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Diversity and genetic structure of three species of *Dioon* Lindl. (Zamiaceae, Cycadales) from the Pacific seaboard of Mexico

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We have estimated levels of genetic diversity and partitioning in the Mexican endemic cycad species *Dioon sonorense*, *Dioon tomasellii*, and *Dioon holmgrenii*, whose populations are exclusively distributed along the Pacific seaboard. For the three species, the patterns of variation at 19 allozyme loci in a total of 11 populations were evaluated. The average number of alleles per locus was in the range 2.05–1.68, corresponding to the northernmost population of *D. sonorense* (Mazatán), and the southernmost population of *Dioon holmgrenii* (Loxicha), respectively. In turn, the percentage of polymorphic loci peaked (94.73) in the El Higueral and Altamirano populations of *Dioon tomasellii*, and was estimated to be lowest (57.89) in the Loxicha population of *D. holmgrenii*. The mean expected heterozygosis varied markedly between taxa, with relatively high indices for *D. sonorense* and *D. tomasellii* ($H_E = 0.314$ and 0.295, respectively) and substantially lower values for *D. holmgrenii* ($H_E = 0.170$). Comparison of the inferred genetic structure based on *F*-statistics for the three species also indicated differences along the north-south Pacific seaboard axis. For *D. sonorense* and *D. tomasellii*, local inbreeding (F_{IS}) was zero but global inbreeding (F_{IT}) values were positive and significantly different from zero (0.130 and 0.116, respectively). By contrast, values of both F_{IT} and F_{IS} were negative and significantly different from zero (–0.116 and –0.201, respectively) for *D. holmgrenii*. The genetic differentiation between populations (F_{ST}) had positive values in all taxa and corresponded with their geographic location along the north-south axis: according to this statistic, *D. sonorense* was the most differentiated species ($F_{ST} = 0.151$), *D. tomasellii* had intermediate values ($F_{ST} = 0.145$), and *D. holmgrenii* was the less differentiated taxon ($F_{ST} = 0.069$). Finally, a phenogram representing Nei's genetic distances among populations displayed three major groups, each one corresponding to each of the studied species. Within *D. tomasellii* (of intermediate geographic distribution), a further division into two clusters corresponded precisely to the pair of populations that are geographically divided by the Trans Mexican Neovolcanic Mountains.

ADDITIONAL KEYWORDS: Allozyme electrophoresis – Nei's genetic distances – population genetics – threatened species – Trans Mexican Neovolcanic Mountains.

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INTRODUCTION

Two main research lines have been practiced to characterize genetic variation between species of cycads of the same genus, or within populations of the same species. The first approach has involved standard molecular systematic analyses, either using character-based (i.e. cladistic parsimony) or distance-based (e.g. Neighbour-joining, maximum likelihood, etc.) methods. Although the genus *Cycas* has been approached from this perspective (Hill, 2004), most cycad molecular systematic studies have been conducted for genera in the Zamiaceae, including *Ceratozamia* (González & Vovides, 2002; Vovides *et al*., 2004; De Castro, Vázquez-Torres & Luca, 2006), *Dioon* (Moretti *et al*., 1993), *Encephalartos* (Van der Bank *et al*., 2001; Treutlein, Vorster & Wink, 2004; Vorster, 2004), and *Zamia* (Caputo *et al*., 2004). The second line of research concerned with the characterization of cycad genetic variation is represented by population genetic studies *sensu stricto*. Most population genetics on cycads has consisted of analyses of the distribution of genetic diversity within and between populations of a single species (Keppel, 2002; González-Astorga *et al*., 2003a, 2005, 2006). However, a few studies have also been conducted for two or more closely-related species within the same genus, namely, *Macrozamia* (Sharma, Jones & Foster, 1998, 1999, 2004) and *Cycas* (Xiao et al., 2005; Xiao & Gong, 2006).

Although the population genetics studies on multiple *Macrozamia* and *Cycas* species mentioned above contain valuable conclusions regarding genetic structure, they do not include a satisfactory discussion of the effects and impact of historical factors of geological nature, as well as those of ecological processes related to habitat fragmentation, upon the current geographical distribution of the cycad populations involved. When the cycad species under scrutiny in population genetics work are rare, these considerations are particularly important because the populations of these species are usually undergoing threats due to change of land use, deforestation, and agricultural expansion (Van Geert *et al*., 2007), as well as illegal extraction and environmental changes (DiBattista, 2007). Over the last century, species in genera such as *Encephalartos* (*Encephalartos relictus* and *Encephalartos woodii*; Donaldson, 2003) have been considered extinct in their wild locations in Africa (Walters, 2003). Likewise, *Lepidozamia* and *Bowenia* in Australia (Hill, 2003), *Cycas* in Asia (Hill, Chen & Loc, 2003), and *Microcycas* and *Dioon*, in Cuba and Mexico, respectively, are also endangered or threatened (Stevenson, Vovides & Chemnick, 2003). If processes such as the Pleistocene glaciations, the formation of floristic refugia, or local orogenic events are taken into account, research that analyzes genetic diversity in cycads can turn into *ad hoc* studies of the effects of both historical (i.e. long-term) and ecological (i.e. short-term) events upon the present distribution of populations. In a series of recent studies, we have already attempted this type of integration for the Mexican cycad species *Dioon edule* and *Dioon angustifolium* (González-Astorga *et al*., 2003a, b, 2005), as well as for *Zamia loddigesii* (González-Astorga *et al*., 2006). However, in all these studies, every cycad species was considered individually; by definition, the interesting possibility of common biogeographical processes affecting more than one species simultaneously has not been investigated.

In the present study, we used allozyme markers to examine the patterns and levels of genetic diversity in three *Dioon* species located along the Pacific seaboard of Mexico. The three species in question (*Dioon sonorense*, *Dioon tomasellii*, and *Dioon holmgrenii*) are allopatric, with a geographic distribution that extends north to south, in the aforementioned order. With a specific interest in interpreting our empirical evidence in the context of the current distribution of the species' populations recognized to date, and following the integrative objectives outlined above, these interpretations include explicit references to known past historical/ecological factors, such as habitat fragmentation, isolation of populations due to climatic changes, and/or colonization of new environments. The present work is part of an exhaustive research program devoted to the explanation of the observed biogeographic distribution of the contemporary diversity of cycads across Mexico. At the same time, this information can enhance the interpretation of phylogenetic reconstructions based on morphological and molecular data. Finally, the conclusions from this project can also be valuable for establishing which criterion of rarity (Rabinowitz, 1981) best fits cycad taxa from the Neotropics, as well as in directing conservation biology efforts for threatened species within this charismatic gymnosperm group (González-Astorga *et al*., 2003a, b, 2005, 2006, Vovides *et al*., 2004).

MATERIAL AND METHODS

STUDY SITES

The study was carried out on populations of *D. sonorense*, *D. tomasellii*, and *D. holmgrenii* from 11 localities along the Pacific seaboard of Mexico, separated by distances in the range 20–1300 km (Fig. 1). All the populations sampled include the known total distribution range of the three species.

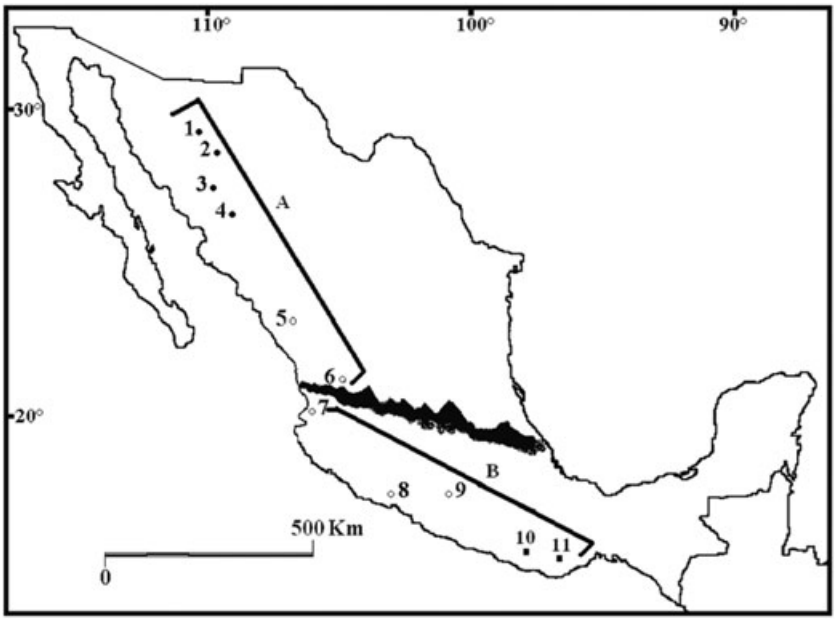

Figure 1. Geographical distribution of *Dioon sonorense* (●) *Dioon tomasellii* (○), and *Dioon holmgrenii* (■) in Mexico. Numbers indicate the geographical position of each population evaluated (Table 2). The Trans Mexican Neovolcanic Mountain Range is illustrated at centre of the map (thick black jagged line). A and B indicate the populations above and below the Trans Mexican Neovolcanic Mountains, respectively.

SPECIES STUDIED

Dioon sonorense (De Luca, Sabato & Vázq.Torres) Chemnick, Gregory & S. Salas-Mor.
This species is located at the extreme north-western distribution for the genus, and is known only from four localities in Sonora. It consists of plants with trunks typically 30–90 cm tall, but not exceeding 150 cm and up to 25 cm in diameter, with a terminal crown of up to 25 ascending, broad lanceolate pinnate leaves, sometimes twisted near the apex, up to 100 cm long and 25 cm wide.

Dioon tomasellii De Luca, Sabato & Vázq.Torres
This species has the greatest distribution range for the genus (approximately 800 km), extending from northern Sinaloa to Guerrero. It consists of plants with variable trunks, from semi hypogeous to arborescent, reaching up to 200 cm tall or more and 30 cm diameter, with a terminal crown of 14–30 or more ascending to spreading elliptic to oblong pinnate leaves that are flat to slightly convex, up to 200 cm long and 36 cm wide.

Dioon holmgrenii De Luca, Sabato & Vázq.Torres
This species has a highly restricted geographic distribution, and is known from only two localities in southern Oaxaca. It is a large arborescent cycad with trunks up to 6 m tall and 40 cm diameter, with a terminal crown of 15 to 30 or more ascending to spreading flat, lanceolate pinnate leaves up to 130 cm long and 25 cm wide.

SAMPLE COLLECTION

For the three *Dioon* species included in this study, 18–79 adults plants per population were sampled at random. Samples of approximately 5 g of leaflet tissue were transported in containers with ice, to avoid protein denaturation, and then stored in a Revco freezer set to –70 °C, until the moment of extraction.

ELECTROPHORESIS

Allozyme electrophoresis was performed on horizontal potato starch gels at 10% V/W. Approximately 300 mg of leaflet tissue from each individual were ground using liquid nitrogen. Subsequently, 250 μL of extraction buffer (0.1 M Tris-HCl, pH 7.5, 4% PVP-40, 0.001 M ethylenediaminetetracetic acid, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, and 0.1% β-mercaptoethanol; González-Astorga *et al.*, 2005) were added to dilute and stabilize the enzyme extracts, which were then stored on filter paper wicks at –70 °C. For each individual plant, allozymic variation was scored for 19 loci (Table 1). Electrophoreses were carried out at 4 °C for 6.5 h to constant current of 50 mA and voltage of 100 V.

Table 1. Allozyme systems resolved in this research

Allozyme	Locus abbreviation	EC No.	Buffer*
Acid phosphatase	Acph	3.1.3.2	R
Alcohol dehydrogenase	*Adh*	1.1.1.1	R
Anodic peroxidase	*Apx1*, *Apx2*, and *Apx3*	1.11.1.7	R
Diaphorase	*Dia*	1.6.99	R
Gliceraldeide-3-phosphate dehydrogenase	*G3pdh*	1.2.1.12	R
Glutamate dehydrogenase	*Gdh*	1.4.1.2	R
Isocitrate dehydrogenase	*Idh*	1.1.1.41	R
Malate-dehydrogenase	*Mdh*	1.1.1.37	R
Menadione reductase	*Mnr*	1.6.99	R
Phosphogluco isomerase	*Pgi1* and *Pgi2*	5.3.1.9	R
6-Phosphogluconate dehydrogenase	*6Pgd*	1.1.1.44	R
Aconitate hydratase	Aco	4.2.1.3	PK
Glutamate oxalacetate transaminase	*Got*	2.6.1.1	PK
Malic enzyme	*Me*	1.1.1.40	PK
Phosphoglucomutase	*Pgm*	5.4.2.2	PK
Shikimate dehydrogenase	*Sdh*	1.1.1.25	PK

*R and PK refer to system buffers of Yang & Meerow (1996) and Chao-Luan *et al*. (1999), respectively.
Nomenclature and abbreviations follow Wendel & Weeden (1989), based on the recommendations of the International Union of Biochemistry.
EC No, Enzyme Commission Number.

Statistical analysis

On the basis of the observed allozyme banding patterns, allelic frequencies and values of observed heterozygosis (H_O) were calculated using the TFPGA 1.3. Program (Miller, 1997). From these data, the average of alleles per locus (A), percent polymorphic loci (P), and average expected heterozygosis (H_E) were estimated under the assumption of Hardy–Weinberg equilibrium (Hartl & Clark, 1997). The parameters of genetic diversity between species were contrasted using analysis of variance (ANOVA), and the results were later compared by Tukey's multiple range test for unequal sample sizes (Zar, 1999). In addition, we performed a linear regression model for the latitude and all the genetic diversity estimators in all populations, with the aim of exploring the relationship between these variables.

The patterns of genetic variation within and among populations in each species (genetic structure) was estimated with F statistics (i.e. F_{IT}, F_{IS}, and F_{ST}) (Wright, 1978), and analyzed by Tukey's multiple range test for unequal sample sizes (Zar, 1999). A chi-square test was used (Weir, 1990) to determine whether the F_{IT} and F_{IS} statistics (local and global inbreeding, respectively) obtained for each species differed significantly from zero. A chi-square test was also performed to verify the significance of genetic differentiation between populations (F_{ST}) in each species. Finally, phenetic clustering of populations within species was performed using Nei's (1972) genetic distances and the unweighted pair group method with arithmetic mean (UPGMA) (Sneath & Sokal, 1973) algorithm, as implemented in TFPGA, version 1.3 (Miller, 1997). With the purpose of exploring correlations between populational parameters available in the literature for the cycads, we performed lineal regression analyses between these quantities (Zar, 1999).

RESULTS

Genetic diversity

A total of 19 enzyme loci were analyzed by starch gel electrophoresis (Table 1). The patterns of allelic distribution and number of alleles per locus were found to vary across populations, with a few of them appearing in restricted populations. The allele *Idh* C was only found in two populations of *D. sonorense* (Mazatán and Novillo), and the allele *Sdh* C was found exclusively in the Nuri and Alamos populations. For *D. tomasellii*, the allele *Got* B was observed only in the El Tuito and El Higueral populations. Finally, the allele *Mdh* C was exclusively found in the Loxicha population of *D. holmgrenii* (Table 2).

The mean of alleles per locus ranged from 2.05 for the Mazatán population of *D. sonorense*, to 1.68 for the Loxicha population of *D. holmgrenii*. The Mazatán and Loxicha populations, respectively, represent the northernmost and southernmost extremes for the three species studied (Table 3). The analysis of

Table 2. Allelic frequencies for all *Dioon* populations analyzed

		Dioon sonorense				*Dioon tomasellii*						*Dioon holmgrenii*
Locus	Allele	1	2	3	4	5	6	7	8	9	10	11
Aco	A	0.9286	0.9211	0.6667	0.5833	0.6500	0.7949	0.9886	0.9262	0.6824	0.9500	1.0000
	B	0.0714	0.0789	0.3333	0.4167	0.3500	0.2051	0.0114	0.0738	0.3176	0.0500	0.0000
Acph	A	0.4048	0.3158	0.1000	0.1389	0.1833	0.4487	0.7614	0.9426	0.6806	1.0000	0.8600
	B	0.5952	0.6842	0.9000	0.8611	0.8167	0.5513	0.2386	0.0574	0.3194	0.0000	0.1400
Adh	A	0.6429	0.8421	1.0000	1.0000	1.0000	0.8974	0.9659	0.5500	0.6103	0.8600	0.9500
	B	0.3571	0.1579	0.0000	0.0000	0.0000	0.1026	0.0341	0.4500	0.3897	0.1400	0.0500
Apx 1	A	0.6579	0.7368	0.9667	0.9167	0.9333	0.9231	1.0000	1.0000	0.9324	0.9800	0.8200
	B	0.3421	0.2632	0.0333	0.0833	0.0667	0.0769	0.0000	0.0000	0.0676	0.0200	0.1800
Apx 2	A	1.0000	1.0000	0.9000	0.6944	0.9500	0.7949	0.9773	0.8115	0.7027	0.8500	0.9100
	B	0.0000	0.0000	0.1000	0.3056	0.0500	0.2051	0.0227	0.1885	0.2973	0.1500	0.0900
Apx 3	A	0.9524	0.9211	0.9667	0.8611	0.9667	0.8846	0.9318	0.7623	0.5135	0.9000	1.0000
	B	0.0476	0.0789	0.0333	0.1389	0.0333	0.1154	0.0682	0.2377	0.4865	0.1000	0.0000
Dia	A	0.8947	0.6429	0.4667	0.7222	0.4815	0.3974	0.5455	0.5574	0.4861	0.5400	0.8261
	B	0.1053	0.3571	0.5333	0.2778	0.5185	0.6026	0.4545	0.4426	0.5139	0.4600	0.1739
G3pdh	A	0.7059	0.9474	0.9333	0.7500	0.9167	0.8974	0.4773	0.6311	0.5608	1.0000	1.0000
	B	0.2941	0.0526	0.0667	0.2500	0.0833	0.1026	0.5227	0.3689	0.4392	0.0000	0.0000
Gdh	A	0.6750	0.6842	0.8000	0.4444	0.5517	0.9605	0.6250	0.8279	0.5764	0.8600	0.8500
	B	0.3250	0.3158	0.2000	0.5556	0.4483	0.0395	0.3750	0.1721	0.4236	0.1400	0.1500
Got	A	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	**0.9091**	**0.9344**	1.0000	1.0000	1.0000
	B	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	**0.0909**	**0.0656**	0.0000	0.0000	0.0000
Idh	A	**0.5000**	**0.8824**	0.5333	0.5000	0.7069	0.6538	0.6591	0.7679	0.5915	0.8900	0.9400
	B	**0.2188**	**0.0000**	0.4667	0.5000	0.2931	0.3462	0.3409	0.2321	0.4085	0.1100	0.0600
	C	**0.2812**	**0.1176**	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Mdh	A	0.5500	0.6471	0.8333	0.8333	0.7000	0.8462	0.8750	0.9344	0.7397	0.5000	**0.5100**
	B	0.4500	0.3529	0.1667	0.1667	0.3000	0.1538	0.1250	0.0656	0.2603	0.5000	**0.4600**
	C	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	**0.0300**
Me	A	0.6500	0.5882	0.7333	0.8056	0.9667	1.0000	0.9091	0.8197	0.7292	0.9100	0.8900
	B	0.3500	0.4118	0.2667	0.1944	0.0333	0.0000	0.0909	0.1803	0.2708	0.0900	0.1100
Mnr	A	0.1904	0.1944	0.0667	0.0833	0.2833	0.3205	0.0682	0.1636	0.0676	0.3152	0.3300
	B	0.4048	0.6112	0.5000	0.5556	0.0500	0.6154	0.5455	0.4818	0.4797	0.6848	0.6700
	C	0.4048	0.1944	0.4333	0.3611	0.6667	0.0641	0.3864	0.3545	0.4527	0.0000	0.0000
Pgi 1	A	0.4762	0.5833	0.7000	0.3611	0.9667	0.2692	0.8182	0.7759	0.5822	1.0000	1.0000
	B	0.2381	0.2500	0.2000	0.4444	0.0333	0.0385	0.1818	0.2241	0.4178	0.0000	0.0000
	C	0.2857	0.1667	0.1000	0.1944	0.0000	0.6923	0.0000	0.0000	0.0000	0.0000	0.0000
Pgi 2	A	0.9524	0.8947	0.6000	0.3889	0.8333	0.8462	0.8977	0.9074	0.3786	1.0000	1.0000
	B	0.1476	0.1053	0.4000	0.6111	0.1667	0.1538	0.1023	0.0926	0.6214	0.0000	0.0000
Pgm	A	0.8333	0.8684	1.0000	0.8056	0.7833	0.6282	0.7727	0.8279	0.7615	0.8700	1.0000
	B	0.1667	0.1316	0.0000	0.1944	0.2167	0.3718	0.2273	0.1721	0.2385	0.1300	0.0000
Sdh	A	0.8571	0.7105	**0.0334**	**0.8333**	1.0000	0.8718	1.0000	0.6967	0.6438	0.9000	0.9300
	B	0.1429	0.2895	**0.2333**	**0.0000**	0.0000	0.1282	0.0000	0.3033	0.3562	0.1000	0.0700
	C	0.0000	0.0000	**0.7333**	**0.1667**	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
6Pgd	A	0.3095	0.3421	0.9667	0.8611	0.8000	0.9103	0.9091	0.8033	0.5746	0.6700	0.9800
	B	0.6905	0.6579	0.0333	0.1389	0.2000	0.0897	0.0909	0.1967	0.4254	0.3300	0.0200

1, Mazatán; 2, Novillo; 3, Nuri; 4, Alamos; 5, Pánuco; 6, Compostela; 7, El Tuito; 8, El Higueral; 9, Altamirano; 10, Rancho El Limón; 11, Loxicha.
Numbers in bold, loci with unique alleles.

Table 3. The genetic variation found in four, five and two populations of *Dioon sonorense*, *Dioon tomasellii*, and *Dioon holmgrenii*, respectively from along the Pacific seaboard of Mexico

Species	Population	D_n	N_i	A	P	H_O	H_E
Dioon sonorense	Mazatán, Sonora (1)	100	20.05	2.05	78.94	0.372	0.344
	Novillo, Sonora (2)	80	18.31	2.00	89.47	0.315	0.311
	Nuri, Sonora (3)	80	15.00	1.95	68.42	0.291	0.264
	Alamos, Sonora (4)	90	18.00	2.00	89.47	0.342	0.337
	Mean ± SD		17.84 ± 2.1	2.00 ± 0.04[a]	81.58 ± 10.1[a]	0.330 ± 0.035[a]	0.314 ± 0.036[a]
Dioon tomasellii	Pánuco, Sinaloa (5)	500	29.5	1.84	68.42	0.241	0.240
	Compostela, Nayarit (6)	600	38.9	2.00	84.21	0.314	0.274
	El Tuito, Jalisco (7)	1000	44.0	1.95	73.68	0.264	0.244
	El Higueral, Michoacán (8)	1000	59.8	2.00	94.73	0.256	0.300
	Altamirano, Guerrero (9)	1000	71.8	2.00	94.73	0.468	0.419
	Mean ± SD		48.80 ± 16.9	1.96 ± 0.07[a]	83.15 ± 12.0[a]	0.309 ± 0.093[a]	0.295 ± 0.073[a]
Dioon holmgrenii	Rancho El Limón, Oaxaca (10)	600	49.8	1.74	68.42	0.237	0.194
	Loxicha, Oaxaca (11)	2000	49.8	1.68	57.89	0.171	0.147
	Mean ± SD		49.8 ± 0.0	1.71 ± 0.04[b]	63.16 ± 7.45[a]	0.204 ± 0.047[a]	0.170 ± 0.034[b]

Values sharing letters are not significantly different at $P < 0.05$.

SD, standard deviation; D_n, estimated number of plants in the field; N_i, average sample size; A, mean number of alleles per locus; P, percentage of polymorphic loci; H_O and H_E, observed and expected mean heterozygosity, respectively.

Table 4. Means and standard deviations of F statistics (F_{IT}, F_{IS}, and F_{ST}) of three species of *Dioon* along the Pacific seaboard of Mexico

Species	N	F_{IT}	F_{IS}	F_{ST}
Dioon sonorense	4	0.130 ± 0.06*[a]	–0.025 ± 0.06[NS, a]	0.151 ± 0.38*[a]
Dioon tomasellii	5	0.116 ± 0.05**[b]	–0.035 ± 0.03[NS, a]	0.145 ± 0.21**[b]
Dioon holmgrenii	2	–0.116 ± 0.15**[c]	–0.201 ± 0.14*[b]	0.069 ± 0.032*[c]

*$P < 0.05$; **$P < 0.01$.

N, Number of populations. Values sharing letters are not significantly different at $P < 0.001$. NS, not significant.

variance between the averages of the number of alleles per locus indicated significant differences between them, grouping *D. sonorense* with *D. tomasellii*, and separating *D. holmgrenii* (Table 3). Also, the results of the linear regression between the mean number of alleles per locus and the latitudinal distribution of populations (i.e. $A = 1.61 + 0.014\beta$; $F_{1,9} = 5.2$, $R^2 = 36.5\%$, $P < 0.05$), show a positive correlation between increase in latitude and mean number of alleles per locus. This correlation was not observed for any other genetic diversity parameter. The percentage of polymorphic loci varied from 94.73 for *D. tomasellii* (El Higueral and Altamirano) to 57.89 for *D. holmgrenii* (Loxicha) (Table 3). On average, the expected heterozygosity was found to differ between taxa, displaying higher values for *D. sonorense* and *D. tomasellii* ($H_E = 0.314$ and 0.295, respectively) compared to *D. holmgrenii* ($H_E = 0.170$) (Table 3).

Partitioning of genetic variation within and among populations

Genetic structure differed considerably in each species of *Dioon* analyzed (Table 4). For *D. sonorense* and *D. tomasellii*, the values of F_{IT} (global inbreeding) were positive and significantly different from zero (0.130 and 0.116, respectively). By contrast, *D. holmgrenii* had a negative value of F_{IT} but significantly different from zero (–0.116), which indicates a heterozygote excess for this species. The analysis of variance revealed significant differences between these parameters (ANOVA: $F_{2,54} = 570.4$, $P < 0.001$). The local inbreeding (F_{IS}) was zero for *Dioon sonorense* and *D. tomasellii* whereas, for *D. holmgrenii*, this value was negative, indicating an excess of heterozygous genotypes ($F_{IS} = –0.201$). Given these F_{IS} values, for this level, the analysis of variance between means for each taxon revealed significant differences (ANOVA: $F_{2,54} = 372.6$, $P < 0.001$). The genetic differentiation (F_{ST}) values for all taxa differed from zero, and the variation due to differences between populations also contrasted between taxa; with *D. sonorense* (northernmost distribution) having a higher value ($F_{ST} = 0.151$) than *D. tomasellii* ($F_{ST} = 0.145$) (intermediate distribution) and *D. holmgrenii* ($F_{ST} = 0.069$) (southernmost distribution) (Table 4). These differences were confirmed with the analysis of variance between the averages of the F_{ST} values for each taxa (ANOVA: $F_{2,54} = 630.2$, $P < 0.001$).

Table 5. Nodes of genetic distances of Nei's (1972) in 11 populations of three species of *Dioon* along the Pacific seaboard of Mexico (Fig. 2)

Node	Genetic distance	Includes populations
a	0.149	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11
b	0.083	5, 6, 7, 8, 9, 10, 11
c	0.072	5, 6, 7, 8, 9
d	0.041	1, 2, 3, 4, 5
e	0.038	7, 8, 9
f	0.032	5, 6
g	0.026	7, 8
h	0.023	1, 2, 3
i	0.020	10, 11
j	0.016	1, 2

Genetic relationships among taxa

The mean of the genetic distances for the three *Dioon* species was 0.05 ± 0.041. For all populations, the UPGMA tree based on Nei's (1972) genetic distances (Fig. 2, Table 5) displayed three major groups, corresponding to each species as originally defined in morphological terms. The populations corresponding to the species distributed in the central region of the Mexican Pacific seaboard (*D. tomasellii*) form two sub-groups, one located to the north (Compostela and Pánuco populations) and the other to the south (Altamirano, El Higueral and El Tuito populations) of the Trans Mexican Neovolcanic Mountains (Figs 1, 2).

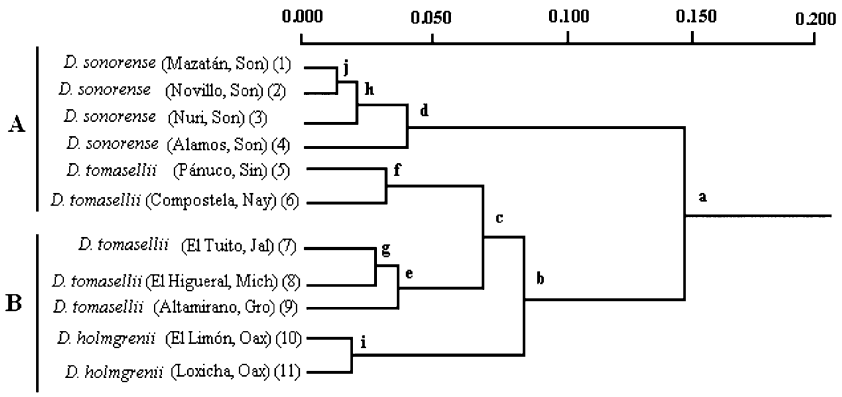

Figure 2. Unweighted pair group method with arithmetic mean phenogram based on Nei's (1972) genetic distances between 11 populations of three species of *Dioon*, estimated from 19 loci (Table 5).

DISCUSSION

Genetic diversity

The three endemic *Dioon* species included in the present population genetics study maintain substantial levels of genetic heterogeneity. This observation is consistent with their extensive geographical distribution along the Pacific Ocean seaboard of Mexico (approximately 1000 km, from north to south), and with the great diversity of habitats wherein they occur. In all cases, the basic indicators of genetic polymorphism show higher values than the averages obtained by Hamrick & Godt (1996) for endemic plants (i.e. $A = 1.39$; $P = 26.3$; $H_E = 0.063$) and Hamrick (2004) for 213 species of woody perennials (i.e. $A = 1.74$; $P = 47.9$; $H_E = 0.144$). When compared to estimates for 26 cycad species studied so far, including data from unpublished observations from several Mexican *Zamia* species ($A = 1.7$; $P = 56.4$; $H_E = 0.189$, in Table 6), the average values of A, P, and H_E for *D. holmgrenii*, *D. sonorense*, and *D. tomasellii* are also in the upper range. In the context of this phylogenetically focused comparison, there exists a positive linear relationship between the number of studied populations and genetic differentiation evaluated from F_{ST} values ($F_{1,23} = 7.58$, $P = 0.011$, $R^2 = 24.8\%$; Table 6). This meta-analysis agrees with the results obtained for the three species studied here. Similar values of genetic diversity were reported in four populations of the cycad *Z. loddigesii*, from the Gulf of Mexico seaboard ($A = 1.8$; $P = 66.6$; $H_E = 0.266$; González-Astorga *et al.*, 2006), which showed a latitudinal cline.

Partitioning of genetic variation within and among populations

In relation to morphological and geographic variation, the distribution pattern of genetic diversity for the three *Dioon* species studied here is similar to that found for *D. edule* and *D. angustifolium*, along the eastern seaboard of the Gulf of Mexico (González-Astorga *et al.*, 2003a, b, 2005). However, certain intertaxa comparisons between the available estimates for the *Dioon* species are not directly in agreement with the hypothesis that species with small population sizes have lower levels of genetic diversity than those with larger sizes, as suggested by Barrett & Kohn (1991); Mateu-Andrés (2004). Of the five *Dioon* species for which population genetic data currently exist (for unpublished data for *Dioon caputoi*, see Table 6), *D. sonorense* has the highest A, P, and H_E values, but also the lowest mean number of plants evaluated because these populations have been drastically reduced in size in recent history (González-Astorga & Núñez-Farfán, 2001; González-Astorga & Castillo-Campos, 2004; Van Geert *et al.*, 2007). On the other hand, genetic variability estimators in *D. sonorense* and *D. tomasellii* are very similar to each other; only N_i, which is the average sample size, turned out to be approximately three times higher in the latter species. Meanwhile, populations of *D. holmgrenii*, which have a similar N_i value to *D. tomasellii*, have a significantly lower F_{ST} value (Table 4). However, despite the absence of a direct correlation between small population sizes and lower genetic variability in the interspecific comparisons, it might still be possible that the relatively high differentiation levels found in *D. sonorense* (approximately 15%) are associated with its small interpopulational sizes. The currently observed N_i values in *D. sonorense* have probably been determined by fragmentation processes driven by human activities (González-Astorga *et al.*, 2006). In this respect, the observations of Ellis *et al.* (2006) are worth considering because these authors have shown that when populations of a species have low levels of genetic structure, the loss of a single population may have little impact on the species-wide genetic variability but, under conditions of high

Table 6. Summary of the genetic diversity and structure of 26 cycad species

Species	*N*	*A*	*P*	H_E	F_{ST}	Reference
Cycas pectinata	11	1.82	58.5	0.076	0.387	Yang & Meerow (1996)
Cycas siamensis	13	1.48	58.9	0.134	0.291	Yang & Meerow (1996)
Cycas panzhihuaensis	3	1.13	14.3	0.061	0.139	Chao-Luan *et al*. (1999)
Cycas guizhouensis	3	1.61	58.3	0.100	0.080	Yang & Meerow (1996)
Cycas taitungensis	2	1.07	2.5	0.013	0.034	Lin *et al*. (2000)
Cycas seemannii	5	1.20	21.3	0.057	0.594	Keppel (2002)
Macrozamia communis	5	1.61	50.0	0.045	0.270	Ellstrand *et al*. (1990)
Macrozamia riedlei	15	2.43	93.0	0.274	0.092	Byrne & James (1991)
Macrozamia parcifolia	2	1.20	17.6	0.037	0.090	Sharma *et al*. (1998)
Macrozamia pauli-guilielmi	3	1.30	31.3	0.081	0.030	Sharma *et al*. (1998)
Macrozamia heteromera	5	1.30	26.0	0.077	0.100	Sharma *et al*. (1999)
Macrozamia plurinervia	9	1.50	36.6	0.111	0.588	Sharma *et al*. (2004)
Zamia loddigesii	4	1.80	66.6	0.266	0.790	González-Astorga *et al*. (2006)
Zamia pumila	4	2.21	16.7	0.041	–	Walters & Decker-Walters (1991)
Zamia cremnophila	2	2.06	100	0.429	0.174	González-Astorga J, Nicolalde-Morejon F, Vovides AP (unpubl. data)
Zamia katzeriana	3	2.13	87.1	0.298	0.191	González-Astorga J, Nicolalde-Morejon F, Vovides AP (unpubl. data)
Zamia lacandona	3	2.04	69.2	0.216	0.108	González-Astorga J, Nicolalde-Morejon F, Vovides AP (unpubl. data)
Zamia variegata	2	2.02	97.3	0.355	0.085	González-Astorga J, Nicolalde-Morejon F, Vovides AP (unpubl. data)
Zamia purpurea	2	2.03	100	0.485	0.025	González-Astorga J, Nicolalde-Morejon F, Vovides AP (unpubl. data)
Microcycas calocoma	7	1.49	48.1	0.170	0.337	Pinares de la Fe A, Gonzalez-Astorga J, Vovides AP (unpubl. data)
Dioon sonorense	4	2.00	81.6	0.314	0.151	Present study
Dioon tomasellii	5	1.96	83.1	0.295	0.145	Present study
Dioon holmgrenii	2	1.7	63.1	0.170	0.069	Present study
Dioon caputoi	4	1.91	79.0	0.350	0.099	Cabrera-Toledo, Gonzalez-Astorga, Vovides (in press)
Dioon edule	8	1.44	54.8	0.240	0.075	González-Astorga *et al*. (2003a)
Dioon angustifolium	3	1.67	52.4	0.218	0.167	González-Astorga *et al*. (2005)
Mean ± SD	5.0 ± 3.5	1.70 ± 0.4	56.4 ± 29	0.189 ± 0.13	0.204 ± 0.2	

SD, standard deviation; *N*, number of populations studied; *A*, mean of alleles per locus; *P*, percentage of polymorphic loci; H_E, expected heterozygosity; F_{ST}, genetic differentiation among populations.

genetic structure, the loss of a single population might significantly reduce overall genetic diversity. This pattern might have implications for conservation, since the extinction of one of the populations of *D. sonorense*, a species with highly fragmented populations, could therefore be critical with respect to its overall genetic diversity.

It is noteworthy that *D. holmgrenii* maintains higher genetic diversity than most endemic or narrowly-distributed plant species (Hamrick & Godt, 1996; Hamrick, 2004), in spite of its restricted distribution. Unlike the other two *Dioon* species investigated in the present study, *D. holmgrenii* presents an excess of heterozygotes, a situation that might be an effect of stabilizing selection (Eguiarte, Pérez-Nasser & Piñero, 1992; Hansson & Westerberg, 2002; Gonzalez-Astorga *et al*., 2003a). Given its levels of genetic diversity and long-life cycle, the observations of

Mitton & Grant (1984) and Stilwell, Wilbur & Taylor (2003), concerning a positive correlation between heterozygote genotypes and greater levels of homeostasis and adaptability to changing environments (Mitton, 1978; Quattro & Vrijenhoek, 1989; Hedrick, 2006), can also apply to this species. In addition to heterozygote excess, the unusual loss of several alleles furthermore suggests that *D. holmgrenii* might be of natural hybrid origin, probably derived from *Dioon merolae* and *D. tomasellii*. In a context of cytological evidence, Johnson (1963) had already discussed hybrid swarms in cycads for the Australian genus *Macrozamia*; aggregation of taxa with such origins could exist in Neotropical cycads. Furthermore, patterns of genetic diversity similar to those displayed by this Mexican cycad species are characteristic of novel hybrid genotypes resulting from the mixing of parental genomes (Rieseberg, 1997, 2006).

Genetic relationships among taxa

Out of the thirteen currently valid species of the cycad genus *Dioon*, the species *D. sonorense*, *D. tomasellii*, and *D. holmgrenii* constitute a morphological and geographical group of well-differentiated taxa (De Luca, Sabato & Vázquez-Torres, 1981, 1984; Sabato & De Luca, 1985). Although the objective of the present study was not an evaluation of taxonomic relationships, the overall results on the variation and genetic structure of these three *Dioon* species are coherent with the distribution of their morphological and geographical variation. The phenogram obtained with the genetic diversity data clearly segregates three groups that correspond to the three morphologically defined species, particularly suggesting that *D. tomasellii* and *D. holmgrenii* maintain a closer relationship to each other than any of the two with *D. sonorense*, notwithstanding that Nei's average genetic distance remains small between all species. Interestingly, these results are in contrast with those reported by Moretti *et al*. (1993), who found a closer relationship between *D. tomasellii* and *D. sonorense* based on chloroplast DNA restriction fragment length polymorphism analysis. The phenogram also indicates that the *D. tomasellii* populations located to the north of the Trans Mexican Neovolcanic Mountains form a separate group from those to the south of this mountain range. In a recent cladistic biogeography study, it was postulated that this mountain belt might have been an outstanding determinant in the spatial evolution of conspicuous Mexican gymnosperms (e.g. pines; Contreras-Medina, Luna & Morrone, 2007). Our phenetic analysis also suggests that this important orographic barrier might have played a role in genetic differentiation processes within *D. tomasellii*. The extent to which differentiation at this level could be reflected in other aspects of the biology of the species can be assessed through a revision of the morphological variation between the known populations, and the systematic evaluation of other sources of information (e.g. nucleotide characters from the chloroplast or the nucleus). It might be possible that *D. tomasellii* at both sides of this transvolcanic mountain range are currently engaged in an incipient speciation process (González-Astorga *et al*., 2005).

In order to corroborate the importance of the Trans Mexican Neovolcanic Mountains for the distribution of genetic variation found in the five populations of *D. tomasellii* an analysis of molecular variance was performed, on group A, to the north of the barrier and group B, to the south (Figs 1, 2). The results indicated that the variation explained among groups was 8% of the total variance (0.737; $P < 0.01$), the variation among populations was 19% (1.68; $P < 0.01$), and within populations was 73% (6.57; $P < 0.01$); all were found significant. These results agree with the incipient speciation hypothesis for *D. tomasellii*.

The geographical distribution of *D. tomasellii* covers a number of states in Mexico, and the species is protected in a private reserve in the state of Jalisco. Therefore, we do not consider it to be critically endangered; in our opinion, the present IUCN category (IUCN, 2005) of endangered (EN A2abd) still holds. However, in view of the small population sizes of *D. sonorense*, which have been drastically reduced over recent decades, we recommend that this species is put under the IUCN (2005) Red List category of critically endangered (CR A1, c, d). The reduction in population sizes for *D. sonorense* has been the consequence of high levels of exploitation for alcohol production (J. Rees, pers. comm.), which in turn have caused high levels of genetic differentiation, inbreeding and genetic drift. Only one of the populations of *D. sonorense* is located within a protected area and, because of the high genetic variation in this species, a loss of any one of the unprotected populations might significantly reduce the overall genetic diversity of the species (Ellis *et al*., 2006). We recommend that *D. holmgrenii* should be listed as CR C2a(ii) in the IUCN Red List (IUCN, 2005) because the populations have continued to decline and the number of mature individuals in the two known populations are estimated to be under 50%. As *D. holmgrenii* is not found in any protected areas, the habitats should be declared as sanctuaries, owing to the narrow endemic status of this cycad species.

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REFERENCES

Barrett SCH, Kohn JR. 1991. Genetic and evolutionary consequences of small population size in plants: Implications for conservation. In: Falk DA, Holsinger KE, eds. *Genetic and conservation of rare plants*. New York, NY: Oxford University Press, 3–30.

Byrne M, James SH. 1991. Genetic diversity in the cycad Macrozamia riedlei. *Heredity* **67:** 35–39.

Caputo P, Cozzolino S, De Luca P, Moretti A, Stevenson D. 2004. Molecular phylogeny of *Zamia* (Zamiaceae). In: Walters T, Osborne R, eds. *Cycads classification concepts and recommendations*. Wallingford: CABI Publishing, 149–157.

Chao-Luan L, Wang Q, Jiang SY, Ge S, Wang KE. 1999. Genetic diversity of allozymes in populations of *Cycas panzhihuaensis* L. Zhou and S. Y. Yang. In: Chen CJ, ed. *Biology and Conservation of Cycads-Proceedings of the Fourth International Conference on Cycad Biology*. Beijing: International Academic Publishers, 323–327.

Contreras-Medina R, Luna I, Morrone JJ. 2007. Gymnosperms and cladistic biogeography of Mexican Transition Zone. *Taxon* **56:** 905–915.

De Castro O, Vázquez-Torres M, De Luca P. 2006. Utility of AFLP markers for the assessment of molecular relationships in *Ceratozamia* Brongn. (Zamiaceae). *Plant Biosystems* **140:** 221–228.

De Luca P, Sabato S, Vázquez-Torres M. 1981. *Dioon holmgrenii* (Zamiaceae), a new species from Mexico. *Brittonia* **33:** 552–555.

De Luca P, Sabato S, Vázquez-Torres M. 1984. *Dion tomasellii* (Zamiaceae), a new species with two varieties from western Mexico. *Brittonia* **36:** 223–227.

DiBattista JD. 2007. Patterns of genetic variation in anthropognically impacted populations. *Conservation Genetics* **9:** 141–156 DOI 10.1007/s10592-007-9317-z.

Donaldson J. 2003. Regional Overview: Africa. In: Donaldson J, ed. *Cycads. Status survey and conservation action plan. IUCN/SS Cycad specialist group*. Gland: IUCN Publications Services Unit, 9–19.

Eguiarte LE, Pérez-Nasser N, Piñero D. 1992. Genetic structure, outcrossing rate and heterosis in *Astrocaryum mexicanum* (tropical palm): implications for evolution and conservation. *Heredity* **69:** 217–228.

Ellis JR, Pashley CH, Burke JM, McCauley DE. 2006. High genetic diversity in a rare and endangered as compared to a common congener. *Molecular Ecology* **15:** 2345–2355.

Ellstrand NC, Ornduff R, Clegg JM. 1990. Genetic structure of the Australian cycad, *Macrozamia communis* (Zamiaceae). *American Journal of Botany* **77:** 677–681.

González D, Vovides AP. 2002. Low intralineage divergence in *Ceratozamia* (Zamiaceae) detected with nuclear ribosomal DNA ITS and Chloroplast DNA *trnL*-F non-coding region. *Systematic Botany* **27:** 654–661.

González-Astorga J, Castillo-Campos G. 2004. Genetic variability of the narrow endemic tree Antirhea aromatica Castillo-Campos and Lorence, (Rubiaceae, Guettardeae) in a tropical forest of Mexico. *Annals of Botany-London* **93:** 521–528.

González-Astorga J, Núñez-Farfán J. 2001. Effect of habitat fragmentation on the genetic structure of the narrow endemic Brongniartia vazquezii. *Evolutionary Ecology Research* **3:** 861–872.

González-Astorga J, Vovides AP, Iglesias C. 2003b. Morphological and geographical variation of the cycad *Dioon edule* Lindl. (Zamiaceae): ecological and evolutionary implications. *Botanical Journal of the Linnean Society* **141:** 465–470.

González-Astorga J, Vovides AP, Cruz-Angón A, Octavio-Aguilar P, Iglesias C. 2005. Allozyme variation in three extant populations of the narrowly endemic cycad Dioon angustifolium Miq. (Zamiaceae) from North-eastern Mexico. *Annals of Botany* **95:** 999–1007.

González-Astorga J, Vovides AP, Ferrer M, Iglesias C. 2003a. Population genetics of *Dioon edule* Lindl. (Zamiaceae, Cycadales): biogeographical and evolutionary implications. *Biological Journal of the Linnean Society* **80:** 457–467.

González-Astorga J, Vovides AP, Octavio-Aguilar P, Aguirre-Fey D, Nicolalde-Morejón F, Iglesias C. 2006. Genetic diversity and structure of the Cycad *Zamia loddigesii* Miq. (Zamiaceae): implications for evolution and conservation. *Botanical Journal of the Linnean Society* **152:** 533–544.

Hamrick JL. 2004. Response of forest trees to global environment changes. *Forest Ecology and Management* **197:** 323–335.

Hamrick JL, Godt MJW. 1996. Conservation genetics of endemic plant species. In: Avise JC, Hamrick JL, eds. *Conservation genetics. case histories from nature*. New York, NY: Chapman & Hall, 281–304.

Hansson B, Westerberg L. 2002. On the correlation between heterozygosity and fitness in natural populations. *Molecular Ecology* **11:** 2467–2474.

Hartl DL, Clark AG. 1997. *Principles of population genetics*, 3rd edn. Sunderland, MA: Sinauer Associates.

Hedrick PW. 2006. Genetic polymorphism in heterogeneous environments: the age of genomics. *Annual Review of the Ecology, Evolution and Systematics* **37:** 67–93.

Hill KD. 2003. Regional overview: Australia. In: Donaldson J, ed. *Cycads. Status survey and conservation action plan. IUCN/SS Cycad specialist group*. Gland: IUCN Publications Services Unit, 20–24.

Hill KD. 2004. Character evolution, species recognition and classification concepts in the Cycadaceae. In: Walters T, Osborne R, eds. *Cycads classification concepts and recommendations*. Wallingford: CABI Publishing, 23–44.

Hill KD, Chen CJ, Loc PK. 2003. Regional overview: Asia. In: Donaldson J, ed. *Cycads. Status survey and conservation action plan. IUCN/SS Cycad specialist group*. Gland: IUCN Publications Services Unit, 25–30.

IUCN. 2005. IUCN red list of threatened species. [cited 21 August 2007.] Available at http://www.redlist.org.

Johnson LAS. 1963. Cytological and taxonomic notes on Zamiaceae. *Contributions from the New South Wales National Herbarium* **3:** 235–240.

Keppel G. 2002. Low genetic variation in a pacific cycad: conservation concerns for *Cycas seemannii* (Cycadaceae). *Oryx* **36:** 41–49.

Lin TP, Sun YC, Lo HC, Cheng YP. 2000. Low genetic diversity of *Cycas taitungensis* (Cycadaceae), an endemics species in Taiwan, revealed by allozyme analysis. Taiwan Journal of Forest. *Science* **15:** 13–19.

Mateu-Andrés I. 2004. Low levels of allozyme variability in the threatened species *Antirrhinum subbaeticum* and *A. pertegasii* (Scrophulariaceae): implications for conservation of the species. *Annals of Botany* **94:** 797–804.

Miller MP. 1997. *Tools for population genetic analyses (TFPGA) 1.3: a windows program for the genetic data*. Computer software distributed by the author.

Mitton JB. 1978. Relationship between heterozygosity for enzyme loci and variation of morphological characters in natural populations. *Nature* **273:** 661–662.

Mitton JB, Grant MC. 1984. Associations among protein heterozygosity, growth rate, and development homeostasis. *Annual Review of Ecology and Systematics* **15:** 479–499.

Moretti A, Caputo P, Cozzolino S, De Luca P, Gaudio L, Gigliano GS, Stevenson DW. 1993. A phylogenetic analysis for *Dioon* (Zamiaceae). *American Journal of Botany* **80:** 204–214.

Nei M. 1972. Genetic distances between populations. *American Naturalist* **106:** 283–292.

Quattro JM, Vrijenhoek RC. 1989. Fitness differences among remnant populations of the endangered Sonoran topminnow. *Science* **245:** 976–978.

Rabinowitz D. 1981. Seven forms of rarity. In: Synge H, ed. *Biological aspects of rare plant conservation*. Chichester: John Wiley & Sons Ltd, 205–217.

Rieseberg LH. 1997. Hybrid origins of plant species. *Annual Review of Ecology and Systematics* **28:** 359–389.

Rieseberg LH. 2006. Hybrid speciation in wild sunflowers. *Annals of the Missouri Botanical Garden* **93:** 34–48.

Sabato S, De Luca P. 1985. Evolutionary trends in *Dion* (Zamiaceae). *American Journal of Botany* **72:** 1353–1363.

Sharma IK, Jones DL, Foster PI. 2004. Genetic differentiation and phonetic relatedness among seven species of the *Macrozamia plurinervia* complex (Zamiaceae). *Biochemical Systematics and Ecology* **32:** 313–327.

Sharma IK, Jones DL, Foster PI, Young AG. 1998. The extent and structure of variation in the *Macrozamia pauli-guilielmi* complex (Zamiaceae). *Biochemical Systematics and Ecology* **26:** 45–54.

Sharma IK, Jones DL, Foster PI, Young AG. 1999. Low isozymic differentiation among five species of the *Macrozamia heteromera* group (Zamiaceae). *Biochemical Systematics and Ecology* **27:** 67–77.

Sneath PHA, Sokal RR. 1973. *Numerical taxonomy*. San Francisco, CA: W. H. Freeman.

Stevenson DW, Vovides A, Chemnick J. 2003. Regional overview: New World. In: Donaldson J, ed. *Cycads. Status survey and conservation action plan. IUCN/SS Cycad specialist group*. Gland: IUCN Publications Services Unit, 31–38.

Stilwell KL, Wilbur HM, Werth CR Taylor DR. 2003. Heterozygote advantage in the American chestnut, *Castanea dentata* (Fagaceae). *American Journal of Botany* **90:** 207–213.

Treutlein J, Vorster P, Wink M. 2004. Molecular relationships in *Encephalartos* (Zamiaceae, Cycadales) based on nucleotide sequences of nuclear ITS 1&2, Rbcl, and genomic ISSR fingerprinting. *Plant Biology* **7:** 79–90.

Van Geert A, Van Rossum F, Triest L. 2007. Genetic diversity in adult and seedling populations of Primula vulgaris in a fragmented agricultural landscape. *Conservation Genetics* DOI 10.1007/s10592-007-9409-9.

Van der Bank H, Wink M, Vorster P, Treutlein J, Brand L, Van der Bank M, Hurter J. 2001. Allozyme and DNA sequence comparisons of nine species of *Encephalartos* (Zamiaceae). *Biochemical Systematics and Ecology* **29:** 241–266.

Vorster P. 2004. Classification concepts in Encephalartos (Zamiaceae). In: Walters T, Osborne R, eds. *Cycads classification concepts and recommendations*. Wallingford: CABI Publishing, 69–83.

Vovides AP, Pérez-Farrera MA, González D, Avendaño S. 2004. Relationships and phytogeography in Ceratozamia (Zamiaceae). In: Walters T, Osborne R, eds. *Cycads classification concepts and recommendations*. Wallingford: CABI Publishing, 109–125.

Walters T. 2003. Off-site collections. In: Donaldson J, ed. *Cycads. Status survey and conservation action plan. IUCN/SS Cycad specialist group*. Gland: IUCN Publications Services Unit, 48–53.

Walters T, Decker-Walters DS. 1991. Patterns of allozyme diversity in the west Indies cycad *Zamia pumila* (Zamiaceae). *American Journal of Botany* **78:** 436–445.

Weir BS. 1990. *Genetic data analysis*. Sunderland, MA: Sinauer Associates.

Wendel JF, Weeden NF. 1989. Visualization and interpretation of plant isozymes. In: Soltis DE, Soltis PS, eds. *Isozymes in plant biology*. Portland, OR: Discorides Press, 5–45.

Wright S. 1978. *Evolution and the genetics of populations*, Vol. 4. Chicago, IL: University of Chicago Press.

Xiao L, Gong X. 2006. Genetic differentiation and relationships of populations in the *Cycas balansae* complex (Cycadaceae) and its conservation implications. *Annals of Botany* **97:** 807–812.

Xiao L, Gong X, Hao G, Ge X, Tian B, Zheng S. 2005. Comparison of the genetic diversity in two species of cycads. *Australian Journal of Botany* **53:** 219–223.

Yang SL, Meerow AW. 1996. The *Cycas pectinata* (Cycadaceae) complex: genetic structure and gene flow. *International Journal of Plant Science* **157:** 468–483.

Zar JH. 1999. *Biostatistical analysis*. 4ª edn. Upper Saddle River, NJ: Prentice Hall.