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Estimation of genetic variation in closely related cycad species in *Ceratozamia* (Zamiaceae: Cycadales) using RAPDs markers

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Abstract: The *Ceratozamia norstogii* complex from Southern Mexico is made up of four closely related taxa and occurs in similar habitats (*Quercus* forest). All have linear-lanceolate leaflets with great similarity between them, especially in juvenile stages, but differentiate with age. There has been debate regarding delimitation of species due to character loss in herbarium specimens. The aim of this study was to determine the genetic variation, and to measure genetic similarity between the four taxa. We studied populations in Cintalapa (Chiapas) for *C. alvarezii* and *C. norstogii*; the Sierra Atravesada (Oaxaca) for *C. chimalapensis*, and Villa Flores (Chiapas) for *C. mirandae*. One population for each taxon was sampled (only one population is known for *C. alvarezii*) 11-15 randomly chosen adult individuals were sampled. Twenty-eight primers were tested of which five were polymorphic using the RAPD'S technique. The data were analyzed using Bayesian methods. Results revealed low genetic diversity, and a differentiation was found between species, suggesting a recent divergence. A previous morphological and anatomical study on the complex has found the taxa to be distinct. However, the results of this study have shown that the *C. norstogii* species complex is in a divergence process, probably through genetic drift and founder effects. Rev. Biol. Trop. 65 (1): 305-319. Epub 2017 March 01.

Key words: cycads, *Ceratozamia norstogii* complex, Zamiaceae, Mexico, genetic variation, speciation, founder effects.

Ceratozamia is a Mexican and Central American cycad genus consisting of 27 species (Osborne, Calonje, Hill, Stanberg, & Stevenson, 2012) and 24 are endemic to Mexico. Both morphological and genetic variations have not yet been studied sufficiently, along the distributional range of the genus, where some taxa form species complexes resulting in problems of species delimitation for some populations (Moretti, Sabato, & Vázquez-Torres, 1980; Stevenson, Sabato, & Vázquez-Torres, 1986; Norstog & Nicholls, 1997; Whitelock, 2002). These problems are partly due to various factors, such as character loss during processing material for herbarium vouchers (Vovides, Avendaño, Pérez-Farrera, & Stevenson, 2012). Frequent collection of juvenile stages (Vovides, Rees, & Vázquez-Torres, 1983;

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Norstog & Nicholls, 1997; Whitelock, 2002), also unpredictable phenology and difficult access to populations of some species hampers fieldwork.

Molecular methods such as isoenzyme electrophoresis, protein sequences, DNA hybridization, restriction fragment length polymorphism (RFLPs) and random amplified polymorphic DNA (RAPDs), have been used as a tools to solve species complexes and/or analyze genetic variation between species and populations. Isoenzymes may have different allelic forms (allozymes), mostly selective neutrals and co-dominants, which could be used to quantify allelic frequencies and different measures of genetic variation. Some analyses have been done on a wide range of cycad species, for instance; *Zamia pumila* (Walters & Deckers-Walters, 1991), *Macrozamia pauli-guilielmi* complex (Sharma, Jones, Forster, & Young, 1998), the *M. heteromera* complex (Sharma, Jones, Forster, & Young, 1999), the *M. plurinervia* complex (Sharma, Jones, & Forster, 2004), nine *Encephalartos* species (Van Der Bank et al., 2001), *Cycas seemannii* (Keppel, Lee, & Hodgskiss, 2002) and five species of *Dioon* have been analyzed (González-Astorga, Vovides, Ferrer, & Iglesias, 2003; González-Astorga, Vovides, Cruz-Angón, Octavio-Aguilar, & Iglesias 2005; Cabrera-Toledo, González-Astorga, & Vovides, 2008; González-Astorga et al., 2008; Cabrera-Toledo et al., 2010). Genetic variation and species relationships have been studied to a lesser extent with RAPD markers, which are dominants and highly polymorphic, examples are *Encephalartos* (Chaiprasongsuk et al., 2004), with AFLP, RFLP and RAPDs in *Cycas* (Mekanawakul & Juntawong, 2004; Sangin, Thongpan, Lindstrom, Sangduan, & Mingmuang, 2004). Dominant molecular markers as well as microsatellites have recently been used on *Cycas micronesica, Cycas rumphii*, *Dioon edule*, *Zamia integrifolia, Z. erosa, Z. portoricensis* and *Z. pumila*, (Moynihan, Meerow, & Francisco-Ortega, 2007; Meerow & Nakamura, 2007; Cibrián-Jaramillo, Marler, De Salle, & Brenner, 2008; Cibrián-Jaramillo, Daly, Brenner, De Salle, & Marler, 2010; Meerow et al., 2012).

González and Vovides (2002) analyzed variation patterns in nuclear DNA (ITS) and chloroplast *trn*L-F non-codified regions in the genus *Ceratozamia* and found homogeneity of characters and low genetic variation among species. In contrast, González-Astorga et al. (2003) using isoenzymes found high genetic variation in *D. edule* through its range along the Gulf of Mexico seaboard.

It has been proposed that *Ceratozamia norstogii* D.W. Stev. forms a complex characterized by narrow linear to linear-lanceolate leaflets, cylindrical stems and erect cones, and comprises four named taxa: *C. norstogii, C. mirandae* Vovides, Pérez-Farr. & Iglesias*, C. alvarezii* Pérez-Farr., Vovides & Iglesias and *C. chimalapensis* Pérez-Farr. & Vovides, all endemic to Mexico. *Ceratozamia norstogii, C. mirandae* and *C. alvarezii* are from the Northern part of the Sierra Madre de Chiapas. Their habitat is oak and pine/oak forests with some species typical of cloud forest in the *C. mirandae* localities. These three species share a narrow and overlapping elevation range: *C. alvarezii* is found at 950 masl, *C. norstogii* between 800-1 600 masl and *C. mirandae* between 910-1 300 masl (Stevenson, 1982; Pérez-Farrera, Vovides & Iglesias, 1999, 2001; Vovides, Pérez-Farrera & Iglesias, 2001, Vovides, Pérez-Farrera, González-Astorga & Iglesias, 2008). *Ceratozamia chimalapensis* is from the Sierra Atravesada of Oaxaca and is found at the lower elevation range of 270-1 000 masl in oak forest habitat. It is associated with cloud forest elements such as *Liquidambar, Nectandra, Elaphoglossum* and *Begonia* as well as elements common to lowland tropical forest environments such as *Cecropia* and *Bursera*.

There are important morphological differences between species within the *C. norstogii* complex. All species show erect female cones except *C. chimalapensis* that are descending. *Ceratozamia mirandae* and *C. norstogii* show a light brown to ochre tomentum on the megasporophyll lobule near the horns, whereas *C. chimalapensis* presents a bluish tomentum

on the megasporophyll margin. *Ceratozamia alvarezii* shows a blue to blue-green tomentum on the megasporophyll lobe between the horns and at times covering the whole lobe. *Ceratozamia mirandae* has a short and thick peduncle whereas in *C. chimalapensis* it is long and thin (Vovides et al., 2008). From eleven anatomical variables perivascular fibers and palisade mesophyll cells accounted for 96.7 % of the variance of all the anatomical data. The four species showing well separated scatter clouds for discriminant functions with consistency between anatomical and morphological data among the species. The multivariate spacing for the four species showed no overlap (Pérez-Farrera, Vovides, & Avendaño, 2014).

Intraspecific morphological variation has been observed among taxa obscuring the boundaries, particularly between *C. mirandae* and *C. chimalapensis* (Pérez-Farrera, Vovides, Hernández-Sandoval, González, & Martínez, 2004). These species have been separated based on morphological and leaflet anatomical characters (Pérez-Farrera et al., 2014), but have not been studied from a genetic variability viewpoint. Therefore, information on genetic diversity can contribute to our knowledge on the circumscription of taxa within this complex. Also, genetic similarities and distances can be instrumental in aiding conservation strategies. With this in mind, this study is aimed to investigate the genetic variation among the taxa that comprises the complex using RAPDs markers. The development of DNA markers has greatly facilitated genetic studies in eukaryotic and prokaryotic genomes (Mullis, 1990; Erlich, Gelfand, & Sninsky, 1991; Archak, Karihaloo, & Jain, 2002). RAPDs analysis was found to be simple and efficient among the available DNA-based techniques (Welsh & McClelland, 1990; Williams, Kubelik, Livak, Rafalski, & Tingey, 1990; Khan, Khan, Awan, Sadaqat, & Bahadur, 2011) not requiring sequence information (Gepts, 1993). Furthermore, RAPD techniques are advantageous because they require smaller quantities of DNA compared to other techniques, and for their ability to reveal a large number of polymorphisms (Cheng, Chang, Su, & Hsu, 1997; Carelli, Gerald, Grazziotin, & Echeverrigaray, 2006). However, we are aware that these results should be considered preliminary, since specific microsatellites for *Ceratozamia* are still under development in order to compare these results with other molecular markers in the future.

MATERIALS AND METHODS

Taxon sampling: One population per named taxon was sampled, and from one to three clean undamaged leaflets per individual were obtained for analysis (Fig. 1). We sampled a total of 11 individuals of *C. alvarezii* and 15 of *C. norstogii* from their respective populations in Cintalapa, Chiapas; besides, 15 individuals of *C. chimalapensis* from the Sierra Atravesada, Oaxaca, and 10 individuals from *C. mirandae* from Villa Flores, Chiapas. Precise locality information has been purposely omitted to discourage illegal commercial collecting. All cycad species in Mexico are considered threatened and endangered and are protected by the Norma Oficial Mexicana NOM-059-SEMARNAT (2010). The genus *Ceratozamia* is especially threatened and is listed in Appendix 1 of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). The leaflets chosen were free from lichens, fungal infections and insect bites. The number of individuals chosen (per population) for sampling was limited to the number of adults present. Criteria for selecting these populations were accessibility and health of each population, showing minimum disturbance and good regeneration. Only one population per named taxon was sampled in order to keep sampling even, since *C. alvarezii* is only known from a single population. Representative vouchers for each population were deposited at HEM (Herbario Eizi Matuda, Universidad de Ciencias y Artes de Chiapas, Tuxtla Gutiérrez, Mexico) (Appendix 1).

DNA extraction: The middle portion of each leaflet was cleaned and processed for DNA extraction according to González and Vovides (2002) for subsequent analysis using

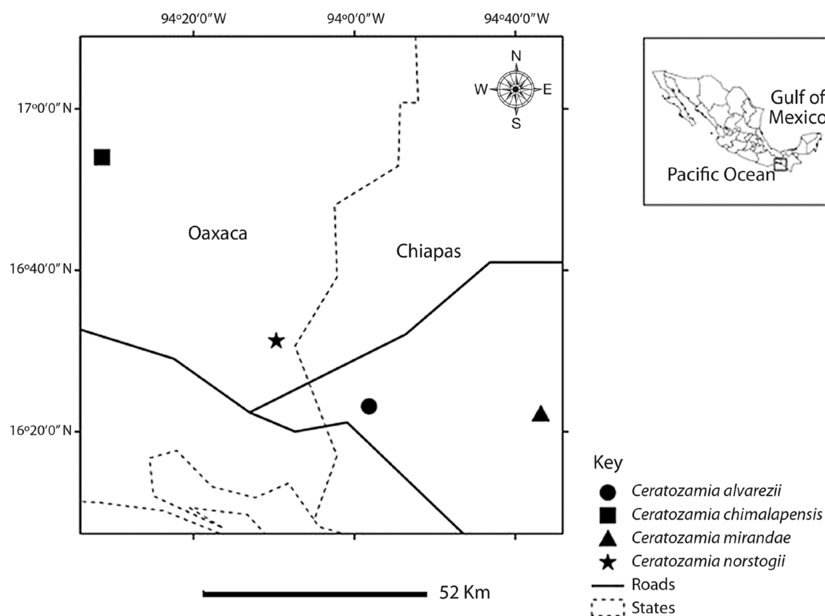

Fig. 1. Geographic distribution of *Ceratozamia norstogii* complex in southern Mexico.

RAPD markers. The tough leaflet cuticle was removed before extraction in order to expose as much soft tissue as possible. The tissue was mixed with a cationic detergent extraction buffer Cetyltrimethyl Ammonium Bromide (CTAB) (10 % in H_2O), 3 mL, 3 %; 5 M NaCl, 2.8 mL, 28 %; 0.5 M EDTA (pH 8.0), 0.4 mL, 4 %; 1 M Tris-Cl (pH 8.0), 1 mL, 10 %; Polyvinylpyrrolidone (PVP) (MW 40 kDa), 0.3 g, 3 %; β - Mercaptoethanol, 0.02 mL, 0.2 %; H_2O, 2.48 mL, 24.8 % and incubated at 65 ^{o} C for 30 min, before adding 750 µL of a mixture of chloroform-isoamyl alcohol (24:1, v/v). The extracted DNA was precipitated with absolute ethanol, washed with 70 % ethanol, dried, and re-suspended in TE buffer (10 mM Tris-Cl, pH 8.0, 1.0 mM EDTA).

Verification and DNA purification: Prior to amplification, the DNA was purified using a low melting-point gel. The DNA band was cut from the gel and extracted with Geneclean^{TM} (BIO 101, La Jolla, CA) according to manufacturer's instructions. The DNA concentration was determined by means of an electrophoresis on a 1 % agarose gel with a DNA standard of known concentration.

Amplification: Pure Taq^{TM} Ready to Go^{TM} PCR Bead (Amersham Biosciences, Little Chalfont Buckinghamshire UK) was used for amplification. This kit was used because it is highly robust and its reproducible performance in standard PCR applications. Five microliters of DNA (at a concentration of 2.5 ng/µL) were placed in each tube and 3 µL of primer (at a concentration of 10 µM) were added along with 17 µL of distilled water for a total reaction volume of 25 µL. Thermocycle parameters were: initial denaturation at 94 ºC for 2.5 min, followed by a primer hybridization to template at 37 ºC for 1 min, and then an extension cycle at 72 ºC for 2 min for 44 cycles, ending with an extension cycle at 72 ºC for 7 min.

Twenty-eight primers were tested from kits B and C (Operon Technologies, Alameda CA) of which five were polymorphic. Amplification primers used were: B7 (5' - GGTGACGCAG - 3'); B11 (5' - GTAGACCCGT - 3'); C2 (5' - GTGAGGCGTC - 3'); C3 (5'- GGGGGTCTTT - 3') and C6 (5'- GAACGGACTC - 3'). The amplified products were run on a 1 % agarose gel with a "DNA Ladder" 123 base pairs (bp) marker (Sigma, St. Louis, MO) as reference,

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to identify allele's sizes in bp. The gels were photographed with a Kodak model DC 120 Zoom digital camera, and photographs analyzed using the Kodak Digital Science ds 1D v. 2.0.3 software for Windows (Eastman Kodak Company). The data were then transferred to an Excel 2000 v.9.0 spreadsheet where a presence (1) absence (0) data matrix of alleles was constructed. For analyses, samples of each species were considered as independent populations. Statistics and measures of diversity and genetic structure assuming Hardy-Weinberg genotypic proportions were obtained using AFLP-SURV 1.0 for Windows (Vekemans, Beauwens, Lemaire, & Roldan-Ruiz, 2002). This program estimates allelic frequencies at each marker locus in each species assuming they are dominant, and have only two alleles (presence of a band = dominant allele; absence of a band = recessive null allele). The estimation of allelic frequencies was computed through a Bayesian method with non-uniform *a priori* distribution as implemented in AFLP-SURV 1.0 (Lynch & Milligan, 1994; Zhivotovsky, 1999).

The following parameters were obtained: number (#loc_P) and proportion (PLP) of polymorphic loci at the 5 % level; expected heterozygosity or Nei's gene diversity (Hj) and its variance components (total variance, Var (Hj)); Measures of population genetic structure were according to Lynch and Milligan (1994), as follows: total genetic diversity (Ht), i.e. expected heterozygosity or gene diversity in the overall sample, average gene diversity within populations (Hw), and average gene diversity among populations in excess of that observed within populations (Hb), which is analogous to Nei's Distance. Wright's fixation index (Fst), and Nei and Raynolds genetic distances were also measured (Raynolds, Weir, & Cockerham, 1983) and a UPGMA cluster analysis presented. Furthermore, an analysis of Molecular Variance (AMOVA) was performed using GENALEX 6 for Windows (Peakall & Smouse, 2006). A Mantel test (Sokal & Rohlf, 1985) was also done to test association significance between matrixes. Additionally, a linear regression was done to evaluate the correlation between genetic and geographic distances, gene flow and geographic distance, and genetic distance vs. altitude using TFPGA v. 1.3 for Windows 2000 (Miller, 1997).

Aware of the problems associated with the RAPD markers, up to three repetitions of the reactions were done to ensure repeatability. Pure Taq^{TM} Ready to Go^{TM} PCR Bead (Amersham Biosciences, Little Chalfont Buckinghamshire UK) were used in all reactions to guarantee homogeneity in the reagents used, thus avoiding variables in the preparation of reactions. Only the alleles that showed clear signals of presence absence were recorded.

Good quality color photographs taken on fresh material in the field can mitigate the problem of character loss in herbarium vouchers. Especially close-ups of reproductive structures and their habit i.e. decumbent, erect (among others) sporophyll, and indument details, and leaf venation whenever possible.

Genetic population studies require a sampling of as many populations as possible per species. However in our case, the cycad species are rare, narrowly distributed and one species, *C. alvarezii* is endemic and known only from one small population, therefore large population based samples are not possible. For this reason, the results and conclusions should be regarded only as possibilities and taken conservatively.

RESULTS

A total of 125 bands were scored, of which in average 92 (73.6 %) were polymorphic. The number of alleles generated per species varied from 76 to 116. An average of (0.22 ± 0.01) of expected heterozygosity also called Nei´s genetic diversity was found in the *Ceratozamia norstogii* complex. *Ceratozamia chimalapensis* showed the highest genetic diversity (Hj/He= 0.25) (± 0.01) (Table 1). The AMOVA revealed that there is a low genetic variation among species, in about 14 %, suggesting that 86 % of the molecular variance was between individuals within species (Table 2).

TABLE 1
Comparison of the number of alleles per locus, heterozygosis, Neis's genetic diversity index and number of polymorphic loci per species of the *Ceratozamia norstogii* complex

Population	n.	#loc	#loc_P	PLP	Hj	S.E. (Hj)	Var (Hj)	VarL %
C. alvarezii	11	125	82	65.6	0.2007	0.015	0.000225	80
C. chimalapensis	15	125	116	92.8	0.2459	0.011	0.000128	65.7
C. norstogii	15	125	76	60.8	0.2118	0.015	0.000216	83.5
C. mirandae	10	125	95	76	0.2359	0.013	0.000172	66.4

n : No. of individuals analyzed; # loc : number of loci; # loc_P : number of polymorphic loci at the 5 % level, i.e. loci with allelic frequencies lying within the range 0.05 to 0.95; PLP : proportion of polymorphic loci at the 5 % level, expressed as a percentage; Hj : expected heterozygosity under Hardy-Weinberg genotypic proportions, also called Nei's genetic diversity (analogous to H or He in most publications); S.E. (Hj) : standard error of Hj; Var (Hj) : variance of Hj; VarL % : proportion of Var (Hj) due to sampling of loci.

TABLE 2
Summary results of AMOVA

Source	df	SS	MS	Est. Var.	%	Stat	Value	P
Among Pops	3	1.878	0.626	0.034	14			
Within Pops	47	9.533	0.203	0.203	86	PhiPT	0.142	0.001
Total	50	11.412		0.236	100			

df, degrees of freedom; SS, sum of squared observations; MS, mean of squared observations; Est. var., estimated variance; % Var., percentage of total variance; PhiPT, proportion of the total genetic variance among individuals within populations of the *Ceratozamia norstogii* complex.

TABLE 3
Nei's genetic distance (above the asterisks) and genetic distance after Reynolds et al., 1983 (below the asterisks) between species of the *Ceratozamia norstogii* complex

	C. alvarezii	*C. chimalapensis*	*C. norstogii*	C. mirandae
C. alvarezii	*	0.014	0.020	0.018
C. chimalapensis	0.047	*	0.013	0.007
C. norstogii	0.072	0.044	*	0.013
C. mirandae	0.061	0.022	0.045	*

Ceratozamia alvarezii and *C. norstogii* showed the highest Nei's genetic diversity index within the complex also that there is almost nil genetic diversity between *C. mirandae* and *C. chimalapensis* (Table 3). The mean gene diversity (Hw), also called mean Nei's gene diversity within populations (analogous to Hs) was 0.22 (± 0.01), while the average genetic diversity among population was 0.011 (± 0.001), showing low variability. The total genetic diversity (H_t) was 0.23 while the differentiation of genetic diversity between species (Fst) was 0.047 (± 0.15), indicating a low divergence among species within the complex.

The results of the UPGMA cluster analysis showed a similarity in genetic distances between species, the most similar being *C. mirandae* and *C. chimalapensis* (Fig. 2).

The value of genetic differentiation (Fst), between all populations of the complex was 0.047. The lowest value was between *Ceratozamia mirandae* and *C. chimalapensis* (Fst= 0.022), separated geographically by 122 km, while the highest value was between

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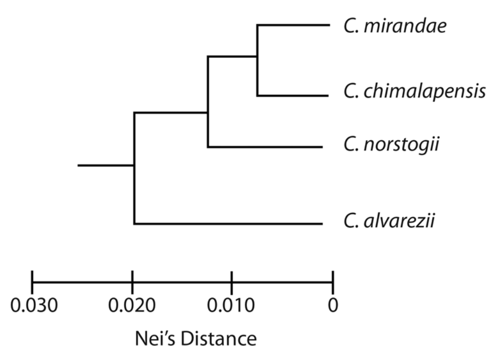

Fig. 2. A UPGMA cluster analysis using Nei's genetic distances.

C. norstogii and *C. alvarezii* (Fst= 0.072) separated by 26.6 km (Table 4). No significant correlation was found between genetic distance/ altitude (Mantel r= - 0.53, p= 0.24) and F_{ST} / altitude (Mantel r= - 0.33, p= 0.41), but a slight and significant correlation was found between genetic distance / geographic distance (Mantel r= - 0.93, p= 0.06) and F_{ST} / geographic distance matrixes (Mantel test, r= - 0.95, p = 0.06) between species of the complex (Table 5). The linear regression analysis between genetic distance, Fst and difference geographic distance was found significant (Fig. 3 and Fig. 4).

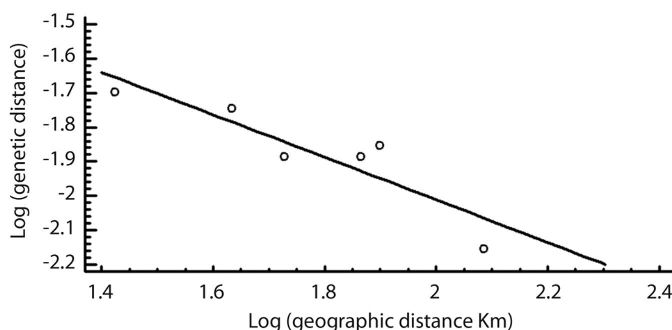

Fig. 3. Correlation between difference of genetic distance and geographic distance between species of the *Ceratozamia norstogii* complex (Y = - 0.768795 - 0.621495 * X; F = 16.86; df = 5; p = 0.01; R = - 0.89; R^2 = 0.80).

TABLE 4
Geographic distance (Km) (above the asterisks) and F_{ST} (below the asterisks) between species of the *Ceratozamia norstogii* complex

	C. alvarezii	*C. chimalapensis*	*C. norstogii*	*C. mirandae*
C. alvarezii	*	79.4	26.6	43
C. chimalapensis	0.046	*	73.4	122
C. norstogii	0.070	0.043	*	53.5
C. mirandae	0.059	0.022	0.044	*

TABLE 5
Elevation difference between the species (m.a.s.l.) (below the asterisks) and Nei's genetic distance after Lynch and Milligan (1994) (above the asterisks) matrix between populations of the *Ceratozamia norstogii* complex

	C. alvarezii	*C. chimalapensis*	*C. norstogii*	*C. mirandae*
C. alvarezii	*	0.014	0.020	0.018
C. chimalapensis	630	*	0.013	0.007
C. norstogii	400	1 030	*	0.013
C. mirandae	50	680	350	*

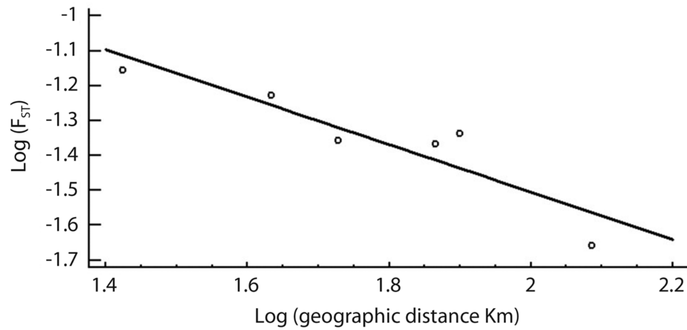

Fig. 4. Correlation between difference F_{ST} and geographic distance between species of the *Ceratozamia norstogii* complex (Y = - 0.143077 - 0.68087 * X; F =19.97; df = 5; p = 0.01; R = -0.91; R^2 = 0.83).

DISCUSSION

The average percentage of polymorphic loci for *Ceratozamia norstogii* complex (73.8 %) was similar to that previously reported for gymnosperms (70.9 %) (Hamrick & Godt, 1989), but higher than other cycads (56.69 %; Pinares, González-Astorga, Vovides, Lazcano, & Vendrame, 2009).

The total genetic diversity (Ht) for the complex (0.23) was lower than that found for the *Macrozamia plurinervia* complex (0.352; Sharma et al., 2004); *Encephalartos barteri* (0.297; Ekué, Gailing, Hölscher, Sinsin, & Finkeldey, 2008) and *Cycas simplicipinna* (0.878; Feng, Wang, & Gong, 2014), but higher than *Macrozamia communis* (0.19; Ellstrand, Ornduff, & Clegg, 1990); *M. heteromera* (0.165; Sharma et al., 1999) and *Cycas guizhouensis* (0.108; Long-Qian, Xue-Jun, Xun, Gang, & Si-Xiang, 2004), and similar to the *Macrozamia pauli-guilelmi* complex (0.229; Sharma, Jones, Forster, & Young, 1998).

Comparisons between the results of this work with other researches were made, taking into account the differences between markers; isoenzymes are co-dominant with low ability to detect genetic variation; RAPDs are dominant and highly variable, and microsatellites segregate in a Mendelian way with high polymorphism and co-dominance. Despite the controversy about the use of RAPDs, the results of this study have been very informative. Additionally, numerous genetic analyses have been performed using this technique (e.g. Chapco, Ashton, Martel, Antonishyn, & Crosb, 1992; Landry, Dextraze, & Boivin, 1993; Demeke, Lynch, Kawchuk, Kozub, & Armstrong, 1996).

The diversity within species (Hs) for the *Ceratozamia norstogii* complex (0.22) was higher than the one reported in most of *Macrozamia* complexes, for example *M. heteromera* (0.149), *M. plurinervia* (0.145) and *M. pauli-guilelmi* complex (0.12), but the diversity among species (Hb) (0.011) was lower than that for *M. communis* (0.1), *M. plurinervia* complex (0.207) (Sharma et al., 2004) and *M. pauli-guilelmi* complex (0.109) (Sharma et al., 1998); nevertheless, it was similar to the *M. heteromera* group (0.016) reports (Sharma et al., 1999).

The genetic differentiation between species for the *Ceratozamia norstogii* complex (Fst= 0.04) was lower than the one reported for most of the *Macrozamia* complexes, for example *M. pauli-guilelmi* complex (0.47) (Sharma et al., 1998), *M. plurinervia* complex (0.588) (Sharma et al., 2004) and in general, for genus *Ceratozamia* (0.757) (De Castro, Vázquez-Torres, & De Luca, 2006) or cycads (0.2) (Pinares et al., 2009). However, its value was similar to the one reported for several species of Mexican cycads, such as *Zamia purpurea* (0.025), *Z. variegata* (0.085) (Pinares et al., 2009), *Dioon merolae* (0.07), *D. caputoi* (0.06) (Pinares et al., 2009), *D. edule* (González-Astorga et al., 2003) and to gymnosperms in general (0.068) (Hamrick & Godt, 1989).

The small genetic distances between species within the *Ceratozamia norstogii* complex could be due to diverse factors such as geographic range, life history and ecological characteristics of the species. Genetic factors including low mutation rates, not accumulated or fixed variability in the species' gene pool, and/or having originated from a genetically depauperate ancestor with a restricted geographical distribution, may have also influenced. The low genetic variability within this complex is in agreement with molecular results presented by González and Vovides (2002) for *Ceratozamia*, that suggests a relatively recent isolation, perhaps during post-Pleistocene climatic changes during the Cenozoic (Burnham & Graham, 1999).

The most significant findings from this study are the low genetic diversity and differentiation between populations of the *Ceratozamia norstogii* complex, suggesting a recent divergence for the four species of the complex. In general, cycads have been reported to show low intrapopulation variation and high interpopulation differentiation, these being biological and evolutionary characteristics of cycads (Yang & Meerow, 1996). These results were based on *in situ* and *ex situ* observations; in the case of the latter, the plants were grown under similar conditions in botanic garden greenhouses for over 10 years. It has been observed that overall morphology is maintained and did not differ from that observed for *in situ* plants, suggesting no phenotypic plasticity effects. *Ceratozamia alvarezii* has the smallest but highly branched stems 10-50 cm long (Pérez-Farrera et al., 1999), *C. chimalapensis* is of medium size with stems 20-100 cm long with scarce branching (Vovides et al., 2008), and *C. mirandae* the most robust with erect stems becoming prostrate and branching with age 32-105 cm long (Vovides et al., 2001). All species have erect female cones at maturity except *C. chimalapensis,* which has descending cones when mature (Vovides et al., 2008). Leaflet articulations are yellow in *C. chimalapensis*, whilst those of *C. mirandae* and *C. alvarezii* are green (Vovides et al., 2008). This suggests the possibility of great morphological variation expressed by the species represented by few genes.

The genetic diversity values found in the complex were surprisingly low, especially when compared to other cycad species with similar biology, as well as long-lived perennials with low dispersion and growth rates (Hamrick & Godt, 1989; Hamrick, 2004; González-Astorga, Vovides, Cabrera-Toledo, & Nicolalde-Morejón, 2009). Paired genetic distance values for populations within species are typically 0.1 or less (Crawford, 1983). However, the range of genetic distance values between species within the *C. norstogii* complex showed a high level of similarity (0.007 and 0.020) which contrasted significantly with values usually found for other genera (0.33) reported by Gottlieb (1981).

In general, cycads present low observed heterozygosis, low genetic diversity and low differentiation between populations, except in *Cycas guizhouensis*, where differentiation was found to be high (Long-Qian et al., 2004). Additionally, Sharma et al. (1998), based on genetic distances, found that species in the *Macrozamia pauli-guilielmi* complex were similar. Van Der Bank et al. (2001) had also reported recent divergence in nine species of *Encephalartos* based on genetic distances; and Sharma et al. (1999) reported similar results for the *Macrozamia heteromera* complex.

Pérez-Farrera et al. (2004) proposed that this species complex might probably have arisen due to a speciation process that assumes a constant evolution rate and allopatric speciation (Grant, 1985), a hypothesis based on Wright (1943) theory of "isolation by distance". According to this hypothesis, populations within a taxon with a continuous distribution pattern, may undergo genetic differentiation proportional to the geographical distances between its populations. Keppel et al. (2002) reported geographic distance proportional to genetic distances in *Cycas seemannii* using 13 enzyme systems. However, this appears not to be the case for the *C. norstogii* complex, where geographical distance was found inversely

proportional to genetic distance, but nevertheless seems to indicate a speciation process.

In a morphological and anatomical study performed on the same populations, Pérez-Farrera, Vovides and Avendaño (2014), showed an ecological speciation process related to altitude differences and in this study is in accordance with the morphology of the taxa (Pérez-Farrera et al., 2014). The leaflet anatomical data indicated that the best discriminators were the palisade mesophyll and perivascular fibers, also to a lesser extent the abaxial epidermis, macrolumen cells and the adaxial cuticle. These results indicated that *Ceratozamia norstogii* is most distant to *C. chimalapensis*, and is in agreement with the gross morphological data, and is nearest to *C. mirandae,* and, to a lesser extent, *C. alvarezii* (Pérez-Farrera et al., 2014).

It is pertinent to mention that in the morphological anatomical study (in Pérez-Farrera et al., 2014) the individuals used were also cultivated under similar conditions in the Francisco Javier Botanic Garden (Xalapa, Veracruz), for over ten years thus eliminating phenotypic plasticity. Some studies appear to demonstrate that natural selection operates through ecological gradients and could be more important than geographical isolation in some taxa (Schneider, Smith, Larison, & Moritz, 1999). For example, Bruschi, Vendramin, Bussati and Grossoni (2003) found relationships between molecular and climatic factors due to altitudinal effects in populations of *Quercus petrea.* Dudley (1978) found six morphological characters to vary along an elevation gradient in the Melastomataceae in Peru and suggested that pleiotropy was involved.

The genetic similarity between the species may be due to a single gene mutation influencing multiple and possibly unrelated phenotypic traits (pleiotropy), not detected by the genetic analysis in our case, but nevertheless conferring selective advantage or adaptation to the species at differing altitudes. Better resolution may be obtained in this respect when microsatellites have been developed for the genus. Thus, the number of genes involved in the speciation process is further complicated by pleiotropy due to a single gene that has multiple effects (Adam, Morris, Pandey, & Schwarzbach, 2005). Factors such as wind, rainfall, humidity, and edaphic conditions such as drainage, mineral content of soils, especially phosphate content, soil temperature and solar radiation, could be selective pressures that determine adaptive radiation (Dudley, 1978). All the species within the *C. norstogii* complex are sympatric with *Quercus* spp. with close geographic distances but differing elevation.

Our results showed low genetic differentiation and diversity within the *C. norstogii* species complex, and also a correlation that is inversely proportional between geographic distance and genetic distance. The Fst values suggested that species with greater geographic distance are genetically closest in spite of morphological and leaflet anatomical differences found by Pérez-Farrera et al. (2014); for example *C. alvarezii* a “dwarf” highly branching species is separated from *C. chimalapensis* a “robust” rarely branched species by about 79 km. It appears that species of the *C. norstogii* complex probably emerged from an allopatric speciation process resulting from recent and ongoing divergence processes due to drift stemming from founder effects, resulting in alleles from the original populations becoming fixed in most of the surviving populations. This has been reported in several species of cycads, for example *Cycas debaoensis* (Qing-Qing, Wang, Gong, & Peng, 2011); *C. taitungensis* (Huang, Chiang, Schaal, Chou, & Chiang, 2001); *Dioon angustifolium* (González-Astorga et al., 2005); *D. edule* (Octavio-Aguilar, González-Astorga and Vovides (2009); *D. sonorense* (González-Astorga et al., 2009). A possible contribution to inbreeding could be that the cycad beetle pollinators do not fly great distances (Norstog, 1987; Norstog & Fawcett, 1989).

In this study, we put forward the hypothesis that the four species within the *Ceratozamia norstogii* complex, despite the low genetic variability between them, probably have originated from a genetically depauperate ancestor through an allopatric speciation process, resulting from recent divergence due to genetic

drift via founder effects. In spite of low genetic differentiation, with both morphological and anatomical differences, we considered that the analyzed taxa and related species are still in the process of differentiation.

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RESUMEN

Variación genética en especies de cícadas estrechamente relacionadas en *Ceratozamia* (Zamiaceae: Cycadales) mediante el uso de RAPDs. Los cuatro taxa que componen el complejo *Ceratozamia norstogii* de especies en el sur de México están estrechamente relacionados y se dan en hábitats similares (bosque de *Quercus*). Todos tienen folíolos linear-lanceolados con gran similitud entre ellos, sobre todo en las etapas juveniles, pero se diferencian con la edad. Ha habido un debate en relación con la delimitación de especies debido a la pérdida de caracteres en especímenes de herbario. Los objetivos de este estudio son determinar la variación genética y medir la similitud genética entre los cuatro taxones en el complejo. Las poblaciones estudiadas están en; Cintalapa, Chiapas para *C. alvarezii* y *C. norstogii*, la Sierra Atravesada, Oaxaca para *C. chimalapensis* y Villa Flores, Chiapas para *C. mirandae*. Se tomaron muestras de una población de cada taxón (sólo una población es conocida para *C. alvarezii*) 11-15 individuos adultos elegidos al azar fueron muestreados. Veintiocho primers fueron probados, de los cuales cinco fueron polimórficos mediante la técnica RAPD's. Los datos fueron analizados utilizando métodos bayesianos. Los resultados revelaron baja diversidad genética y la diferenciación encontrada entre las especies sugiere una divergencia reciente. Un estudio morfológico y anatómico anterior en el complejo encontró que los taxa son distintos. Sin embargo, los resultados del presente estudio han demostrado que el complejo *C. norstogii* aun se encuentra en un proceso de divergencia, probablemente a través de deriva genética y efectos de fundador.

Palabras clave: complejo de *Ceratozamia norstogii*, Zamiaceae, México, variación genética, especiación, efecto fundador, cícadas.

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APPENDIX 1

Voucher and botanic garden (JBC) living collections accessions information for the taxa used in this study. Voucher specimens have been deposited and other vouchers examined in the following herbaria: CAS = California Academy of Sciences, San Francisco, USA; CHAPA = Colegio de Postgraduados, Chapingo, Mexico; CHIP = Instituto de Historia Natural, Tuxtla Gutiérrez, Mexico; F = Field Museum of Natural History, Chicago, USA; FTG = Fairchild Tropical Botanic Garden, Miami, USA; HEM = Universidad de Ciencias y Artes de Chiapas, Tuxtla Gutiérrez, Mexico; MEXU = Universidad Nacional Autónoma de México, Mexico City; MO = Missouri Botanical Garden, Saint Louis, USA; UAMIZ = Universidad Autónoma Metropolitana, Iztapalapa, Mexico City; XAL = Instituto de Ecología, A.C., Xalapa, Mexico.

Ceratozamia alvarezii Pérez-Farr., Vovides & Iglesias: MEXICO. CHIAPAS: Cintalapa, *M .A. Pérez-Farrera 889 d* CHIP, MEXU, MO, JBC accession 1996-012; *M. A. Pérez-Farrera 1260* XAL, JBC accession 1996-061, 064; *M. A. Pérez-Farrera 64, 67* CHIP. Other vouchers examined: *Breedlove 70956, 60309* CAS, *Castillo-Hernández 624, 445* CHIP.

Ceratozamia chimalapensis Pérez-Farr. & Vovides: MEXICO, OAXACA, Chimalapa, *M. A. Pérez-Farrera 2622* HEM, JBC accession 2002-006, 007. Other vouchers examined: *E. H. Xolocotzi & A. J. Sharp X-1277* MEXU.

Ceratozamia mirandae Vovides, Pérez-Farr. & Iglesias: MEXICO, CHIAPAS, Villaflores, *R. De La Cruz 66* CHIP; *De la Cruz. R.* 20, 24, *76* CHIP, XAL, MEXU; *M. A. Pérez Farrera 26[a], 37, 126, 129, 163, 352, 465* CHIP, JBC accession 1993-055. *A. P. Vovides 1261* XAL, JBC accession 1995-154. Other vouchers examined: *A. R. Lopez, F. A. Espejo & A. Flores 507* UAMIZ; *J. J. Castillo Hdez 230, 548, 595* CHIP; *Chamberlain s.n.* (F); *S. K. Kiem s.n.* FTG; *J. Watson s.n.* FTG; *Breedlove 23999* CAS; *U. Bachem & C. Ricardo Rojas 819* CHAPA.

Ceratozamia norstogii D.W. Stev.: MEXICO, CHIAPAS; Cintalapa, *M. A. Pérez-Farrera,. 71*, *775* (CHIP); *A .P. Vovides, 1230, 1233, 1237,* XAL, JBC accession 1993-008, 011, 012. Other vouchers examined: *E. Palacios 375* CHIP; *Breedlove 4431* CAS; *Breedlove & Smith 21813*, *Breedlove 24709* (CAS).