

Allozyme Variation in the Three Extant Populations of the Narrowly Endemic Cycad *Dioon angustifolium* Miq. (Zamiaceae) from North-eastern Mexico

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• **Background and Aims** *Dioon angustifolium* was considered within *D. edule*. Recent morphometric and allozyme studies on *D. edule* have shown that *D. angustifolium* has originated from geographic isolation and is therefore considered to be a separate species. This cycad is endemic to north-eastern Mexico and is known only from three populations in the Sierra Madre Oriental mountain chain. Its populations are small when compared with its southern relative *D. edule*. In this study, genetic variation was determined within and between populations of *D. angustifolium* and the genetic consequences of habitat fragmentation and isolation of populations of this species were assessed.

• **Methods** Allozyme electrophoresis of 14 presumptive loci was used. The data were analysed with statistical approximations for estimating genetic diversity, structure, gene flow and recent genetic bottlenecks.

• **Key Results** Means and standard deviations of genetic diversity estimators were: number of alleles per locus ($A = 1.67 \pm 0.23$), percentage of polymorphic loci ($P = 52.4 \pm 23\%$) and expected heterozygosity ($H_E = 0.218 \pm 0.093$). The genetic variation attributable to differences among populations was 16.7%. Mean gene flow between paired populations was $Nm = 1.55 \pm 0.67$, which is similar to that reported for endemic plant species of narrow geographical distribution and species with gravity-dispersed seed. A recent bottleneck is detected in the populations studied.

• **Conclusions** *Dioon angustifolium* presents high levels of genetic diversity compared with other cycad species, in spite of small population sizes. The recent bottleneck effect did not effectively reduce the genetic variation to the extent of eliminating these populations. The distribution of *D. angustifolium* appears to be the result of historical biogeographical effects related to the Pleistocene glaciations. It is recommended that this species be catalogued in the IUCN Red List of Threatened Species and conservation efforts be made to preserve it.

Key words: Conservation, cycads, endemic narrow distribution, genetic bottleneck, genetic structure, Mexico, Pleistocene refugia, population genetics.

INTRODUCTION

The distribution of genetic variability within and among populations is thought to arise primarily from the combined effects of four evolutionary forces: inbreeding, genetic drift, mutation and gene flow. The description of genetic structure of wild plant populations provides insights into how the history of past and contemporary species was influenced by these evolutionary forces (Hutchinson and Templeton, 1999; Epperson, 2003). Also, the distribution and extent of plant species and genetic variation are related to factors such as taxonomic status (Gitzendanner and Soltis, 2000; Cole, 2003), life history (Loveless and Hamrick, 1984) and biogeographic history (Cox and Moore, 2000).

Pleistocene glacial events affected the biogeographical distribution patterns of many plant species in both the northern and southern hemispheres (Graham, 1975; Prance, 1982; Toledo, 1982). Marchelli and Gallo (2004) attribute the distribution patterns of the genetic variation of *Nothofagus obliqua* (Mirb.) Oerst. and *N. nervosa* (Phil.) Dim. et Mil. to post-glacial migration routes from different glacial refugia. Plana *et al.* (2004) have shown that certain African *Begonia* lineages have a pre-Pleistocene origin, while others

resulted from recent Pleistocene radiations. In addition, Contreras-Medina and Vega (2002) discuss the congruence of areas of gymnosperm endemism and suggested Pleistocene refuges to explain them. Endemic species of *Polygonella* in North America that maintain high genetic diversity in known Pleistocene refuges have important conservation implications where species richest in gene diversity may be the most endangered (Lewis and Crawford, 1995). Al-Rabab'ah and Williams (2004) have shown that the Lost Pines population of *Pinus taeda* of central Texas had undergone drastic contractions in population size since Pleistocene and post-Pleistocene times as evidence of an ancient bottleneck.

Cycads are the oldest group of living seed plants (Norstog and Nicholls, 1997), the group's fossil record goes back to the Permian (approx. 290 million years) and possibly the Carboniferous (approx. 350 million years) (Mamay, 1976; Norstog and Nicholls, 1997). They are long-lived perennials, dioecious, pachycaulous plants that are insect-pollinated with little or no evidence of wind-pollination (Tang, 1987; Norstog and Fawcett, 1989; Vovides, 1991; Vovides *et al.*, 1997) and appear to be predominately outcrossing. Approximately 300 species (Donaldson, 2003a, b; Hill *et al.*, 2004) have been described from tropical and subtropical

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locations in both hemispheres (Norstog and Nicholls, 1997; González-Astorga *et al.*, 2003a, b; Vovides *et al.*, 2004), a number that is likely to increase in the future as more botanical explorations take place (Hill *et al.*, 2004). Cycads are regarded by some popular texts as 'living fossils' that were abundant during the Jurassic, 'the age of cycads and dinosaurs' (Jones, 1993), and Knut Norstog, an eminent cycadologist, refers to them as 'the dinosaurs of the plant world' (Norstog and Nicholls, 1997). Cycads are not to be considered as evolutionary remnants since they are specialized rather than primitive (Thomas and Spicer, 1987) and are heterobathmic (Moretti, 1990) in that they contain ancestral as well as derived characters. Recently, molecular studies appear to indicate that some extant genera originated during relatively modern times (during Miocene and Pleistocene times) (Schneider *et al.*, 2002).

Some species of cycads show high levels of genetic diversity within populations but little genetic differentiation among populations (e.g. *Macrozamia riedlei*, Byrne and James, 1991; *Dioon edule*, González-Astorga *et al.*, 2003a). Species in disturbed or fragmented populations have shown lower genetic variability, possibly caused by fragmentation leading to bottleneck effects (e.g. Sharma *et al.*, 1998, 1999; Chao-Luan *et al.*, 1999; Keppel, 2002). In addition, isolation by increased geographical distances, as a result of habitat fragmentation, probably caused a high genetic differentiation among populations (Ellstrand *et al.*, 1990; Walters and Decker-Walters, 1991; Yang and Meerow, 1996; Keppel *et al.*, 2002).

Cycads have been considered as threatened, rare or endangered, and have been listed in the IUCN Red List (The World Conservation Union, 2003) because of their narrow distributions, small population sizes and various threats to populations and their habitats (Donaldson, 2003a, b; Hill, 2003; Stevenson *et al.*, 2003). In Mexico many species are restricted to areas where Pleistocene refugia have been hypothesized (González and Vovides, 2002; González-Astorga *et al.*, 2003a, b; Vovides *et al.*, 2004). Contrary to restriction of range and habitat reduction, populations of *Cycas seemannii* on the Pacific Islands (New Caledonia, Vanuatu, Fiji and Toga) were considered to stem from recent colonization events, and it has been suggested that this fact should be taken into account when devising conservation strategies (Keppel, 2002; Keppel *et al.*, 2002).

Dioon angustifolium Miq. (Zamiaceae) is an arborescent cycad with trunks up to 1.5 m tall and is distributed only in north-east Mexico, specifically in the *Sierra Madre Oriental* of Nuevo León state and in central Tamaulipas State (Stevenson and Sabato, 1986; González-Astorga *et al.*, 2003a) (Fig. 1). *Dioon angustifolium* is closely related to *D. edule* Lindl. which is the type species of the genus. *Dioon angustifolium* was described by Miquel (1848) who based the description on leaflet characters. Later he assigned it as a variety of *D. edule*. Lemaire (1855) listed this variety, amongst others, under the synonymy of *D. edule*. Leaflet characteristics are often used in cycad taxonomy which early authors have often emphasized or minimized in their treatments of *Dioon*. A detailed discussion of this can be found in De Luca *et al.* (1982). A molecular phylogenetic

analysis of *Dioon* using DNA restriction fragment length polymorphism (Moretti *et al.*, 1993) also confirms the close relationship of the two.

Dioon edule has a far greater distribution range than *D. angustifolium*, though discontinuous, from southern Tamaulipas to central Veracruz amongst scattered populations of varying sizes. These have become greatly reduced during recent years (De Luca *et al.*, 1982; González-Astorga *et al.*, 2003a, b). The two species are not sympatric and *D. angustifolium* is found at the northernmost edge of the distribution range of the genus in eastern Mexico (Fig. 1). Though no pollination or seed dispersal studies have been carried out on *D. angustifolium*, curculionid beetles (weevils) have been found in dehiscent male cones, suggesting an insect pollination syndrome similar to that found in other cycads (Norstog and Fawcett, 1989; Vovides, 1991; Norstog and Nicholls, 1997; Norstog *et al.*, 1995). Dispersal of *Dioon* seeds is largely by gravity, and the rodent *Peromyscus mexicanus* is known to disperse *D. edule* seeds (González-Christen, 1990; Vovides, 1990).

Habitat destruction and elimination of cycads by cattle ranchers owing to their toxicity to cattle (Norstog and Nicholls, 1997) are among the main threats of both *D. angustifolium* and *D. edule* wild populations. Cycads are also considered a priority by the Mexican conservation authorities for conservation and study (INE-SEMARNAP, 2000).

In this study the variation and genetic structure of *D. angustifolium* was assessed. Allozyme loci were used to examine the population genetics of the three known populations of this cycad. The aims were to: (a) evaluate the genetic diversity and determine the amount and distribution of genetic diversity within and among populations; (b) estimate levels of gene flow between population pairs; (c) contrast the results and conclusions with other plant species possessing similar ecological and evolutionary characteristics, particularly with respect to speciation mechanisms; (d) detect recent bottlenecks in each population from allele frequency data; and (e) evaluate the conservation status of the extant populations of *D. angustifolium*, such as the consequences of habitat fragmentation.

METHODS

Study sites

Three extant populations were sampled in the states of Nuevo León and Tamaulipas (Fig. 1). The first population at San Carlos has approx. 150 adult plants in an area of approx. 1 ha in spiny scrub vegetation with oak. The second population at Alamos consists of approx. 50 adult plants scattered along a river valley, covering approx. 1 ha, in remnants of pine-oak forest. About 20 seedlings were found in this area. The third at Iturbide-Linares consists of about 100 adult plants in approx. 1 ha of oak forest.

Sample collection

Populations are separated by distances that range from approx. 19 to 96 km (Table 3 and Fig. 1). Sampling of

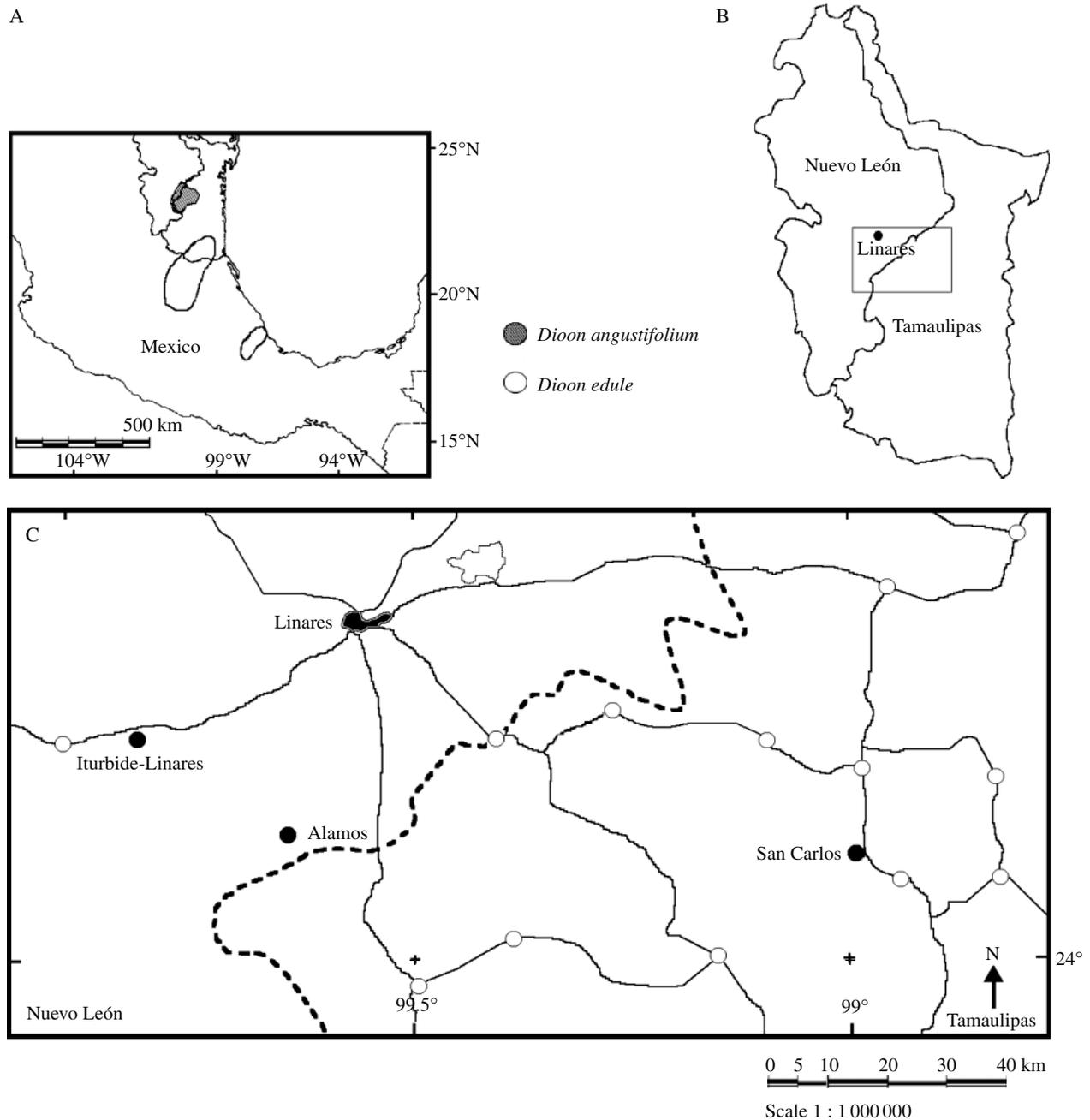


FIG. 1. Geographical distribution of *Dioon edule* and *Dioon angustifolium*: (A) map of Mexico showing distribution ranges of both taxa; (B) States of Nuevo León and Tamaulipas; (C) the three populations of *Dioon angustifolium* examined (black dots).

leaflets was done in three populations along the distribution range of *D. angustifolium*. The collected leaflets were transported on ice, to avoid protein denaturalization, and subsequently stored in a Revco freezer set to -70°C until the moment of allozyme extraction.

Electrophoresis

Approximately 300 mg of fresh leaflet tissue were ground with liquid nitrogen. About 250 μL of extraction buffer

(0.1 M Tris-HCl pH 7.5, 4 % PVP-40, 0.001 M EDTA, 0.01 M CaCl_2 , 0.01 M MgCl_2 and 0.1 % β -mercaptoethanol; Chao-Luan *et al.*, 1999) were added to dilute and stabilize the enzyme extracts, which were stored on filter paper wicks at -70°C until used for analyses. Multilocus genotypes of individuals from each population were obtained through horizontal starch gel electrophoresis (10 % w/v). For each individual plant, allozymic variation was scored for 14 loci (Table 1). Electrophoresis was carried out at 4°C for 8 h (constant current of 50 mA, and voltage of 80 V).

TABLE 1. Allozyme systems resolved in this study

Allozyme	Locus abbreviation	EC no.	Buffer*
Malate dehydrogenase	<i>Mdh</i>	1.1.1.37	R
Phosphoglucosomerase	<i>Pgi</i>	5.3.1.9	R
6-Phosphogluconate dehydrogenase	<i>6Pgd</i>	1.1.1.44	R
Diaphorase	<i>Dia</i>	1.6.99.-	R
Isocitrate dehydrogenase	<i>Idh</i>	1.1.1.41	R
Esterase	<i>Est</i>	3.1.1	9
Leucine aminopeptidase	<i>Lap</i>	3.4.11.1	9
Anodic peroxidase	<i>Apx1</i> , <i>Apx2</i> , <i>Apx3</i> and <i>Apx4</i>	1.11.1.7	9
Glutamate oxaloacetate transaminase	<i>Got</i>	2.6.1.1	9
Aminopeptidase	<i>Amp</i>	3.4.11.1	9
Alcohol dehydrogenase	<i>Adh</i>	1.1.1.1	9

Nomenclature and abbreviations follow Wendel and Weeden (1989), based on IUBNC (Nomenclature Committee of the International Union of Biochemists).

EC no. = Enzyme Commission number.

* R and 9 refer to system buffers (gel/electrode) of Chao-Luan *et al.* (1999) and Soltis *et al.* (1983), respectively.

Statistical methods

The alleles were inferred from observed banding patterns and the genotypes constructed. The TFGPA 1.3 package (Miller, 1997) was used to estimate the genetic variation. Genotypic frequencies at each population were used to estimate the observed mean heterozygosity (H_O). The observed allelic frequencies for each population were used to estimate the mean number of alleles per locus (A), the percentage (at 95 %) of polymorphic loci (P) and expected mean heterozygosity (H_E) with respect to the Hardy-Weinberg expectation (Hartl and Clark, 1997; Hedrick, 2000).

Partitioning of genetic variation was done with F statistics (Wright, 1965, 1978) estimated according to the equations of Weir and Cockerham (1984). These statistics measure genetic structure by partitioning variation in the same way as a regular analysis of variance. The F_{st} statistic measures the divergence in allele frequencies among populations, whereas F_{is} and F_{it} measure heterozygote excess (<0) and deficit (>0) relative to the Hardy-Weinberg expectation in local populations and the total set of populations, respectively. To determine whether F_{is} and F_{it} estimations for each locus were significantly different from zero, chi-square statistics [$\chi^2 = F(2N)(k - 1)$] were obtained, with $k(k - 1)/2$ degrees of freedom, where N is the sample size and k is the number of alleles (Weir, 1990; Berg and Hamrick, 1997). To determine the significance of the F_{st} statistic per locus, the chi-square statistic was used: $\chi^2 = (2N)F_{st}(k - 1)$, with $(k - 1)(s - 1)$ degrees of freedom, where s is the number of populations (Workman and Niswander, 1970). The confidence intervals (at 95 %) of the F statistics were obtained by bootstrapping over loci for the multilocus estimate, and then jackknifing over populations for the single-locus estimates (Weir and Cockerham, 1984; Weir, 1990).

The exact test of Raymond and Rousset (1995), which is analogous to Fisher's exact test (Fisher, 1935) but uses Markov chains to explore all potential states of an $m \times n$ contingency table based on m populations and n genotypes, was employed to test for genetic differentiation among populations. The test was for all pairwise combinations and was conducted using TFGPA 1.3 software (Miller, 1997) and 10 000 Markov steps.

The average gene flow among populations (Nm) was estimated from F_{st} values, as $F_{st} = 1/(4Nm\alpha+1)$; where $\alpha = [s(s - 1)]^2$ and s the number of analysed populations (Crow and Aoki, 1984). Nm is interpreted as the number of migrants per generation between two given populations (Slatkin, 1993, 1994).

Finally the gene frequency data were analysed using BOTTLENECK software (Piry *et al.*, 1999) to assess evidence of recent bottlenecks. Deviations from expected heterozygosity were computed for each locus for each population. The infinite allele model (Kimura and Crow, 1964) was used since it is the most appropriate for allozyme data (Piry *et al.*, 1999). To determine the significance of deviations a two-tailed Wilcoxon sign-rank test (Luikart *et al.*, 1998) was conducted. This method tests whether the expected gene diversity (H_E) is higher than the expected equilibrium gene diversity (H_{eq}) 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 and Cornuet, 1998).

RESULTS

Genetic variation

Allelic frequencies for 14 loci were scored for each population (Table 2). The average number of alleles per locus was 1.67 ± 0.23 . Percentage of polymorphic loci per population varied from 35.7 % (Iturbide-Linares, NL population) to 78.6 % (San Carlos, Tam population), with an average of 52.4 % (Table 3). Observed mean heterozygosity was 0.215 ± 0.132 (range 0.121–0.366) and expected mean heterozygosity was 0.218 ± 0.093 (range 0.134–0.318) (Table 3).

Genetic structure

The mean of F_{it} value was significantly different from zero (0.165 ± 0.045). Specifically, for F_{it} statistics (global inbreeding) in 14 loci, six were positive and significantly different from zero (*Mdh*, *6Pgd*, *Dia*, *Idh*, *Est* and *Lap*) and the other eight loci were not different from zero (Table 4). Only two loci (*Dia* and *Idh*) had F_{is} values (local inbreeding) significantly different from zero; the first was positive and second was negative, indicating an excess and deficiency of homozygotes, respectively. The mean of the F_{is} values did not significantly differ from zero (Table 4). Most loci showed F_{st} estimates to be positive and significantly different from zero, with the exception of loci *Apx 4*, *Got*, *Amp* and *Adh*. For all loci, the mean F_{st} was 0.167 ± 0.045 (95 % confidence interval: 0.095–0.247). This result indicated that 16.7 % of the genetic diversity

TABLE 2. Allelic frequencies of 14 allozyme loci of three populations of *Dioon angustifolium* in northern Mexico

Locus	Allele	Population		
		San Carlos (Tam)	Alamos (NL)	Iturbide-Linares (NL)
<i>Mdh</i>	A	0.3265	0.3472	0.0000
	B	0.6735	0.6528	1.0000
<i>Pgi</i>	A	0.3605	0.0000	0.0000
	B	0.6395	1.0000	1.0000
<i>6Pgd</i>	A	0.3545	0.4730	0.7273
	B	0.6455	0.5270	0.2727
<i>Dia</i>	A	0.2872	0.2805	0.5385
	B	0.4468	0.3171	0.1923
	C	0.2660	0.4024	0.2692
<i>Idh</i>	A	0.3611	0.1277	0.1000
	B	0.1204	0.6596	0.8714
	C	0.5185	0.2127	0.0286
<i>Est</i>	A	0.4825	0.1463	0.2400
	B	0.5175	0.8537	0.7600
<i>Lap</i>	A	0.5208	0.6000	0.8235
	B	0.4792	0.4000	0.1765
<i>Apx 1</i>	A	0.9123	1.0000	1.0000
	B	0.0877	0.0000	0.0000
<i>Apx 2</i>	A	0.8860	1.0000	1.0000
	B	0.1140	0.0000	0.0000
<i>Apx 3</i>	A	0.8772	1.0000	1.0000
	B	0.1228	0.0000	0.0000
<i>Apx 4</i>	A	0.8333	1.0000	1.0000
	B	0.1667	0.0000	0.0000
<i>Got</i>	A	1.0000	1.0000	1.0000
	B	0.0000	0.0000	0.0000
<i>Amp</i>	A	1.0000	1.0000	1.0000
	B	0.0000	0.0000	0.0000
<i>Adh</i>	A	1.0000	1.0000	1.0000
	B	0.0000	0.0000	0.0000
No. of alleles/locus (mean \pm SD)		1.93 \pm 0.62	1.57 \pm 0.76	1.50 \pm 0.76

within *D. angustifolium* was distributed among populations (Table 4). Also, the exact test of allelic differentiation among populations was significant (Table 5).

Gene flow

Indirect estimates of gene flow (Nm) for *D. angustifolium* indicated an average of 1.55 ± 0.67 migrant individuals per generation between population pairs. The lowest Nm value was obtained between populations of San Carlos and Iturbide-Linares ($Nm = 0.78$) separated by 96 km, and the highest one between the Alamos and Iturbide-Linares populations ($Nm = 2.07$) separated by 19 km.

Bottleneck effect

Table 6 shows the significant test results for a recent bottleneck by polymorphic loci in each population. For the San Carlos population four loci showed significant differences between expected heterozygosity (H_E), which was found to be higher than the expected heterozygosity at mutation equilibrium drift (H_{eq}). For the Alamos population this was found for two loci and for the Iturbide-Linares population for one locus.

DISCUSSION

The effects of geographical and geological events, e.g. glaciations (Lewis and Crawford, 1995; Hannan and Orick, 2000; Nason *et al.*, 2002; Coleman *et al.*, 2003; González-Astorga *et al.*, 2003a, b; Marchelli and Gallo, 2004; Plana *et al.*, 2004) and island formation (Batista *et al.*, 2001; Batista and Sosa, 2002; Keppel *et al.*, 2002), on genetic structure, extinction and colonization of local populations have been analysed recently. The drastic reduction in population sizes during Pleistocene and post-Pleistocene times may have given rise to the 'recent' bottleneck effect observed in the present study.

The mean percentage of polymorphic loci in *D. angustifolium* was slightly higher (52.4 %) than that reported for other long-lived perennial and endemic plant species (48.1 %; Hamrick and Godt, 1996), including other cycad species (Table 7). The mean expected heterozygosity within populations for *D. angustifolium* was also higher (0.218), than those values reported for other regionally distributed (*sensu* Hamrick and Godt, 1989), long-lived tropical trees, and even higher than those for long-lived temperate trees (0.125 and 0.145, respectively; Hamrick *et al.*, 1994). When compared with other cycads, *D. angustifolium* also showed a higher mean expected heterozygosity (Table 7). It is also higher than those of other long-lived perennial and endemic species (0.105; Hamrick and Godt, 1996). In concordance with high levels of heterozygosity, the BOTTLENECK test results (Table 6) indicate an excess of heterozygosity relative to allele numbers at several gene loci studied (see Piry *et al.*, 1999), which indicates that founder effects (bottlenecks) may have played a role in the history of the species (cf. Young *et al.*, 1996; González-Astorga and Núñez-Farfán, 2001; González-Astorga and Castillo-Campos, 2004; González-Astorga *et al.*, 2004). Of course, high levels of genetic diversity are not normally expected in a species that underwent founder events. However, high levels of genetic diversity in *D. angustifolium* may be related to the plants' history and reproductive system. For example, the dioecy in *D. angustifolium* may ameliorate the effects of local inbreeding (Berg and Hamrick, 1997; Hedrick, 2000). High genetic diversity has been reported in other cycads that have narrow distributions and relatively low population densities [cf. *Macrozamia riedlei*, Byrne and James (1991) and *D. edule*, González-Astorga *et al.* (2003a)].

At the population level, the genetic diversity of *D. angustifolium* was higher ($A = 1.67$, $P = 52.4$ and $H_E = 0.218$; Table 8) than the mean of 11 cycad species ($A = 1.47$, $P = 38.8$, $H_E = 0.107$) (see Tables 7 and 8). However, the levels of genetic diversity are relatively similar when compared with the mean values for six species in the Zamiaceae ($A = 1.48$, $P = 40.5$ and $H_E = 0.125$) (Table 8), with the exception of H_E , which is greater in *D. angustifolium*. In contrast, *D. edule* (González-Astorga *et al.*, 2003a) has higher genetic diversity than *D. angustifolium*. This can be attributed to the wider geographical distribution of *D. edule*, greater population sizes and higher population densities than *D. angustifolium*. These findings are consistent with Gitzendanner and Soltis (2000) and Cole (2003)

TABLE 3. Geographic location and levels of genetic variation for three populations of *Dioon angustifolium* in Mexico

Population	Geographical position	Altitude (m a.s.l.)	N_i	P	H_O	H_E
San Carlos (Tam)	24°39'04"N 98°58'54"W	524	53.7	78.6	0.366	0.318
Alamos (NL)	24°41'09"N 99°41'32"W	560	43.8	42.8	0.159	0.203
Iturbide-Linares (NL)	24°44'55"N 99°48'29"W	815	37.2	35.7	0.121	0.134
Mean ± SD			44.9 ± 8.3	52.4 ± 23.0	0.215 ± 0.132	0.218 ± 0.093

N_i = average sample size; P = percentage of polymorphic loci; H_O and H_E = observed and expected mean heterozygosity, respectively.

TABLE 4. Wright's F statistics for three populations of *Dioon angustifolium* in Mexico

Locus	F_{it}	F_{st}	F_{is}
<i>Mdh</i>	0.1443*	0.1636**	-0.0230
<i>Pgi</i>	0.1732	0.1540*	0.0227
<i>6Pgd</i>	0.1858**	0.1728***	0.0157
<i>Dia</i>	0.2597***	0.1899***	0.0862***
<i>Idh</i>	0.0829***	0.1304***	-0.0546**
<i>Est</i>	0.1957**	0.1683***	0.0329
<i>Lap</i>	0.1658**	0.1775***	-0.0142
<i>Apx 1</i>	0.1635	0.1685*	-0.0060
<i>Apx 2</i>	0.1646	0.1684*	-0.0046
<i>Apx 3</i>	0.1528	0.1685**	-0.0190
<i>Apx 4</i>	0.1629	0.1676	-0.0056
<i>Got</i>	0.1680	0.1664	0.0019
<i>Amp</i>	0.1680	0.1664	0.0019
<i>Adh</i>	0.1680	0.1664	0.0019
Mean ± SD	0.1649** ± 0.1279	0.1670* ± 0.0451	-0.0067 ± 0.1114
CI 95 %	-0.0160-0.3922	0.0952-0.2468	-0.1630-0.2146

* $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$.

TABLE 5. Exact χ^2 test for genetic differentiation among three populations of *Dioon angustifolium*

Contrast among populations	χ^2 test	Probability (P)
San Carlos (Tam) vs. Alamos (NL)	125.5	0.00001
San Carlos (Tam) vs. Iturbide-Linares (NL)	174.3	0.00001
Alamos (NL) vs. Iturbide-Linares (NL)	70.9	0.00001

The test was done for 10 000 Markov steps.

TABLE 6. Genetic analysis of population bottlenecks in *Dioon angustifolium*

Population	Locus	H_E	H_{eq}	Probability (P)
San Carlos (Tam)	<i>Dia</i>	0.654	0.322	0.013
	<i>Idh</i>	0.592	0.323	0.047
	<i>Est</i>	0.504	0.178	0.022
	<i>Lap</i>	0.504	0.189	0.016
Alamos (NL)	<i>6Pgd</i>	0.505	0.200	0.020
	<i>Dia</i>	0.667	0.336	0.006
Iturbide-Linares (NL)	<i>Dia</i>	0.608	0.331	0.049

who described these patterns of genetic variation between congeneric rare and widespread plant species.

The impact of the Pleistocene glaciations on the distribution of plants has been well documented (Pielou, 1991; Lewis and Crawford, 1995; Huang *et al.*, 2001; González and Vovides, 2002; González-Astorga *et al.*, 2003a, b; Sosa *et al.*, 2003). It is now known that plant species with a northern distribution were forced southward completely out of their previous ranges during the glacial advance (Toledo, 1982). Among these species are *Pinus banksiana* (Critchfield, 1984), 11 species of *Polygonella* (Lewis and Crawford, 1995), 81 gymnosperm genera (Contreras-Medina and Vega, 2002) and *D. edule* (González-Astorga *et al.*, 2003a). Also, the Pleistocene glaciations have had an effect on the formation of species, such as adaptive radiation from Pleistocene refugia during glacial retreat, which appears to be the case for the cycad genus *Ceratozamia* (González and Vovides, 2002) and other plant species [e.g. *Begonia salaziensis* and *B. comorensis* (Plana *et al.*, 2004) and *Nothofagus nervosa* (Marchelli and Gallo, 2004)].

A similar scenario may apply to *D. angustifolium*. González-Astorga *et al.* (2003a) suggested that this species was probably derived, as a product of the fragmentation and subsequent adaptive radiation, from one or more *D. edule* populations, which is in accord to the phylogenetic scheme of Moretti *et al.* (1993) and the authors' unpublished data.

The age of some large individuals (>3 m tall) of *D. edule* in the field has been estimated to be greater than 2000 years (Vovides, 1990). Male plants of *D. edule* produce cones at 15 years of age under cultivation and this can be doubled for reproduction under field conditions (unpubl. res.). Females, on the other hand, take considerably longer to cone; at a guess, anything between 60 and 120 years under field conditions and therefore approx. 83 generations would have passed since the end of the Pleistocene (10 000 years). This may be sufficient time for the separation of *D. angustifolium* from *D. edule*. Models exist that explain speciation processes from a population genetics approach (Templeton, 1981; Coyne and Orr, 1998). Based on the genetic data analysis, it is suggested that *D. angustifolium* probably emerged from a parapatric or allopatric speciation process (*sensu* Mayr, 1963) resulting from the spread and divergence due to drift stemming from founder effects. Two scenarios may be envisaged here: a pre-Pleistocene distribution of *D. edule* that became fragmented during the Pleistocene glaciations leaving sheltered pockets in north-eastern

TABLE 7. Genetic variation of cycads plant species as reported in the literature

Species	Reference	Site of study	<i>N</i>	<i>A</i>	<i>P</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{st}</i>
<i>Cycas panzhihuaensis</i>	Chao-Luan <i>et al.</i> (1999)	Jinsha River, China	3	1.13	14.3	0.043	0.061	0.139
<i>Cycas pectinata</i>	Yang and Meerow (1996)	China, India, Thailand and Vietnam	11	1.82	58.5	0.066	0.076	0.387
<i>Cycas seemanii</i>	Keppel (2002)	South Pacific Islands	5	1.80	50.0	0.048	0.138	0.418
<i>Cycas siamensis</i>	Yang and Meerow (1996)	Thailand and Vietnam	13	1.48	58.9	0.114	0.134	0.291
<i>Cycas taitungensis</i>	Lin <i>et al.</i> (2000)	Taiwan	2	1.07	2.5	0.014	0.013	0.034
<i>Dioon edule</i>	González-Astorga <i>et al.</i> (2003b)	East coast of Mexico	8	1.44	54.8	0.273	0.240	0.075
<i>Macrozamia heteromera</i>	Sharma <i>et al.</i> (1999)	New South Wales, Australia	5	1.30	29.4	0.072	0.077	0.100
<i>Macrozamia parcifolia</i>	Sharma <i>et al.</i> (1998)	Southern of Queensland	2	1.20	17.6	0.02	0.037	0.090
<i>Macrozamia pauli-guilielmi</i>	Sharma <i>et al.</i> (1998)	Southern of Queensland	3	1.30	31.3	0.043	0.081	0.030
<i>Macrozamia riedlei</i>	Byrne and James (1991)	Western Australia	15	2.43	93	0.263	0.274	0.092
<i>Zamia pumila</i>	Walters and Decker-Walters (1991)	Great Antilles, northern Bahamas Islands, Florida, and south-eastern Georgia	4	1.21	16.7	0.047	0.041	—

N = Number of populations; *A* = number of alleles per locus; *P* = percentage of polymorphic loci; *H_O* and *H_E* = mean observed and expected heterozygosity, respectively; *F_{st}* = genetic divergence between populations.

TABLE 8. Abstract of the genetic variation for cycads species found in the literature

	No. of species	<i>A</i>	<i>P</i>	<i>H_E</i>	<i>F_{st}</i>
<i>Dioon angustifolium</i>	1	1.67 ± 0.23	52.4 ± 23.0	0.218 ± 0.09	0.167 ± 0.04
Zamiaceae species	6	1.48 ± 0.43	40.5 ± 29.2	0.125 ± 0.10	0.077 ± 0.28
All species of cycads	11	1.47 ± 0.40	38.8 ± 26.7	0.107 ± 0.08	0.166 ± 0.14

The data represent the mean and standard deviation of values estimated.

Mexico where small populations of the cycads could hold out during the glaciation, then splitting off to form *D. angustifolium*. This scenario resembles that of *Begonia* where a significant proportion of diversity is of pre-Pleistocene origin but with evidence of recent (Pleistocene) speciation in some lineages (Plana *et al.*, 2004). Alternatively, post-glacial expansion of *D. edule* may have occurred from the south as climates warmed up, thus facilitating migration when conditions at the periphery of the species range became favourable for the establishment of newly founded populations (Lewis and Crawford, 1995). Gregory and Chemnick (2004) suggest altitudinal migration resulting in disjunction and rapid speciation within river canyon systems as well as latitudinal north–south migrations following the transition zone favoured by most *Dioon* spp. of tropical dry forests and oak/pine forests. This has resulted in senescent populations consisting of mature plants with little or no regeneration in drier habitats (tropical dry/thorn forests) with no higher elevation habitats (pine/oak forest) to migrate to. Genetic drift associated with bottlenecks generated by vicariance or founder effects may have driven or accompanied rapid speciation; the genetic variation of both *D. angustifolium* and *D. edule* compared with other cycad species is unexpectedly high.

Conservation implications

The existence of small endemic populations, as in *D. angustifolium*, in what might be termed micro-refugia are

a conservation concern. Small endemic populations rich in genetic diversity are especially vulnerable to extinction through change of land use, habitat destruction, and removal of plants for trade, amongst other factors (Gilbert, 1984; Lewis and Crawford, 1995; Donaldson, 2003a). Practical conservation strategies have been implemented for *D. edule* aimed at its sustainable utilization (Vovides *et al.*, 2002). Because no such strategies are being contemplated for *D. angustifolium* it is highly recommended that the areas in which the two most conserved populations, San Carlos (Tam) and Iturbide-Linares (NL), are found, should be turned into sanctuaries or Protected Areas as they contain high numbers of adult individuals and are the least disturbed habitats.

Finally, given the restricted distribution of *D. angustifolium*, vulnerability and land-use changes, we recommend that this species should be listed in the IUCN Red List category (IUCN, 2003) as vulnerable (VU C, 2a) (Mace and Lande, 1991). Since cycads are considered charismatic and threatened (Given, 1994) we therefore recommend that this species be considered for future conservation and sustainable utilization.

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