatened and endangered (IUCN, 2007).

Cycads, with their palm-like habit, are popular ornamentals and commonly used in landscaping architecture. The international cycad trade generates several million dollars annually (Gilbert, 1984; Vovides *et al.*, 2002). Their populations have experienced inadequate management practices ranging from extraction of whole plants and seeds (e.g. most *Dioon* species; www.cycadpalm.com), decapitation of leaf crowns and illegal extraction for sale (Vovides, 1990), to outright habitat destruction. Continuing attempts at sustainable management of several cycad species are ongoing and aimed at stopping or reversing the negative effects of these poor management practices (Vovides & Iglesias, 1994; Vovides *et al.*, 2002; Donaldson *et al.*, 2003).

Dioon caputoi de Luca, Sabato & Vázq. Torres has erect trunks up to 2 m or more in height and 20–25 cm in diameter. Its conservation status is critically endangered (CR) according to the International Union for Conservation of Nature Red List (IUCN, 2007). At present, there are only four known remaining populations of this species, all of which occur within the Tehuacán–Cuicatlán Biosphere Reserve (TCBR) in the Mexican states of Puebla and Oaxaca.

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These populations range in size from *c*. 50 to 120, mostly adult individuals. During the 1980s, the local inhabitants observed massive illegal extraction by the truck load. This extraction probably focused on juvenile plants as this life-cycle stage is currently under-represented in all populations. Poor natural recruitment has been observed in these populations, possibly as a result of prolonged drought periods (Vovides, 1990) and/or goat grazing.

To develop and implement effective conservation strategies, it is therefore necessary to integrate information drawn from several areas of expertise, including genetics, ecology, sociology and economics (Young, Boshier & Boyle, 2000). Determining how much genetic diversity exists in a species and explaining this in terms of its origin, organization and maintenance is thus of fundamental significance to conservation biology (Yeh, 2000). Population genetics and structure (Wright, 1951) is influenced by a number of factors, such as mutation, genetic drift, inbreeding, gene flow and natural selection (Falconer & Mackay, 1996; Hartl & Clark, 1997). The relevance of these factors in the shaping of genetic diversity is related to the biological and ecological traits. Traits such as breeding system, dispersal mode and lifespan have been related to genetic variation and genetic structure in several plant groups (Hamrick & Godt, 1996; Duminil *et al.*, 2007). Therefore, depending on their biological traits, some plants can be genetically more resilient than others (Hamrick, 2004). For instance, in spite of the well-documented effects that small population sizes have on the genetic pool of many plant species (Hedrick, 2000), studies of long-lived, perennial shrubs and arborescent species with relatively restricted distributions and small population sizes have revealed unexpectedly high levels of genetic variation (e.g. Martínez-Palacios, Eguiarte & Furnier, 1999, *Agave victoriae-reginae* T. Moore; González-Astorga & Núñez-Farfán, 2001 *Brongniartia vazquezii* O. Dorado; Zawko *et al.*, 2001 *Leucopogon obtectus* Benth.; Fu & Dane, 2003 *Castanea pumila* var. *pumila* (L.) Mill.; González-Astorga & Castillo-Campos, 2004 *Antirhea aromatica* Castillo-Campos & Lorence).

Among cycad species, life-history traits (i.e. dioecy, longevity, polinization and dispersion modes) are very similar; however, patterns of genetic diversity are not still defined in this group. It has been argued that, unlike other gymnosperms, low genetic variation is typical for cycads (Xiao *et al.*, 2004, 2005). In fact, this has been reported for most Asian species of *Cycas* such as *C. seemannii* A. Braun (Keppel, 2002), *C. guizhouensis* K. M. Lan & R. F. Zou (Xiao *et al.*, 2004), *C. parvula* S. L. Yang ex D. Y. Wang and *C. balansae* Warb. (Xiao *et al.*, 2005). However, a low level of genetic variation does not characterize some of the New World *Dioon* species (González-Astorga *et al.*, 2003, 2005, 2008), where high levels of genetic variation within populations have been found. Also, a high level of genetic diversity at the DNA level was found in the South African *Encephalartos latifrons* (also Zamiaceae; da Silva *et al.*, unpubl. data).

Historical factors are usually invoked in instances where levels of genetic diversity are difficult to explain based on life-history traits (Lewis & Crawford, 1995). In this sense, it is plausible to hypothesize that *D. caputoi* possesses levels of genetic diversity comparable only with its congeners in spite of its highly reduced populations. This is owing to similar biological traits but also similar evolutionary histories which are shared among them. However, as *D. caputoi* has only recently been described (1980), historical population level data are not available, other than what is known regarding illegal extraction and fragmentation as a result of agricultural expansion. These activities have probably contributed to the reduction of previously much larger, continuous or separate, pristine populations. Although agriculture shows great impact on plant biodiversity in several cycad mega-diversity countries such as Africa (Daniels, Rebelo & Raimondo, 2008); it is likely that the principal decline of population size in most cycads is mainly as a result of illegal extraction (Donaldson & Bsenberg, 1999; Ye, 1999). In this context, we are unaware whether anthropogenic disturbances have impacted the genetic pool of *D. caputoi*. Since the establishment of the Tehuacán–Cuicatlán Biosphere Reserve in 1998, *D. caputoi* has been protected by a sustainable utilization programme for priority species aimed at conservation and rescue (INE-SEMARNAP, 2000) through local farmer-run registered rural nurseries known as Units for Wildlife Management (UMAS: Unidad de Manejo y Aprovechamiento de la Vida Silvestre). A goal of this programme is to create awareness and ownership value of the cycad habitat among farmers through the benefit of additional income. This involves management of the populations through seed extraction, cultivation and seedling reintroduction and, therefore, information on population genetics is desirable to base an effective long-term conservation strategy for this threatened species (Lande, 1988).

The goals of the present study were: (1) to determine the amount and distribution of genetic diversity within and among the four known populations of *D. caputoi*; (2) to investigate whether populations have experienced a recent reduction in effective population size; and (3) to contrast these results with those of other recently studied cycads including *Dioon* species.

MATERIAL AND METHODS

STUDY AREA

The four known populations of *D. caputoi* are La Grana, El Guayabo, Cerro Grande and Loma Pachona, with *c*. 55, 77, 121 and 114 individuals, respectively. All four areas are located in the state of Puebla. La Grana, El Guayabo and Cerro Grande are located south of San Luis Atolotitlán and Loma Pachona is located near Santiago Coatepec (Fig. 1).

All localities lie on the sub-province Sierra Central de Oaxaca, which is part of the Sierra Madre del Sur. This is a semi-arid region with great environmental heterogeneity containing at least 29 vegetation types (Valiente-Banuet *et al.*, 2000). This area is recognized for its floristic richness and high level of endemism and for this reason was declared a biosphere reserve (Dávila *et al.*, 2002). The climate is characterized by summer rains and low temperature fluctuations [BWhw(w)(i')gw], with a mean annual precipitation of 319.1 mm and mean annual temperature of 18.1 °C (García, 2004). The *D. caputoi* habitat has a complex topography where flat areas and slight slopes are rare. The dominant vegetation consists of thorn scrub with secondary vegetation shrubs (Valiente-Banuet *et al.*, 2000).

SAMPLE COLLECTION

Samples of leaflets from 31 to 55 adult individuals from three hectare plots per population were collected. The sample percentage fluctuated between 40 and 70% of the total number of individuals per population (Table 2). The leaflets were transported on ice to the laboratory to avoid protein denaturalization and then stored at –70 °C until protein extraction.

ENZYME EXTRACTION AND ELECTROPHORESIS

Enzymes were extracted according to the protocol of González-Astorga *et al.* (2003). Enzyme extraction

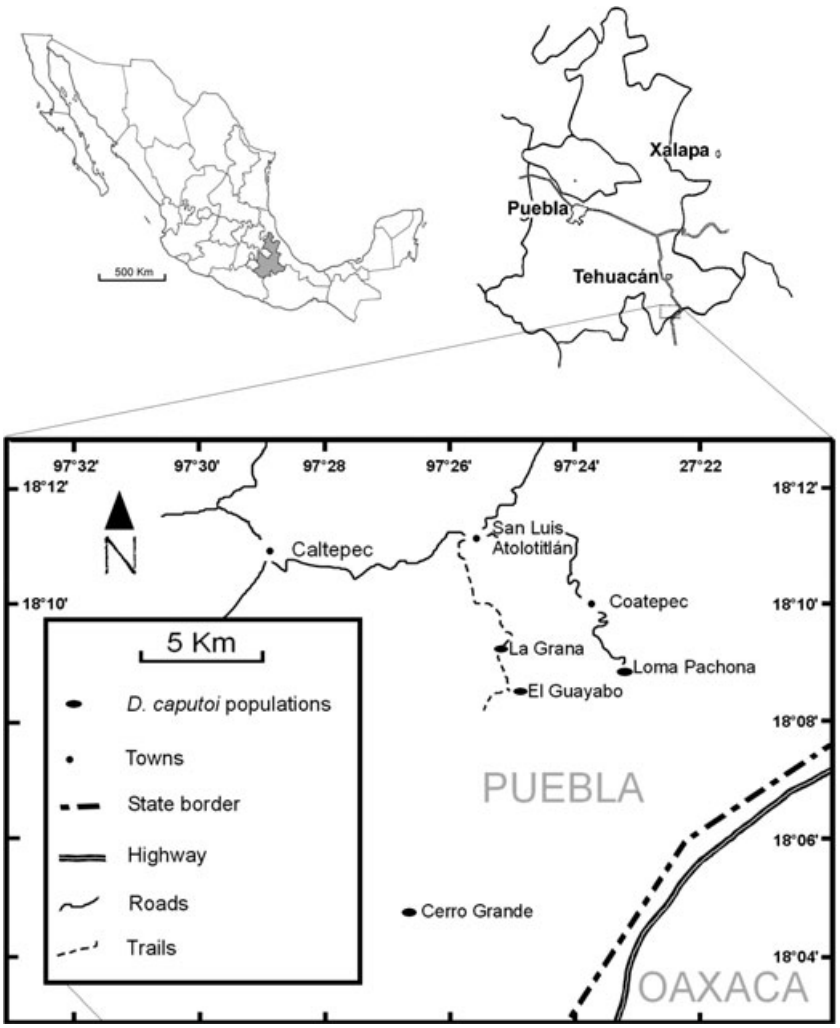

Figure 1. Geographic distribution of the *Dioon caputoi* populations in Puebla, Mexico.

Table 1. Allozyme systems [nomenclature and abbreviations follow Wendel & Weeden 1989, based on IUBNC Enzyme Commission number (E.C. no.)] and allele frequencies of 19 allozyme loci in four populations of *Dioon caputoi*: La Grana (LG), El Guayabo (EG), Loma Pachona (LP) and, Cerro Grande (CG)

			Population			
Allozyme/E.C. no./buffer system	Loci	Alleles	LG	EG	LP	CG
Anodic peroxidase/1.11.1.7/R	APX 1	1	0.533	0.880	0.698	0.453
		2	0.467	0.120	0.302	0.547
	APX 2	1	1.000	0.930	1.000	1.000
		2	0.000	0.070	0.000	0.000
	APX 3	1	1.000	1.000	0.980	1.000
		2	0.000	0.000	0.020	0.000
	APX 4*	1	0.952	0.870	0.745	0.906
		2	0.016	0.130	0.071	0.094
		3	0.032	0.000	0.183	0.000
Malate-dehydrogenase/1.1.1.37/R	MDH 1*	1	0.452	0.400	0.448	0.472
		2	0.548	0.600	0.552	0.528
	MDH 2*	1	0.586	0.378	0.333	0.417
		2	0.414	0.622	0.667	0.583
Menadione reductase/1.6.99/R	MNR*	1	0.032	0.078	0.122	0.083
		2	0.483	0.500	0.520	0.481
		3	0.483	0.422	0.357	0.435
Isocitrate dehydrogenase/1.1.1.41/R	IDH 1*	1	0.565	0.457	0.745	0.733
		2	0.435	0.543	0.255	0.267
	IDH 2*	1	0.387	0.765	0.394	0.361
		2	0.613	0.235	0.607	0.639
6-Phosphogluconate dehydrogenase/5.3.1.9/R	6PGD*	1	0.500	0.480	0.468	0.482
		2	0.500	0.520	0.532	0.519
Alcohol dehydrogenase/1.1.1.1/R	ADH*	1	0.685	0.857	0.542	0.596
		2	0.315	0.143	0.458	0.404
Acid phosphatase/3.1.3.2/R	ACPH	1	1.000	1.000	0.448	0.482
		2	0.000	0.000	0.552	0.519
Glutamate oxaloacetate transaminase/2.6.1.1/PK	GOT	1	1.000	1.000	1.000	0.954
		2	0.000	0.000	0.000	0.046
Phosphogluco isomerase/5.3.1.9/R	PGI*	1	0.548	0.460	0.449	0.482
		2	0.452	0.540	0.551	0.519
Diaphorase/1.6.99/PK	DIA	1	0.710	0.745	0.848	0.959
		2	0.290	0.255	0.152	0.041
Glutamate dehydrogenase/1.4.1.2/PK	GDH	1	0.423	0.713	0.480	0.667
		2	0.577	0.287	0.520	0.333
Leucine aminopeptidase/3.4.11.1/R	LAP	1	0.554	0.806	0.959	0.685
		2	0.446	0.194	0.040	0.315
Esterase/3.1.1/R	EST*	1	0.129	0.083	0.541	0.406
		2	0.484	0.469	0.459	0.594
		3	0.000	0.448	0.000	0.000
Aconitate hydratase/4.2.1.3/PK	ACO	1	0.839	0.480	0.859	0.844
		2	0.161	0.520	0.141	0.156

*Indicates statistical significance from Hardy–Weinberg deviations, using the conventional Monte Carlo method (10 batches, 10 000 permutations per batch and 100 000 total permutations) $P < 0.01$.

was stored on filter paper wicks at –70 °C until use. Multilocus genotypes from the sampled individuals were obtained through horizontal starch gel electrophoresis (12% w/v). For each individual sample, allozyme variation was scored for 14 enzyme systems (Table 1). Electrophoresis was carried out in two buffer systems: (1) R (Li *et al.*, 1999), which was run at 4°C for 8.5 h (constant current of 35 mA and

voltage of 200 V); and (2) PK (Walters & Decker-Walters, 1991), which was run at 4 °C for 7 h (constant current of 50 mA and voltage of 200 V).

Data analyses

Multilocus genotypes were used to estimate allele frequencies. The following genetic analyses were carried out using TFPGA 1.3 (Miller, 1997): (1) the observed mean heterozygosity (H_O); (2) the mean number of alleles per locus (A); (3) the percentage of polymorphic loci at the 95% probability criterion (P); and (4) the expected mean heterozygosity (H_E) under Hardy–Weinberg equilibrium (Hedrick, 2000). Chi-square (χ^2) tests were used to test for deviations from the expected genotypic frequencies under Hardy–Weinberg equilibrium (Snedecor & Cochram, 1967).

Population genetic structure was estimated using F-statistics (Wright, 1951) with TFPGA. Chi-square tests were used to determine whether F_{IS}, F_{IT} and F_{ST} estimates for each locus were significantly different from zero (Workman & Niswander, 1970; Weir, 1990). Also, F_{ST} values between pairs of populations were obtained by running TFPGA with only two population data sets (i.e. data set of population 1 vs. data set of population 2; population 1 vs. population 3, etc.), repeating the comparison for each pair of populations.

We performed bottleneck genetic analysis to determine whether the populations had experienced a recent reduction in their effective population size using the program BOTTLENECK version 1.2 (Cornuet & Luikart, 1996; Piry, Luikart & Cornuet, 1999). This method tests whether the expected gene diversity (H_E) is higher than the expected equilibrium gene diversity (Heq) calculated from the observed number of alleles for each locus in each population under the assumption of a mutation–drift equilibrium and the infinite allele model (Luikart & Cornuet, 1998).

Finally, average estimations of genetic variation and differentiation were compared between the populations of *D. caputoi* and its congeners *D. edule* Lindl., *D. angustifolium* Miq., *D. sonorense* (de Luca, Sabato & Vázq. Torres) Chemnick, T. J Greg. & S. Salas-Mor. and *D. tomasellii* de Luca, Sabato & Vázq. Torres, using analysis of variance (ANOVA). Pairwise comparisons were performed with Tukey Honest Significant Difference test for unequal N comparisons (Sokal & Rohlf, 1995).

RESULTS

Genetic diversity

All nineteen loci were polymorphic for at least one population (Table 1). The χ^2-test (d.f. = 1 and $\alpha = 0.01$) for heterogeneity of allelic frequencies among populations showed that nine loci, APX1, APX2, APX3, ACPH, GOT, DIA, GDH, LAP and ACO, were in Hardy–Weinberg equilibrium, while the remaining, APX4, MDH1, MDH2, MNR, IDH1, IDH2, 6PGD, ADH, PGI and EST, significantly deviated from Hardy–Weinberg equilibrium (Table 1).

The mean number of alleles per locus was 1.91 ± 0.12 (Table 2). Two exclusive alleles were identified in El Guayabo (APX2 and EST), one in Loma Pachona (APX3) and one in Cerro Grande (GOT; Table 1). The mean percentage of polymorphic loci was 78.95 ± 4.3. The observed mean heterozygosis and expected mean heterozygosity was 0.49 ± 0.04 and 0.35 ± 0.01, respectively (Table 2).

Genetic structure

The estimations of Wright's F statistics (F_{IS}, F_{IT} and F_{ST}) were significantly different from zero for all loci

Table 2. Estimates of genetic variation at 19 allozyme loci in all four *Dioon caputoi* populations

						H-W deviations		
	N* (sample percentage)	P	A	H_O	H_E	Test	–	+
La Grana	29.8 (54%)	73.68	1.74	0.501	0.356	7	2	5
El Guayabo	49.5 (65%)	84.21	1.95	0.433	0.334	6	0	6
Loma Pachona	48.1 (42%)	78.95	2	0.496	0.358	11	1	10
Cerro Grande	52.3 (43%)	78.95	1.95	0.526	0.360	8	0	8
Mean ± SD	44.9 ± 10.2	78.95 ± 4.3	1.91 ± 0.12	0.49 ± 0.04	0.35 ± 0.01			

*Represents the sample which is finally computed; missing genotypes are captured as zero, this decreases the collected sample.

N, number of populations; P, per cent of polymorphic loci; A, number of alleles per locus; H_O, observed mean heterozygosity; H_E, mean expected heterozygosity; H-W, Hardy–Weinberg deviations; Test, number of loci deviated; –, number of loci with less heterozygotes than expected; +, number of loci with more heterozygotes than expected.

($P < 0.001$) and F_{ST} pairwise comparisons were all significant (Table 3), except for the comparison between Loma Pachona vs. Cerro Grande. The mean F_{ST} value for all loci was 0.099 ± 0.027 with 0.053 to 0.153 (confidence intervals at 95%). This indicated that *c*. 10% of the variation was because of allozyme differences among the four populations. F_{IS} and F_{IT} were negative at all loci, indicating heterozygote excess in all populations. Means and standard deviations were $F_{IS} = -0.379 \pm 0.076$ and –0.515 to –0.228, $F_{IT} = -0.242 \pm 0.089$ and –0.400 to –0.081, respectively (confidence intervals at 95%).

Table 3. Estimated genetic differentiation values (F_{ST}) and geographic distances (km) between population pairs of *Dioon caputoi* in the Tehuacán–Cuicatlán Biosphere Reserve

Population pair	F_{ST}	km
La Grana vs. El Guayabo	0.08*	1.37
La Grana vs. Loma Pachona	0.11*	3.6
La Grana vs. Cerro Grande	0.08*	8.14
El Guayabo vs. Loma Pachona	0.14*	3.00
El Guayabo vs. Cerro Grande	0.14*	7.24
Loma Pachona vs. Cerro Grande	0.03	9.41
Mean ± SD	0.096 ± 0.043	5.46 ± 3.23

*$P < 0.05$.
F_{ST}, genetic differentiation among populations.

Bottleneck

All populations contained loci that deviated from the mutation–drift equilibrium because of an excess of heterozygotes ($P < 0.001$). Four of these loci were identified in La Grana, two in El Guayabo and five in both Cerro Grande and Loma Pachona (Table 4).

Congeneric comparison

No significant differences were found in most of the mean genetic variation values among *D. caputoi* and its congeners ($P > 0.05$; Table 6). The exceptions to this were: (1) mean number of alleles per locus where *D. caputoi* exhibited more (1.91 ± 0.12) than *D. edule* (1.44 ± 0.24) and, (2) expected heterozygosity, which was higher in *D. caputoi* (0.35 ± 0.01) than in *D. angustifolium* (0.22 ± 0.09).

DISCUSSION

Genetic diversity

Most estimates of genetic variation for *D. caputoi* were high compared to those reported in the allozyme literature for endemic plants (Hamrick & Godt, 1996; Hamrick, 2004). These results are remarkable, since they appear to confirm that rarity is compatible with high levels of genetic diversity (Gitzendanner & Soltis, 2000; Cole, 2003). We suggest that the combination of longevity and mating system may have influenced levels of genetic diversity, or that such levels may be due to an evolutionary history that is shared by other *Dioon* species, which also exhibit high levels of genetic diversity.

Table 4. Genetic bottleneck values in populations of *Dioon caputoi* in the Tehuacán–Cuicatlán Biosphere Reserve

Population	Locus	H_E	H_{eq}	*P*-value (locus)	*P*-value (Wilcoxon's test)
La Grana	APX1	0.506	0.212	0.021	0.00015
	MDH1	0.503	0.204	0.043	
	6PGD	0.508	0.204	0.017	
	PGI	0.503	0.199	0.036	
El Guayabo	IDH1	0.502	0.197	0.032	0.00019
	PGI	0.184	0.038	0.038	
Loma Pachona	MDH1	0.500	0.191	0.045	0.000064
	6PGD	0.503	0.195	0.03	
	PGI	0.500	0.197	0.034	
	GDH	0.504	0.188	0.028	
	EST	0.502	0.188	0.038	
Cerro Grande	APX1	0.500	0.185	0.032	0.00021
	MDH1	0.503	0.179	0.02	
	6PGD	0.504	0.185	0.012	
	ACPH	0.504	0.189	0.025	
	PGI	0.504	0.179	0.023	

H_E, expected gene diversity; H_{eq}, expected equilibrium gene diversity.

Table 5. Mean genetic variation and ranges for cycad families [†‡statistical significance ($P < 0.05$) among groups using Tukey Honest Significant Difference test for unequal N comparisons (Sokal & Rohlf, 1995)]. These data represent the mean and standard deviation of values estimated from: Byrne & James (1991); Walters & Decker-Walters (1991); Yang & Meerow (1996); Sharma *et al.* (1998); Li *et al.* (1999); Sharma *et al.* (1999); Yang & Meerow (1999); Lin *et al.* (2000); Keppel (2002); Pinares (unpubl. data); González-Astorga *et al.* (2003); González-Astorga *et al.* (2005); González-Astorga *et al.* (2008)

Groups	No. of species	*A*	*P*	H_E	F_{ST}
All cycads*	16	1.59 ± 0.35	48.72 ± 24.61	0.144 ± 0.09	0.19 ± 0.16
Zamiaceae	9	1.60† ± 0.36	50.87† ± 24.76	0.167†±0.105	0.185† ± 0.153
		1.2 to 2.43	16.7 to 93	0.041 to 0.274	0.03 to 0.588
Cycadaceae	7	1.56† ± 0.35	44.73† ± 25.76	0.101† ± 0.06	0.196† ± 0.168
		1.07 to 1.82	2.5 to 70.6	0.013 to 0.138	0.02 to 0.387
Genus *Dioon*	4	1.77† ± 0.26	67.98† ± 16.65	0.267‡ ± 0.045	0.135† ± 0.041
		1.44 to 2.00	52.4 to 83.15	0.218 to 0.314	0.075 to 0.167
Dioon caputoi	1	1.91 ± 0.12	78.95 ± 4.3	0.35 ± 0.01	0.099 ± 0.027

*As genus *Dioon* is being compared with other cycad species, the cycad species group includes Zamiaceae and Cycadaceae species but with the exception of *Dioon*.

A, number of alleles per locus; *P*, per cent of polymorphic loci; H_E, mean expected heterozygosity; F_{ST}, genetic differentiation among populations.

Long-lived, woody perennials usually show high levels of genetic diversity (Hamrick, Godt & Sherman-Broyles, 1992). Individual plants of *Dioon* are among the oldest living plants, with long life cycles and ages that have been estimated over 2,000 years (Chamberlain, 1919; Vovides & Peters, 1987; Vovides, 1990). The average minimum age at which wild reproductive female plants of *D. edule* have been observed is about 500 years and 40–50 cm tall (Vovides, 1990). This is in agreement with the reproductive ages found by Octavio-Aguilar, González-Astorga & Vovides (2008). We have calculated similar ages for *D. caputoi* (Cabrera-Toledo, unpubl. data). The long life cycle and obligate outcrossing mating system (dioecy) of *D. caputoi* may be contributing factors which explain the high levels of allozyme diversity found in this study. It has been found that in systems where selfing is completely prevented, through dioecy (Costich & Meagher, 1992; Oostermeijer & De Knegt, 2004) or self-incompatibility (Segarra-Moragues & Mateu-Andrés, 2007), allozyme diversity is higher within than among populations.

Hedrick (2000) suggested that it would take a population of all heterozygotes (i.e. $H_O = 1$) undergoing continuous full-sib mating approximately 15 generations to reach a low level of heterozygosity (e.g. $H_T \sim 0.02$). During such a hypothetical situation for any *Dioon* species, it would likely take several millennia to reach such a low level of heterozygosity. Analogous to *Dioon*, long-lived tree species have high levels of genetic diversity, even in fragmented environments (e.g. González-Astorga & Castillo-Campos, 2004; Lowe *et al.*, 2005; Feyissa *et al.*, 2007) where some impact on genetic variation would be expected given the reduction of population size. This evidence may be explained by the effect of the slow rate of genetic diversity-loss, which renders historic genetic pool still detectable. In other words, the time lapse between the initial habitat disturbance and actual time is short compared to the longevity and generation span of the species (Feyissa *et al.*, 2007).

In this study, bottleneck analysis showed that all populations have experienced a recent reduction of their effective population sizes (Table 4). However, the high levels of allozyme variation suggest that recent drift has not been important (cf. Martínez-Palacios *et al.*, 1999). This also suggests that present populations are relicts of historically much larger populations that were established when environmental conditions were distinct from those of the present. These conclusions support the notion that allozyme variation may be an echo of past environmental conditions (Premoli, Kitzberger & Veblen, 2000).

Alternatively, high levels of genetic diversity in *D. caputoi* could be explained by shared evolutionary histories among its congeners. Most mean values of *D. caputoi* ($A = 1.91$, $P = 78.9$, $H_E = 0.35$) are within the range of values found among them (Table 6). The genus *Dioon*, mostly endemic to México, shows the highest mean expected heterozygosity compared with the mean for other cycad groups, especially *Cycas* spp. (i.e. Cycadaceae, Table 5). *Cycas* is an Asian genus, the most divergent group in the cycads (Hill *et al.*, 2003; Bogler & Francisco-Ortega, 2004). Cycad families and subfamilies have probably maintained their mutual distinctions at least since mid-Mesozoic times

(Norstog & Nicholls, 1997) and thereafter speciation occurred through vicariance (Keppel, Hodgskiss & Plunkett, 2008). Thus, it is expected that different geological histories and paleoclimatic events could have influenced the evolutionary histories of both lineages (i.e. *Cycas* and *Dioon*) in different ways.

Species diversity is consistent with the genetic diversity patterns between these cycad groups, where more cycad species are known from Mexico than from China and Vietnam together. Genetic and biological diversity may be related in several ways, especially if locality characteristics (e. g. spatial/temporal heterogeneity, environment, isolation or any common cause) influence the two levels of diversity (genes and species) in a parallel manner where a positive correlation between them may result (Vellend & Geber, 2005).

Genetic structure

The negative inbreeding values are consistent in all loci evaluated and reflect a non-panmictic mating system with heterozygote excess (Wright, 1951). Other allozyme studies have identified heterozygote excesses for other cycads (Walters & Decker-Walters, 1991 *Zamia pumila* L.; Lin *et al.*, 2000 *Cycas taitungensis* C. F. Shen, K. D. Hill, C. H. Tsou & C. J. Chen; González-Astorga *et al.*, 2003, 2008. *D. edule* and *D. holmgrenii*, respectively), as well as for other long-lived perennial plants (e.g. Eguiarte, Pérez-Nasser & Piñero, 1992 *Astrocaryum mexicanum* Liebm.; Alvarez-Buylla *et al.* 1996a *Cecropia obtusifolia* Bertol.). Overdominance or heterozygote advantage is a frequent and persuasive explanation for the maintenance of genetic variation in natural populations (Alvarez-Buylla *et al.*, 1996b). Nevertheless, the origin of heterozygote excess remains poorly studied (Gemmell & Slate, 2006; Stoeckel *et al.*, 2006).

Cases of natural selection in biochemical traits, such as allozymes, are comparatively rare, probably because they are selectively neutral or weakly coupled to fitness (Endler, 1986). Also, the long life cycle and scarcity of individuals from each stage category in natural populations prevents the design of methods that may assess the mechanisms that are responsible for heterozygote excess in *D. caputoi*. Considering these limitations, we will discuss heterozygote excess from a neutral evolutionary perspective and suggest working hypotheses for further research.

Bottleneck analyses showed that *D. caputoi* has experienced a reduction in population sizes (Table 4) and the high level of allozyme variation suggests that drift has not played a strong role. However, bottlenecks can also increase demographic stochasticity (Luikart *et al.*, 1998). We noticed, mainly, three demographic traits that support this: (1) predominance of the adult stage in all populations; (2) low recruitment rate; and (3) atypical sex ratios (1 : 1 male to female in La Grana and Loma Pachona and 2 : 1 in El Guayabo). Other *Dioon* species' sex ratios are typically 3 : 1 male to female (*D. edule* Vovides, 1990 and *D. merolae*: Cabrera-Toledo, unpubl. data). The higher frequency of male cones in a population may be advantageous to the population by ensuring the presence of ample pollen during the female plant's coning period (Vovides, 1990). However, the low percentage of coning of both sexes in two populations (La Grana 14.5% and El Guayabo 3.9%) (Cabrera-Toledo, unpubl. data) is even more conspicuous. Also, pollinators are rarely found (Cabrera-Toledo, pers. observ.) as they are active for only a brief period during receptivity of female cones (10–15 days). Thus, the possible absence of synchrony and the differing frequency among coning events in both sexes could have modified the sex ratios (1 : 1, 2 : 1). Under these unpaired sex ratios and low frequency of coning events, it is reasonable to expect low recruitment and effective population size (cf. Octavio-Aguilar *et al.*, 2008).

Outcrossing in populations with low effective sizes may act as a mechanism for the accumulation of heterozygote excess where the allelic frequencies between males and females in a small population differs by chance alone; that is, by a drift process resulting in more frequent crosses between individuals bearing different alleles (Pudovkin, Zaykin & Hedgecock, 1996; Balloux, 2004). Moreover, in dioeceous as well as in self-incompatible species, the missing proportion of homozygotes because of outcrossing also contributes to heterozygote excess (cf. Stoeckel *et al.*, 2006).

These combined effects will probably reflect heterozygote excess in future generations and, if so, this should be detectable in the seedling stage (Stoeckel *et al.*, 2006). In order to test this hypothesis, we analysed the allozyme variation of some seedlings obtained from three *D. caputoi* populations (30 seedlings). The seedling F_{IT} and F_{IS} were found to be –0.327 and –0.615, respectively; this appears to support that heterozygote excess occurs in this stage. But the low number of cones available did not allow us to take this as a conclusive result; however, further work could confirm or reject this hypothesis.

The genetic differentiation of *D. caputoi* ($F_{ST} = 0.099$) is lower than the average for Zamiaceae and Cycadaceae species studied to date (see Table 5). Restricted seed and pollen flow generally promotes differentiation in insect-pollinated species with gravity-dispersed seeds and these are expected to show significant differentiation among populations (Loveless & Hamrick, 1984). For cycads, the highest values of genetic differentiation have been reported

for *Cycas pectinata* Buch.-Ham. (F_{ST} = 0.387, Yang & Meerow, 1996), *C. siamensis* Miq (F_{ST} = 0.291, Yang & Meerow, 1996) and *C. seemannii* (F_{ST} = 0.594, Keppel, 2002), which agree with the fact that populations are isolated on islands. In this study, allozyme subdivision (F_{ST}) between pairs of populations did not increase as geographical distance decreased (data not shown). As the lowest genetic differentiation among all pairwise comparisons was between the two largest and most geographically distant (9.41 km, Table 3) populations (i.e. Loma Pachona, $N \sim 114$ individuals; Cerro Grande, $N \sim 121$ individuals), these results may indicate that the genetic differentiation of the smaller populations (i.e. La Grana, $N \sim 55$; El Guayabo, $N \sim 77$) may have been historically influenced mainly by genetic drift, which increases differentiation between populations (Hedrick, 2000). Finally, it is notable that, in spite of their differing geographic ranges, species level F_{ST} values are similar among the *Dioon* spp. so far studied (Table 6) and agree with Cole (2003), where a common ancestor explains non-significant genetic differentiation among congeners.

Conservation implications

Being genetically diverse, *D. caputoi* has great potential for sustainable management. Moreover, this genetic diversity is distributed mainly within populations. In this context, the partial reduction in distribution that could be given by stochastic environmental events (e.g. habitat fragmentation and global climate changes) does not imply a major loss of genetic diversity (Hamrick, 2004); this scenario shows how a low population structure can be advantageous in long-lived plant species, such as *D. caputoi.*

The regional analyses of Donaldson (2003) showed that cycads in the New World, Africa and Asia are far more threatened than those in Australia. Our results suggest that at least inbreeding and genetic drift do not represent an immediate danger for the extinction of *D. caputoi.* However, as populations are composed of mainly adult individuals that were established in ancient environmental and demographic conditions, we do not know if the actual conditions will maintain this healthy genetic scenario in future generations. This could have implications on conservation plans for *D. caputoi* populations, which should focus on demographic threats that are of more immediate importance than the loss of genetic diversity. Several examples of this dilemma were shown by Lande (1988); these could be aggravated if the population decreases.

In this context, demographic studies in the African cycads *Encephalartos cycadifolius* with similar life-history parameters has shown that harvesting of seeds had minimal impact on population growth rates, whereas harvesting simulations of adult plants

Table 6. Estimates of genetic variation in *Dioon* species

Species	Reference	Site of study	GR	*N*	*A*	*P*	H_E	F_{ST}
Dioon edule	González-Astorga *et al.* (2003)	East coast of Mexico	*c.* 950 km (*c.* 1400 ind.)	8	1.44†‡ ± 0.24	54.8† ± 10.95	0.24†‡ ± 0.04	0.075
D. angustifolium	González-Astorga *et al.* (2005)	North-Eastern Mexico	*c.* 96 km (*c.* 600 ind.)	3	1.67†‡ ± 0.23	52.4† ± 22.99	0.218† ± 0.093	0.167
D. sonorense	González-Astorga *et al.* (2008)	Sonoran coast of Mexico	*c.* 295 km (*c.* 600 ind.)	4	2.00† ± 0.04	81.6†‡ ± 10.07	0.314†‡ ± 0.036	0.153
D. tomasellii	González-Astorga *et al.* (2008)	Meridian west Mexican coast	*c.* 800 km (*c.* 1000 ind.)	5	1.96† ± 0.069	83.15‡ ± 12.00	0.295†‡ ± 0.073	0.142
D. caputoi	This study	Centre of Mexico	*c.* 10 km (*c.* 400 ind.)	4	1.91† ± 0.12	78.95†‡ ± 4.30	0.35‡ ± 0.012	0.099

Data represent the mean and standard deviation of values estimated.
GR, geographic range; ind., entire number of individuals in each species; *N*, number of populations; *A*, number of alleles per locus; *P*, per cent of polymorphic loci; H_E, mean expected heterozygosity; F_{ST}, genetic differentiation among populations.
†‡Statistical significance among species ($P < 0.05$) using Tukey Honest Significant Difference test for unequal *N* comparisons (Sokal & Rohlf, 1995).

showed that recovery from a major loss may take between 100 and 300 years (Raimondo & Donaldson, 2003). The persistence of adult plants was also critical for the population dynamics of *D. edule* (Octavio-Aguilar *et al.*, 2008). Urgent conservation actions to reduce demographic threats in *D. caputoi* populations are: (1) to halt illegal extraction and further deforestation within the present populations; (2) to protect and guarantee the presence of reproductive adults; (3) to monitor the population dynamics of the species as a long-term management plan; (4) to increase the presence of seedlings and juveniles by (a) controlling goat grazing and (b) reintroduction of artificially propagated plants. This reintroduction should consider three criteria: (1) the genetic structure of populations – this needs to be maintained by reintroducing plants obtained from seeds from mother plants of the same populations; (2) plants of 8–10 years old should be introduced, by which time they will have higher survival and reproduction probabilities; and (3) plants should be reintroduced to various microhabitats of the populations; this has been found to be successful with the long-lived palm *Pseudophoenix sargentii* by Maschinski & Duquesnel (2006), where multi-site reintroductions were found to buffer against stochastic losses. This is a potential strategy for our case.

Although it is hoped that this will eventually maintain the populations at a stable growth rate over short- and long-term periods, we should remember that the relative importance of demographic and genetic factors in determining extinction probabilities in natural populations is still unclear (Alvarez-Buylla *et al.*, 1996b). Moreover, recent applications of phylogenetic evidence showed that the origin and diversity at the species level should also be integrated to set indices for conservation priorities criteria (Delgado *et al.*, 2008). Therefore, we recommend that further analysis of cycad conservation priorities follow this advice.

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