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MORPHOLOGY AND LEAFLET ANATOMY OF THE *CERATOZAMIA NORSTOGII* (ZAMIACEAE, CYCADALES) SPECIES COMPLEX IN MEXICO WITH COMMENTS ON RELATIONSHIPS AND SPECIATION

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Premise of research. Many *Ceratozamia* species are closely related and form species complexes with great similarity among the component taxa, especially in juvenile stages. This, coupled with character loss in herbarium specimens, has resulted in debate regarding recognition and validation of the species. This study, focused on the four species comprising the *Ceratozamia norstogii* species complex from a morphological and leaflet anatomy viewpoint, was undertaken to clarify relationships among the four taxa in the complex.

Methodology. For morphological variation, 29 individuals from one population of each of the four species was sampled for nine vegetative and six reproductive variables. For leaflet anatomical variation, leaflets were taken from five adult plants of each species held under cultivation for >5 yr under uniform conditions. Cross sections of leaflets and cuticular peels were obtained using standard plant histological techniques and examined under bright-field light microscopy. Cross sections of leaflet anatomy and cuticular features are described. Both morphological and anatomical variables were analyzed by multivariate statistical methods taking habitat information into account, especially elevation.

Pivotal results. Consistency between anatomical and morphological data among the species was found. The multivariate spacing for the four species showed no overlap.

Conclusions. The four taxa comprise distinct species. Effects of ecological gradients, especially distinct differences in elevation between the populations rather than geographical distances, appear to explain the morphological and leaflet anatomical differences found between the species, which supports a scenario for an ecological speciation process in the *C. norstogii* complex.

Keywords: adaptive radiation, *Ceratozamia*, cycads, morphological variation, ecological speciation.

Introduction

Ceratozamia is a Neotropical cycad genus of 27 species (Osborne et al. 2012), the majority endemic to Mexico. It can be identified among other cycads by morphological characters such as entire leaflets and two horns present on the sporophylls of both male and female strobili, from which the genus name was derived (horned *Zamia*). The rachis presents two parallel grooves or depressions running along the adaxial surface at the level of the leaflet articulations.

There is debate regarding recognition and validation of some species (Stevenson et al. 1986; Norstog and Nicholls 1997; Whitelock 2002) due to many being closely related and forming species complexes (Moretti et al. 1980; Vovides et al. 2004). Their similarity in appearance, especially in the immature and juvenile stages, adds to difficulty in identification (Norstog and Nicholls 1997; Whitelock 2002). Other problems that add to the confusion are character loss when herbarium specimens are processed (Pérez-Farrera 2005; Vovides et al. 2012) and the fact that many herbarium vouchers are sterile or have been prepared from juvenile plants (Vovides et al. 1983). There are also problems with accessibility to some remote populations, and phenology is unpredictable for some species (Pérez-Farrera 2005). Finally, along their distribution range several populations of *Ceratozamia* either have not been delimited or constitute species complexes that may have arisen from introgressive hybridization (Johnson 1963; Vovides et al. 2004).

The *Ceratozamia norstogii* D.W.Stev. complex is characterized by narrow linear to linear-lanceolate leaflets and comprises *Ceratozamia norstogii*, *Ceratozamia mirandae* Vovides, Pérez-Farr. & Iglesias, *Ceratozamia alvarezii* Pérez-Farr., Vovides & Iglesias, and *Ceratozamia chimalapensis* Pérez-Farr. & Vovides, all endemic to Mexico. *Ceratozamia norstogii*, *C. mirandae*, and *C. alvarezii* are from the northern part of the

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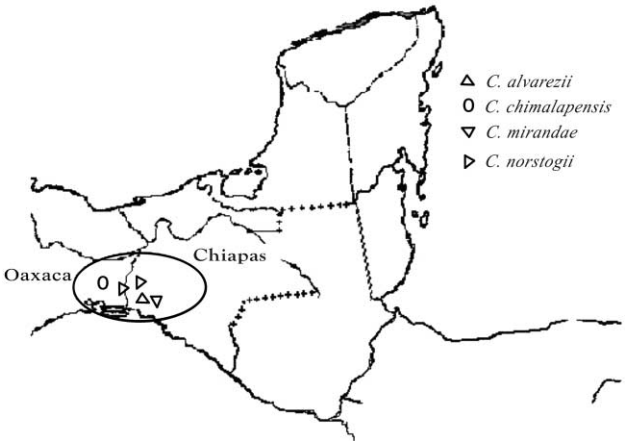

Fig. 1 Geographic distribution of *Ceratozamia norstogii* complex in southern Mexico.

Sierra Madre de Chiapas, and *C. chimalapensis* is from the Sierra Atravesada of Oaxaca. Detailed descriptions can be found in Stevenson (1982), Pérez-Farrera et al. (1999), and Vovides et al. (2001, 2008). It is suspected that some intraspecific variation exists among the four taxa, obscuring especially the boundary between *C. mirandae* and *C. chimalapensis* (Pérez-Farrera et al. 2004).

The general leaflet anatomy of the genus consists of hypostomatic leaflets with stomatal bands alternating with nonstomatal interbands corresponding to veins. Mucilage canals are inconspicuous and are not associated with the vascular bundles but rather are dispersed among the mesophyll tissue. Consistent with most other Zamiaceae, *Ceratozamia* has an epidermis consisting of two basic types of cell: (*a*) empty cells with thick walls and (*b*) smaller living cells with thin walls arranged in uniseriate rows (Pant and Nautiyal 1963), which is distinctive of *Ceratozamia*. The aim of this study is to determine the morphological and leaflet anatomical ranges along elevation gradients to improve our understanding of the relationships in this complex.

Habitat Notes

The habitats of *C. norstogii*, *C. mirandae*, and *C. alvarezii* are oak and pine/oak forests, with some elements of cloud forest in the *C. mirandae* localities. These three species share a narrow and overlapping elevation range: 950 m for *C. alvarezii*, 800–1600 m for *C. norstogii*, and 910–1300 m for *C. mirandae* (Pérez-Farrera et al. 1999, 2001; Vovides et al. 2001). *Ceratozamia chimalapensis* is from the lower elevation range of 270–1000 m in oak forest habitat. It is associated with cloud forest elements, such as *Liquidambar*, *Nectandra*, *Elaphoglossum*, and *Begonia*, as well as elements common to more tropical forest environments, such as *Cecropia* and *Bursera*.

Material and Methods

Taxon Sampling for Morphological Variation

One population from each species of the *Ceratozamia norstogii* complex was sampled, with 29 randomly chosen adult individuals measured from one of the two populations of the *C. norstogii* population, 20 from one of the two populations of *C. alvarezii*, 30 from one of the three populations of *C. mirandae*, and 20 individuals from the only known population of *C. chimalapensis* (fig. 1). *Ceratozamia norstogii*, *C. mirandae*, and *C. alvarezii* are from the Sierra Madre de Chiapas, whereas *C. chimalapensis* is from the Sierra Atravesada of Oaxaca. The number of individuals chosen for sampling per population was limited to the number of adults present. One measurement per variable per individual was taken from a total of 99 individuals for 15 morphological variables (9 vegetative and 6 reproductive), all measurements being taken in situ (table 1). For the trunk perimeter measurement, the greatest value was taken (middle part of trunk); leaflets were chosen from the midportion of mature leaves, and measurements were taken from the midportion of the leaflet of linear leaflets and the widest portion of linear-lanceolate leaflets (slightly off-center); micro- and megasporophylls were chosen from the midportion of mature cones (fig. 2). Criteria for selecting these populations were accessibility and health of each population, showing minimum disturbance

Table 1

Morphological Variables Used for Analysis of Species in the *Ceratozamia norstogii* Complex

No.	Character	Character (linear measurement in cm)
1	LARGTR	Trunk length
2	PERMTR	Trunk perimeter
3	NHOJA	Leaves (no. per crown)
4	LARGOPEC	Petiole length
5	LARGORAQ	Rachis length
6	NFOLIOL	Leaflets (no. per leaf)
7	LARGOFOL	Leaflet length
8	ANCHOFOL	Leaflet width
9	NVENAS	Veins (no. per leaflet)
10	LARGOMICRO	Microsporophyll length
11	ANCHOMICRO	Microsporophyll width
12	ANCHOMEGAS	Megasporophyll distal face width
13	LARGOMEGAS	Megasporophyll distal length
14	DIAMSEMI	Seed diameter
15	LARGSEMI	Seed length

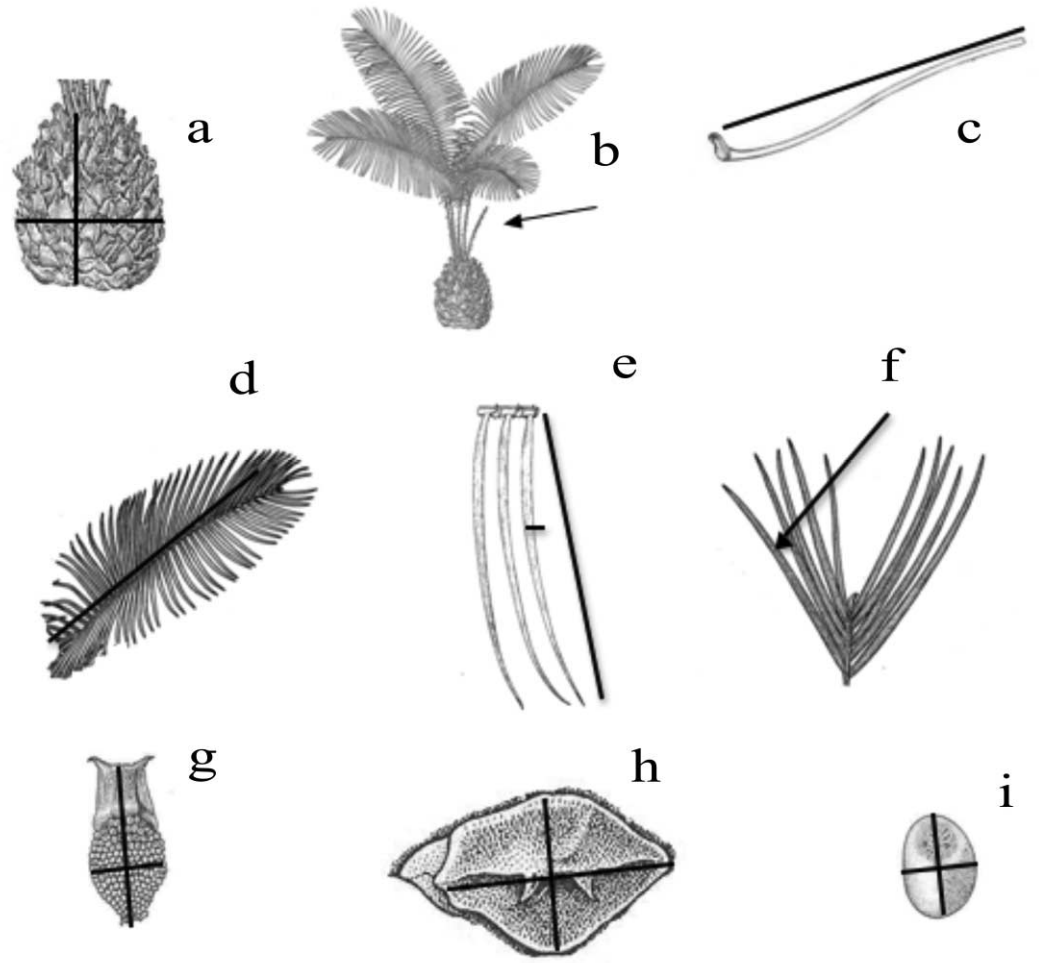

Fig. 2 Position on plant organs where measurements were taken for morphological data analysis of the *Ceratozamia norstogii* species complex: *a*, length and perimeter of trunk; *b*, leaf number; *c*, petiole length; *d*, rachis length; *e*, leaflet length and width; *f*, distance between veins; *g*, length and width of microsporophyll; *h*, length and width of megasporophyll face; *i*, length and diameter of seed (without fleshy sarcotesta).

and good regeneration. Within-plant variation was not analyzed because observation of the same species under uniform greenhouse conditions for >5 yr did not show any phenotypic plasticity. The herbarium vouchers deposited as being representative of the taxa in these populations are listed in the appendix. Measurements were taken with a 3-m flexometer and a digital vernier, and data were transferred to an Excel for Windows 2010 spreadsheet.

The following analyses were performed: (*a*) ANOVA to determine morphological variation of parameters among the species of the complex, (*b*) discriminant analysis to separate two or more groups on the basis of measurements of the variables for each species studied (data were transformed to natural logarithms, and Mahalanobis distances were obtained), and (*c*) correlation analysis between elevation and leaflet length. All data were analyzed using the programs Statistica (ver. 8) and Statgraphics (ver. 5.1) for Windows. Missing data, such as male/female characters, were handled by the mean substitution option of the program. The analysis was focused to evaluate differences among species only, considering sex to be independent at this level.

Taxon Sampling for Leaflet Anatomical Variation

Five healthy adult plants of each species held in the Mexican National Cycad Collection of the Jardín Botánico Francisco Javier Clavijero cultivated between 5 and 10 yr under uniform conditions were chosen for anatomical sampling. The median part of fresh leaflet tissue (~1 cm) was taken from the median part of mature leaves from five replicate individuals of each taxon. The following transverse sectional (TS) measurements were taken on 25 randomly chosen replicates of each cell or tissue type (character) for each of the five leaflets sampled per taxon: thickness of adaxial and abaxial cuticle (measurement taken from the top anticlinal limit of the epidermal cell, where the cuticle is thinnest), TS dimensions of adaxial and abaxial epidermal cells, TS dimensions of epidermal cells with macrolumen, height and width of palisade mesophyll cells, TS di-

Table 2

Eleven Leaflet Anatomical Variables Used for Discriminant Analysis

Variable abbreviation	Transverse sectional measurements (μm)
PE_L	Palisade mesophyll cell height
PE_A	Palisade mesophyll cell width
CutAd_gr	Adaxial cuticle thickness
EAb_L	Abaxial epidermal cell height
CEAd_L	Adaxial epidermal cell height
BUL_L	Macrolumen cell height
FPV_L	Perivascular fiber height
FIV_L	Intervascular fiber height
FIV_A	Intervascular fiber width
NoFPV	No. perivascular fibers
NoFIV	No. intervascular fibers

mensions and number of perivascular and intervascular fibers, and diameter and number of mucilaginous canals (table 2). Vouchers are deposited at XAL and HEM, with JBC accession numbers listed in the appendix.

Sectioning, Staining, and Slide Preparation

TS were taken with both hand microtome and sliding microtome, and the sections were suspended in distilled water. The best sections were selected and subjected to histochemical staining with phloroglucinol-HCl for lignin (Chamberlain 1932) and a mixture of Sudan III and IV for cuticles. Permanent sections were obtained by double staining in safranin and fast green and mounting in Canada balsam (Purvis et al. 1966).

Cuticular Peel Preparation

To expose the cuticle, ~1 cm^2 of the median part of a leaflet from each representative species was taken and macerated in 70% sodium hypochlorite (commercial bleach) until digestion of mesophyll and other lignified tissues. Peels were stained with 1% aqueous Bismark brown (modified from Purvis et al. 1966) for 10 to 20 min, dehydrated through ethanol stages from 70% ethyl alcohol in increments of 10% followed by two changes

Table 3

Summary of Discriminant Analysis for the Morphological Variables of the *Ceratozamia norstogii* Species Complex

Derived function	Wilks's λ	χ^2	df	*P*
1	.0008463	626.1042	45	.00000
2	.0211933	341.0851	28	.00000
3	.229245	130.3573	13	.00000

in absolute ethyl alcohol, cleared for 2–3 min in methyl salycilate, and mounted in Canada balsam (Purvis et al. 1966). The abaxial epidermal cuticle only has been described, and a digital camera was used for microphotography. Stomatal indexes were calculated from one individual for each species with five replicates. The stomatal index is expressed by

$$\text{SI} = \frac{\text{no. stomata}}{\text{no. stomata} + \text{no. epidermal cells}} \times 100.$$

Maceration of Leaflet Tissue

To observe whole fibers, sclereids, and so on, fine slivers were taken from the median part of a leaflet and macerated by boiling in equal parts hydrogen peroxide (20 vol.) and glacial acetic acid using the reflux method by means of a Liebig condenser under a fume hood (according to Purvis et al. 1966) for 24 to 40 h. The macerated tissue was washed in water for several hours, teased apart, and stained with safranin for observation.

Analysis

For discriminant analysis of the anatomical data, Statgraphics (ver. 5.1) for Windows 2007 was used. This procedure is designed to develop a set of discriminant functions that can be used to classify the operational taxonomic units (OTUs) on the basis of quantitative values. A further a posteriori analysis was done to test the robustness of the classification by dividing each of the four populations into two randomly generated

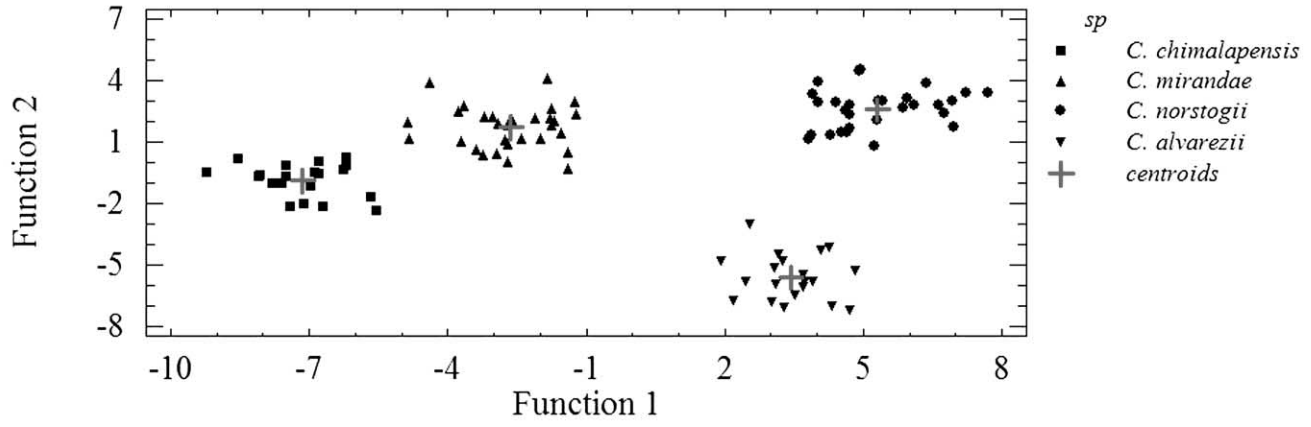

Fig. 3 Discriminant functions derived from the analysis of 15 morphological variables in the *Ceratozamia norstogii* species complex.

Table 4

Squared Mahalanobis Distances between Species (below Ellipses) and *F* Values (above Ellipses) for the Morphological Variables of the *Ceratozamia norstogii* Complex

	C. alvarezii	*C. chimalapensis*	*C. norstogii*	*C. mirandae*
C. alvarezii	...	75.04	92.66	50.26
C. chimalapensis	80.97	...	73.7965	170.13
C. norstogii	49.56	114.26	...	142.71
C. mirandae	62.23	33.76	61.73	...

Note. For all cases, df = 15.80 and $P < 0.001$. Underscoring indicates highest and lowest Mahalanobis distances (see text).

subsets of equal size and one subset chosen at random for a first discriminant analysis, and the functions of this analysis were used to classify the second subset. Eleven anatomical predictive variables were introduced. Wilks's λ test and Mahalanobis squared distances were used to determine significance levels. Two-discriminant functions with $P \leq 0.05$ were considered statistically significant at the 95% confidence level for the anatomical variables. A one-way ANOVA and correlation analysis was done on stomatal index versus elevation (data not shown).

Results

Morphological Data Analysis

Of the 15 variables analyzed, 14 showed highly significant differences ($P < 0.0001$) of the means between species; only the seventh variable, microsporophyll length, did not. *Ceratozamia mirandae* and *Ceratozamia chimalapensis* are the more morphologically robust species in that they present single longer trunks with a greater diameter and higher number of leaves, while *Ceratozamia alvarezii* is the smallest of the complex by having small branching trunks with few leaves per crown.

The data derived from the discriminant functions shows that the four species separate along two spatial axes with no overlap between groups (fig. 3). All OTUs were 100% correctly classified with no overlap, and Wilks's λ test was highly significant ($P < 0.0001$) for the three factors (table 3). These results were confirmed by the a posteriori robustness of the classification test applied to the discriminant analysis (data not shown). The Mahalanobis distance results suggest that *C. mirandae* is very close to *C. chimalapensis* morphologically (table 4). The farthest quadratic Mahalanobis distance was between the *Ceratozamia norstogii* and *C. chimalapensis* populations. Of the 15 variables that included standardized canonical discriminant functions, only two showed the highest value for factor 1 (leaflet width and megasporophyll face length). For factor 2, the highest values were megasporophyll face width and megasporophyll face length. For factor 3, the highest values were number of leaves and trunk perimeter, and the first canonical variable accounts for >60% of the morphological variation (table 5). The correlations for all variables measured showed differences among species and the reproductive variables; seed length and megasporophyll face length were the most prominent between species. The correlation analysis (fig. 4) between leaflet

Table 5

Standardized Discriminant Functions for Each of the Morphological Factors in the *Ceratozamia norstogii* Species Complex

		Factor		
No.	Variable	1	2	3
1	ANCHOFOLIO	.8189	−.0193	.5304
2	ANCHOMEGAS	.2046	−.6229	−.5817
3	ANCHOMICRO	.1129	−.0019	−.4143
4	DIAMSEMILL	−.0712	.1424	−.1588
5	LARGOFOLIO	−.3268	.3553	.0563
6	LARGOMEGAS	.5157	.5169	−.0145
7	LARGOMICRO	.1851	−.1008	.0871
8	LARGOPECIO	.1528	−.0549	.1362
9	LARGORAQUI	−.1290	−.0288	−.2967
10	LARGOSEMIL	.1536	.3429	−.0484
11	LARGOTRONC	.2876	−.0220	.4364
12	NFOLIOLOS	.1190	.0539	.6567
13	NHOJA	.2124	−.2645	.7287
14	NVENAS	.1503	−.1403	−.4789
15	PERIMTRONC	.1497	.4764	−.7627
	Eigenvalue	24.0420	9.8169	3.3621
	% relative	64.5900	26.3700	9.0300
	Canonic correlation	.9798	.9527	.8779

Note. Underscoring indicates highest variance values.

Table 6

Standardized Discriminant Functions for Each of the Leaflet Anatomical Characters in the *Ceratozamia norstogii* Species Complex

	Factor		
Variable	1	2	3
EAb_L	.0704	1.2669	−.2205
Bul_L	.4031	−1.7343	.3089
CutAd_gros	.5188	.7540	1.1540
CEAd_L	−.7945	1.5686	−.8755
PE_L	−.5194	−.0582	.4993
PE_A	1.2531	.2886	−.2884
FPV_L	−.0627	−.6222	.4069
FIV_L	3.1859	−2.8284	1.6821
FIV_A	−2.1803	4.0220	−1.2013
NoFPV	−.0564	−.2445	−.2787
NoFIV	.7666	−2.1254	.4892
Eigenvalue	41.45	17.16	2.01
% relative	68.38	28.31	3.31
Canonic correlation	.9882	.9721	.8169

Note. Underscoring indicates highest variance values.

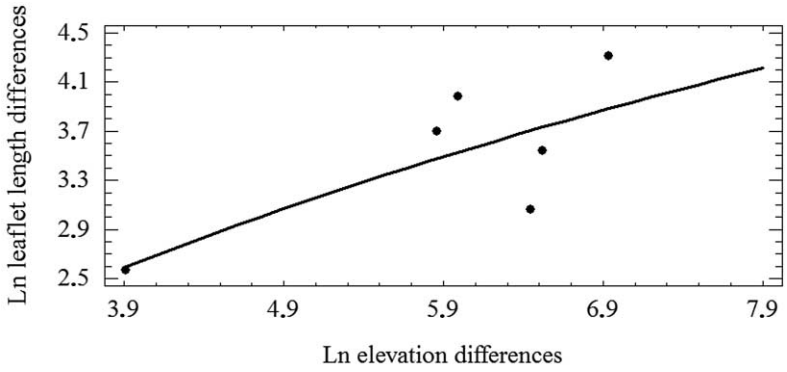

Fig. 4 Correlation between leaflet length and elevation between species of the *Ceratozamia norstogii* species complex ($\ln Y = 1/(0.93 + 1.144/\ln X)$, $R^2 = 66.88$, $R = 0.82$, $F = 8.08$, $P = 0.04$).

length and elevation gave a significant positive correlation; r^2 explained 77% of the variability in leaflet length versus elevation, and the correlation coefficient (0.82) indicated a moderately strong relationship between the two variables.

Leaflet Anatomy Analysis

The anatomical variables that best discriminated between species complex members were palisade mesophyll cell width and intervascular fiber TS height. The first factor accounted for 68.4% of the variance, and the second factor accounted for 28.3% of the variance (table 6). The sum of the two factors explained 96.7% of the variance of all of the data.

The dispersion derived from the discriminant functions of the leaflet anatomical variables of the *Ceratozamia norstogii* complex indicated that all OTUs were 100% correctly classified (fig. 5). The four species show well-separated scatter clouds with no overlap between groups. Wilks's λ test was highly significant ($P < 0.0001$) for two of the three factors (table 7), and the squared Mahalanobis distances between species were also significant ($P < 0.01$) for all cases (table 8). The ANOVA for differences in stomatal index was not found to be significant ($F = 2.91$, $P = 0.16$); however, the correlation coefficient (−0.65) showed an observed pattern between the variables, and the r^2 value indicated that 42.1% of the variation was accounted for by the stomatal index.

Anatomical Description of Leaflet TS and Abaxial Cuticular Peels (Figs. 6–8)

All species show a thicker adaxial cuticle than the abaxial—the thickest in *C. mirandae* (2.5–13 μm) and the thinnest in *C. alvarezii* (2.5–5 μm)—except when it is overlying the thin-walled epidermal cells, where it can be up to 13 μm thick (figs. 9, 10); the abaxial cuticle thickness is even throughout the species (2.5 μm). The adaxial epidermal cells are isodiametric to oblong in TS, and walls are lignified with thick anticlinal and periclinal walls, with the periclinal wall adjacent to the cuticle being the thickest. *Ceratozamia alvarezii* showed the thickest epidermal cell wall (7.5–10 μm), and *C. mirandae*

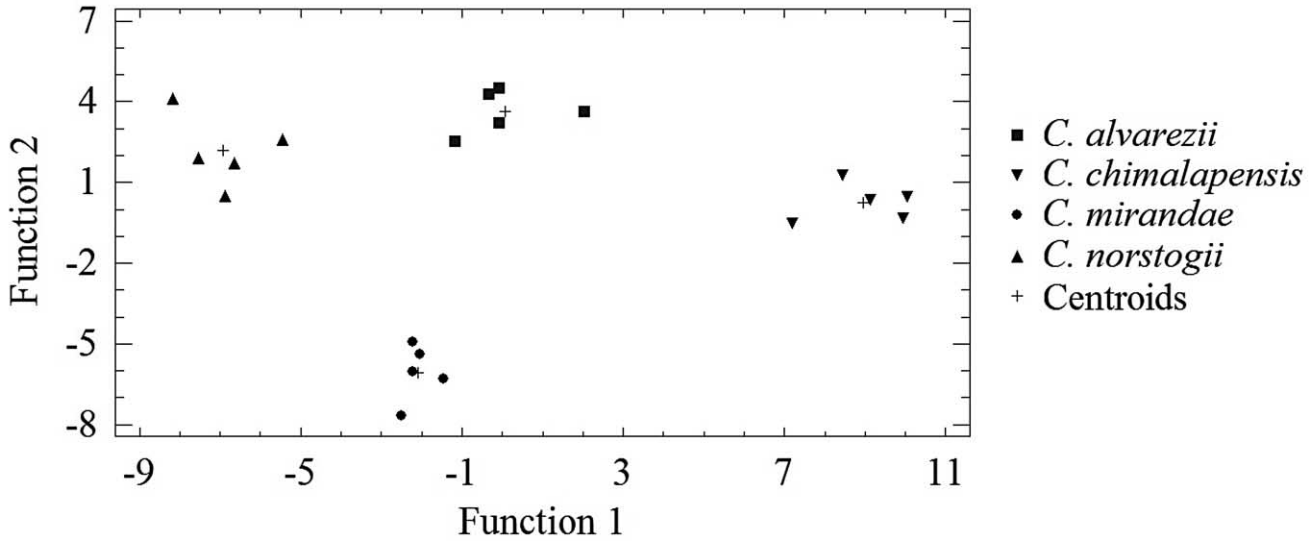

Fig. 5 Discriminant functions derived from the analysis of the 11 leaflet anatomical variables of the *Ceratozamia norstogii* species complex.

Table 7

Summary of the Discriminant Analysis of the Leaflet Anatomical Variable of the *Ceratozamia norstogii* Complex

Derived function	Wilks's λ	χ^2	df	*P*
1	.00043	89.0988	33	.0000
2	.01833	45.9943	20	.0008
3	.33272	12.6552	9	.1788

showed the thinnest (2.5 μm). Hypodermis is generally absent, but when present (in *C. alvarezii* and *C. norstogii*) it is discontinuous and consists of isodiametric to oblong thick-walled sclerenchymatous fibers except at the leaflet subrevolute margins; there it is continuous, with one to two layers of fibers in *C. alvarezii* and *C. norstogii* and up to three layers in the remaining two species, only the innermost layer being discontinuous. Girder sclerenchyma is absent except in *C. norstogii*, where it is only incipient (fig. 6). The palisade mesophyll consists of one layer of oblong cells, the tallest and widest in *C. chimalapensis* (52–105 × 27–53 μm) and the shortest and narrowest in *C. mirandae* (43–90 × 20–25 μm). The vascular bundles are surrounded with thick-walled lignified perivascular fibers in all species and range from three to seven in *C. alvarezii* and from four to nineteen in *C. mirandae*. The spongy mesophyll shows one to two mucilaginous canals and one to six acicular thick-walled lignified intervascular fibers interspersed between adjacent vascular bundles in *C. alvarezii*, one to three mucilaginous canals and one to seven acicular thick-walled intervascular fibers in *C. chimalapensis* and *C. mirandae*, and two to three mucilaginous canals and one to six thick-walled lignified acicular intervascular fibers in *C. norstogii*. The abaxial epidermis consists of lignified epidermal cells that are variable in TS from isodiametric to oblong, ranging from 18–45 × 13–30 μm for all species (excluding cuticle), and is interspersed with larger epidermal cells with macrolumens (fig. 11), the greatest being 35–70 × 30–50 μm in *C. chimalapensis* and the least being 28–53 × 25–40 μm in *C. mirandae*. We do not know the function of these macrolumen cells.

Cuticular peels of the abaxial epidermises of all the species (fig. 8) show distinct wide stomatal bands, the narrowest in *C. alvarezii* not exceeding 670 μm wide and the widest in *C. norstogii* exceeding 1000 μm wide, and narrower nonstomatal interbands, the narrowest in *C. alvarezii* not exceeding 200 μm wide and the widest in *C. mirandae* exceeding 400 μm wide. The stomatal bands show long oblong to spindle-shaped cells linearly aligned with the leaflet veins, with abutting end walls straight to sinuous when not overlapping. The cells of the stomatal bands are shorter and wider than those of the interband, irregularly angular, square, and oblong to wedge shaped, with abutting end walls straight to sinuous when not overlapping, giving a nonlinear appearance. Both areas show uniseriate rows of short, narrow, highly cutinized cells with the cuticular outline appearing thicker than the rest of the epidermal cells (fig. 7). These rows range from one to six cells in line in *C. chimalapensis* and *C. mirandae*, one to nine in *C. alvarezii*, and one to twelve in *C. norstogii*, and sometimes there is overlapping of adjacent rows toward their ends in *C. norstogii*. These cells correspond to the living cells containing cytoplasm with thin cellulose walls and are seen to be heavily cutinized in TS owing to the anticlinal cuticular flanges reaching deep between adjacent epidermal cells (figs. 9, 10). The guard cells (long axis) run parallel to the leaflet veins and epidermal cells. The stomata are generally surrounded by one ring of recognizable subsidiary cells and can be termed monocyclic (Florin 1951; Pant and Mehra 1964), although some stomata in *C. chimalapensis* appear to be amphicyclic in that there are two rings of subsidiary cells (fig. 8*B*), but this must be taken as a tentative observation until a wider exploration is made. Calcium oxalate druses were present in the leaflet tissues of *C. norstogii* but were found to be rare in the remaining species.

Discussion

Morphological Data Analysis

Ceratozamia alvarezii has the longest and narrowest microsporophyll as well as the shortest and narrowest leaves, while *Ceratozamia mirandae* presents the longest trunks with the greatest perimeter and the longest petioles with the greatest diameter in the complex. A perfect separation was found when a partial morphological characterization was done on three species of the complex: *Ceratozamia norstogii*, *C. alvarezii*, and *C. mirandae* (Pérez-Farrera et al. 2004). The morphological data and the squared Mahalanobis distances showed *Ceratozamia chimalapensis* to be close to *C. mirandae*. Both *C. alvarezii* and *C. norstogii* have short and narrow leaflets and share water-stressed environments with frequent fires; the *C. norstogii* habitat is also very windy, owing to its proximity to the Isthmus of Tehuantepec in an area known as "La Ventosa." *Ceratozamia chimalapensis* and *C. mirandae* share more mesic habitats and show longer and wider leaflets than *C. alvarezii* and *C. norstogii*, in agreement with Stevenson et al. (1986);

Table 8

Squared Mahalanobis Distances between Species (below Ellipses) and *F* Values (above Ellipses) for the Leaflet Anatomical Variables of the *Ceratozamia norstogii* Complex

	C. alvarezii	*C. chimalapensis*	*C. norstogii*	*C. mirandae*
C. alvarezii	...	97.93	61.46	100.42
C. chimalapensis	8.35	...	256.4	164.09
C. norstogii	5.24	21.85	...	95.48
C. mirandae	8.56	13.99	8.14	...

Note. For all cases, df = 11.6 and $P < 0.01$. Underscoring indicates highest and lowest Mahalanobis distances (see text).

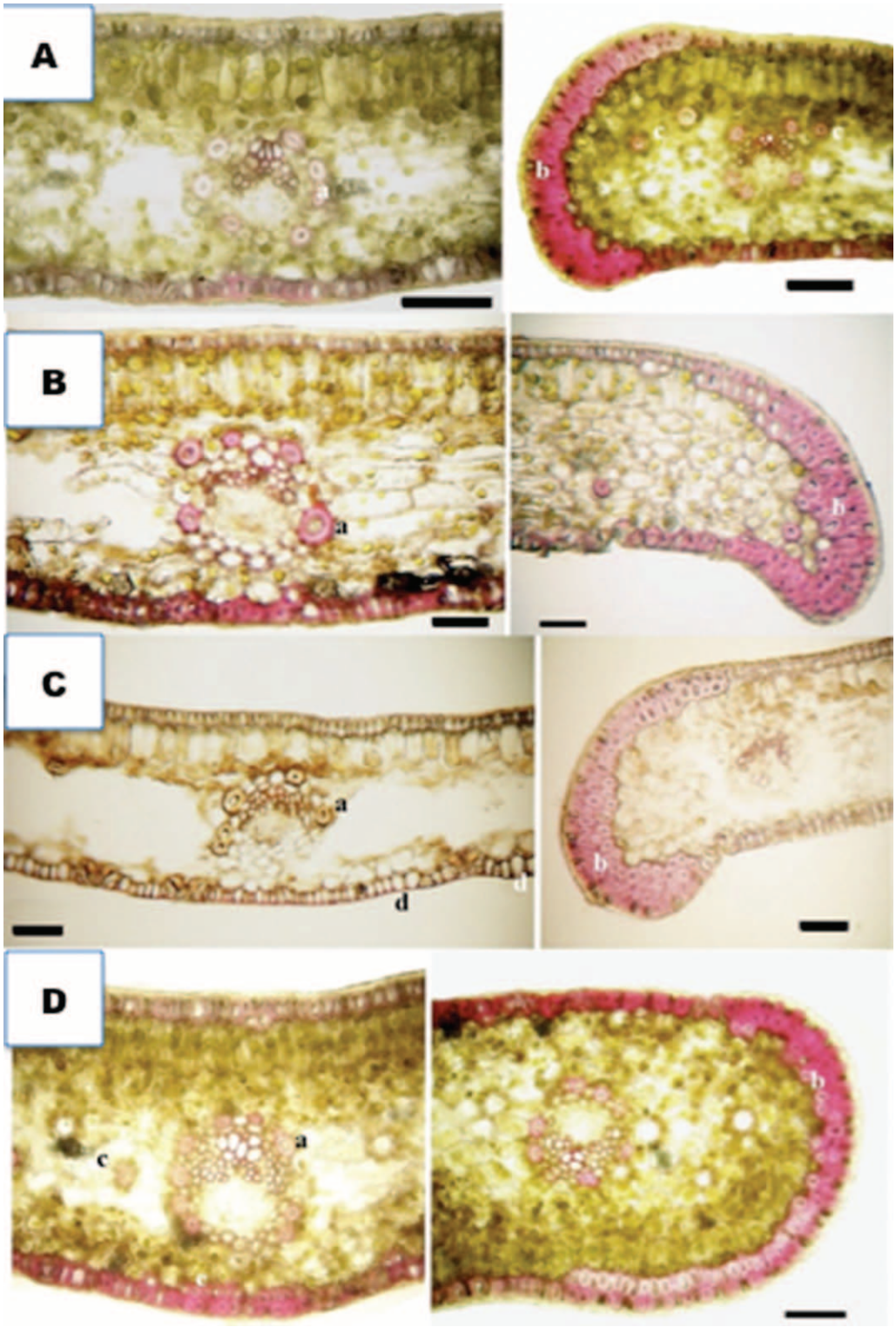

Fig. 6 Transverse sections through midportion of leaflets and subrevolute margins. *A*, *Ceratozamia alvarezii*. *B*, *Ceratozamia chimalapensis*. *C*, *Ceratozamia mirandae*. *D*, *Ceratozamia norstogii*. a = perivascular fibers, b = lignified hypodermis, c = intervascular fibers, d = macrolumen cells, e = slight girder sclerenchyma. All bars = 100 μm.

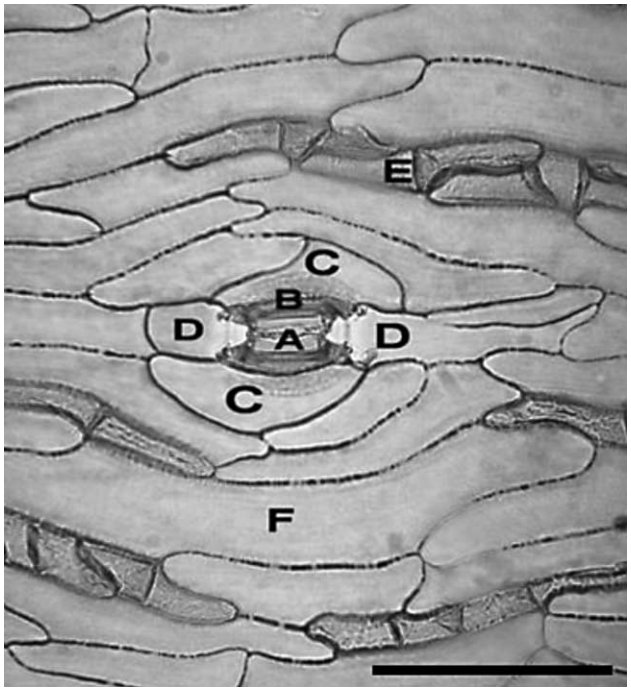

Fig. 7 Detail of stomatal band region of abaxial cuticle of species of the *Ceratozamia norstogii* species complex. A = guard cells, B = cuticular ledge of guard cells, C = subsidiary cells, D = polar subsidiary cells, E = short narrow heavily cutinized cells, F = epidermal cells.

in addition, a strong correlation exists between leaflet length and elevation (fig. 4). These characters do not change when cultivated under uniform conditions in the botanic garden for >10 yr, thus ruling out phenotypic plasticity. We consider that speciation in this species complex is in accordance with an ecological speciation scenario.

The analysis described above clearly shows the morphological differences between species in the *Ceratozamia norstogii* complex. Although some results of the univariate analysis on the morphological data show overlap between OTUs, the multivariate space for the four species shows no overlap whatsoever. This analysis indicates that the taxa analyzed are distinct species. The measurements of leaflet width and length of the megasporophyll face were particularly well represented, and it is not surprising that the differences in forms between these characters emerged as important discriminators between the species.

Leaflet Anatomy Analysis

The discriminant functions of the leaflet anatomical variables clearly show the anatomical differences between the four OTUs (fig. 5). The best discriminators were the abaxial epidermis, macrolumen cells, the palisade mesophyll, perivascular fibers, and the adaxial cuticle. These results indicated that *Ceratozamia norstogii* is most distant to *C. chimalapensis*, in agreement with the gross morphological data, and is nearest to *C. mirandae* and, to a lesser extent, *C. alvarezii*. The differences were in adaxial cuticle thickness, the thickest being presented by *C. mirandae* (mean = 10 μm) and the thinnest being presented by in *C. alvarezii* (mean = 4 μm) and *C. norstogii* (mean = 6 μm). Both *C. chimalapensis* and *C. mirandae* show similarity in the hypodermal fibers of the leaflet margin, which are two to three cell layers thick and lignified (fig. 6*B*, 6*C*). Differences are seen in the palisade mesophyll height and epidermal cell TS, both of which are smaller in *C. mirandae*.

The macrolumen cell TS is greatest in *C. chimalapensis* (mean = 45 μm) and smallest in *C. mirandae* and *C. norstogii* (mean = 40 and 43 μm, respectively). There is a discontinuous one-layered fibrous hypodermis and slight girder sclerechyma present in *C. norstogii* but absent in the other taxa. The habitat of *C. norstogii* is subject to high winds all year round, and the presence of girder sclerenchyma, incipient hypodermis, and narrow canalled leaflets is very likely a reflection of wind stress not present in the other habitats. The cross-sectional diameter of the intervascular fibers is smallest in *C. norstogii* (30 μm) and greater in the other taxa. On the other hand, *C. norstogii* and *C. mirandae* share similarity in TS diameter of peri- and intervascular fibers. The closeness of these OTUs is in accordance with the gross morphological data in that both taxa present channelled leaflets and share similarities in microsporophyll and seed dimensions.

Cuticular peel morphology is similar for the four species in that the common stomatal type is monocyclic. The general epidermal cell shape is similar; however, in *C. norstogii* the epidermal cell cuticles of the stomatal band are visibly wider than in the other three species, some up to two times wide as long (fig. 8*D*). The short, narrow, heavily thickened anticlinal cuticle flanges in both stomatal bands and interbands give the appearance of thick-walled cells. Pant and Nautiyal (1963), in their detailed study of cycad epidermis where epidermal cell walls were described for *Ceratozamia mexicana*, *Ceratozamia brevifrons*, *Ceratozamia kuesteriana*, and *Ceratozamia fuscoviridis*, refer to these short, narrow cells as thin walled and having cell content—confirmed also by Greguss (1968)—that was assimilatory in function compared with that of thick-walled stomatal band epidermal cells. These epidermal cells (as shown in leaflet TS in this study) show the presence of lignin and are considered to provide mechanical strength to the leaflet. The short, narrow, thin-walled cells (sensu Pant and Nautiyal 1963) correspond in this study to the thicker cuticle covering these cells with thick, anticlinal, cuticular flanges penetrating deep between theses cells and adjacent epidermal cells in both stomatal bands and interbands (figs. 9, 10). These thin-walled epidermal cells of assimilatory nature are also present in *Ceratozamia*, *Dioon*, and *Zamia* (Greguss 1968). The stomatal index analysis, weakened by the lack of data on more individuals and repetitions per individual per species, nevertheless shows an observed pattern in the data between stomatal index and elevation. This could be an interesting avenue of research for further studies of the genus.

Pérez-Farrera et al. (2004) proposed that this species complex might have arisen from a speciation process that assumes a constant evolution rate and allopatric speciation (Grant 1985), a hypothesis based on Wright's (1943) theory of "isolation by distance." However, this does not appear to be so in the case of the *C. norstogii* complex, where the elevation gradient re-

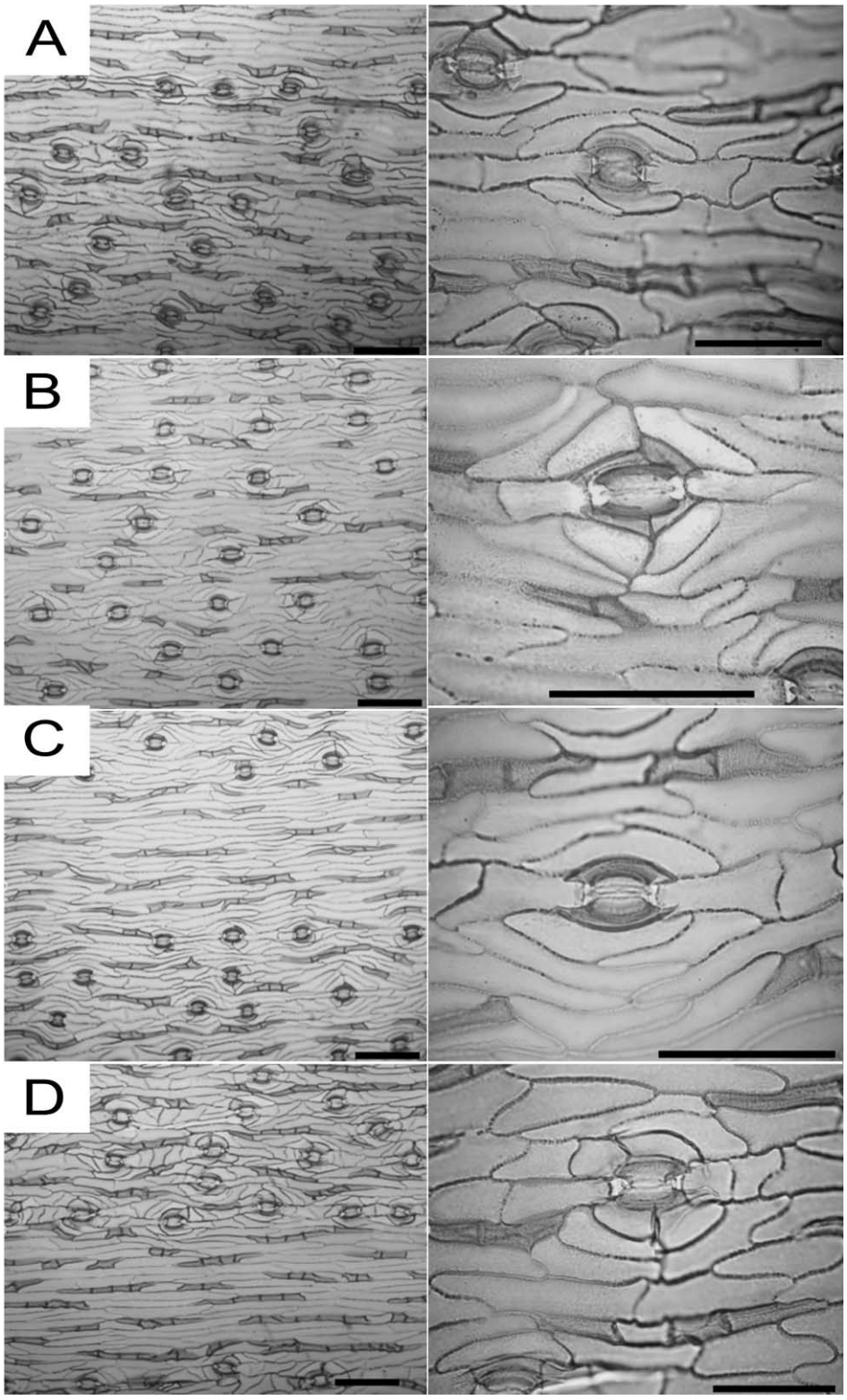

Fig. 8 Cuticles of abaxial leaflet surfaces of the *Ceratozamia norstogii* species complex. *A*, *Ceratozamia alvarezii*. *B*, *Ceratozamia chimalapensis*. *C*, *Ceratozamia mirandae*. *D*, *Ceratozamia norstogii*. Bars = 100 μm.

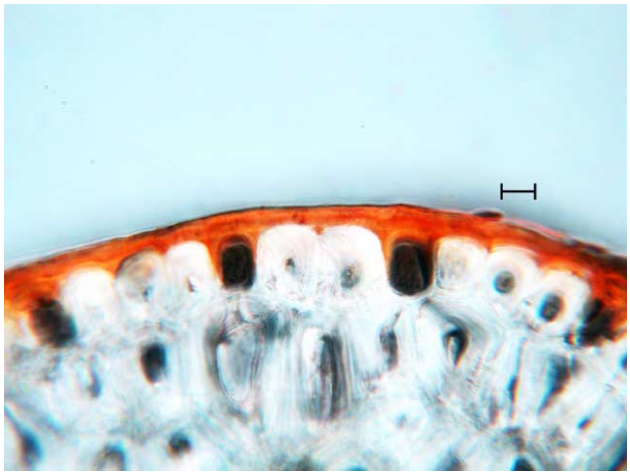

Fig. 9 Transverse section of adaxial epidermis of *Ceratozamia miradae* indicating cuticle stained with Sudan III and IV (orange) showing cuticle thickness above thin-walled epidermal cells and deep cuticular flanges between adjacent thick-walled epidermal cells. Bar = 10 μm.

lationship was evident (although weak) but indicated an ecological speciation process in accordance with genetic distance as found by Pérez-Farrera (2005). Studies appear to demonstrate that natural selection operates through ecological gradients and could be more important than geographical isolation (Schneider et al. 1999). Dudley (1978) found six morphological characters to vary along an elevation gradient in the Melastomataceae in Peru. Dudley (1978) also suggested that factors such as wind, rainfall, humidity, edaphic conditions like drainage and mineral content of soils (especially phosphate), soil temperature, and solar radiation could be selective pressures that determine adaptive radiation. It is notable that all of the species in the *C. norstogii* complex are sympatric with *Quercus* species and that geographic distances between species are relatively close, although differing in elevation and local climatic conditions, especially wind and habitat.

Conclusion

In this study, we have confirmed that the four species in the *C. norstogii* complex are morphologically distinct. They occur at distinct elevations, and it is most likely the secondary effects of these differences impinging on environmental factors that make up the cycads' microhabitats that are responsible for the morphological and leaflet anatomical variation and differences observed. Some anatomical traits appear to correlate with microhabitat, such as girder sclerenchyma and the windy habitat of *C. norstogii*. This supports a scenario for ecological speciation in the *C. norstogii* complex.

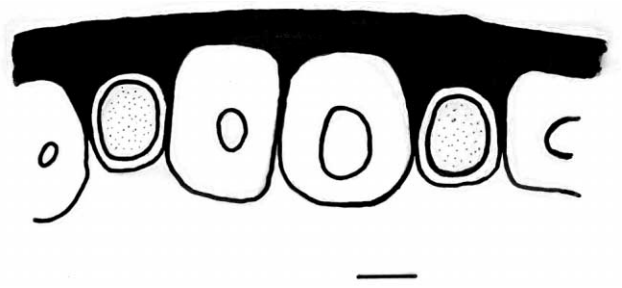

Fig. 10 Camera lucida drawing of transverse section of adaxial epidermis of *Ceratozamia miradae* illustrating thick-walled epidermal cells with small empty lumen and thin-walled epidermal cell with content (dotted). Cuticle and interpenetrating flanges are black. Bar = 10 μm.

Acknowledgments

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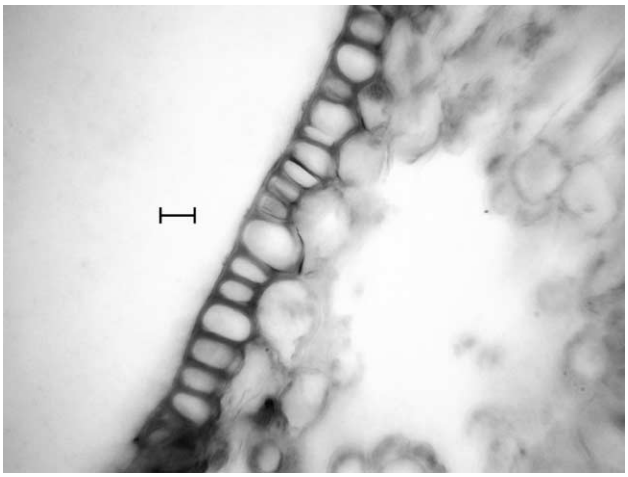

Fig. 11 Transverse section of abaxial epidermis of *Ceratozamia miradae* stained with phloroglucinol HCl (dark areas) indicating lignified epidermal cells and a macrolumen epidermal cell (opposite scale bar). Bar = 10 μm.

Appendix

Voucher and botanic garden (JBC) living collections accession information for the taxa used in this study is provided. Voucher specimens have been deposited and other vouchers examined in the following herbaria: CAS (California Academy of Sciences, San Francisco), CHAPA (Colegio de Postgraduados, Chapingo, Mexico), CHIP (Instituto de Historia Natural, Tuxtla Gutiérrez,

Mexico), F (Field Museum of Natural History, Chicago), FTG (Fairchild Tropical Botanic Garden, Miami), HEM (Universidad de Ciencias y Artes de Chiapas, Tuxtla Gutiérrez, Mexico), MEXU (Universidad Nacional Autónoma de México, Mexico City, Mexico), MO (Missouri Botanical Garden, St. Louis), UAMIZ (Universidad Autónoma Metropolitana, Iztapalapa, Mexico City Mexico), and 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, *De La Cruz*, *R. 66* CHIP; *De La Cruz*, *R. 20*, *24*, *76* CHIP, XAL, MEXU; *M. A. Pérez Farrera 26A*, *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.

Literature Cited

Chamberlain CJ 1932 Methods in plant histology. 5th ed. University of Chicago Press, Chicago.

Dudley EC 1978 Adaptive radiation in the Melastomataceae along an altitudinal gradient in Peru. Biotropica 10:134–143.

Florin R 1951 Evolution in *Cordaites* and conifers. Acta Hortic Berg 15:285–388.

Grant V 1985 The evolution process: a critical review of evolutionary theory. Columbia University Press, New York.

Greguss P 1968 Xylotomy of the living cycads. Akadémiai Kiadó, Budapest.

Johnson LAS 1963 Cytological and taxonomic notes on Zamiaceae. Contrib N S W Natl Herb 3:235–240.

Moretti A, S Sabato, M Vázquez Torres 1980 The distribution of *Ceratozamia* (Zamiaceae). Delpinoa 20:13–21.

Norstog KJ, TJ Nicholls 1997 The biology of cycads. Cornell University Press, Ithaca, NY.

Osborne R, M Calonje, K Hill, L Stanberg, DW Stevenson 2012 The world list of cycads. Mem N Y Bot Gard 106:480–508.

Pant DD, B Mehra 1964 Development of stomata in leaves of three species of *Cycas* and *Ginkgo biloba* L. Bot J Linn Soc 58:491–497.

Pant DD, DD Nautiyal 1963 Cuticle and epidermis of recent Cycadales: leaves, sporangia and seeds. Senckenb Biol 44:257–347.

Pérez-Farrera MA 2005 Estudio sistemático de los complejos *Ceratozamia norstogii* D.W.Stev. y *Ceratozamia miqueliana* H. Wendl. (Cycadales, Zamiaceae). PhD diss. Universidad Autónoma de Querétaro, Santiago de Querétaro, Mexico.

Pérez-Farrera MA, AP Vovides, L Hernández-Sandoval, D González, M Martínez 2004 A morphometric analysis of the *Ceratozamia norstogii* complex (Zamiaceae). Pages 127–136 *in* T Walters, R Osborne, eds. Cycad classification: concepts and recommendations. CABI, Wallingford.

Pérez-Farrera MA, AP Vovides, C Iglesias 1999 A new species of *Ceratozamia* (Zamiaceae, Cycadales) from Chiapas, Mexico. Novon 9: 410–413.

——— 2001 The cycad *Ceratozamia norstogii* Stevenson (Zamiaceae) from southern Mexico: new information on distribution, habitat and vegetative morphology. Bot J Linn Soc 137:71–76.

Purvis MJ, DC Collier, D Walls 1966 Laboratory techniques in botany. Butterworths, London.

Schneider CJ, TB Smith, B Larison, C Moritz 1999 A test of alternative model of diversification in tropical forest: ecological gradients vs. rainforest refugia. Proc Natl Acad Sci USA 96:13869–13873.

Stevenson DW 1982 A new species of *Ceratozamia* (Zamiaceae) from Chiapas, Mexico. Brittonia 34:181–184.

Stevenson DW, S Sabato, M Vázquez Torres 1986 A new species of *Ceratozamia* (Zamiaceae) from Veracruz, Mexico with comments on species relationships, habitats, and vegetative morphology in *Ceratozamia*. Brittonia 38:17–26.

Vovides AP, S Avendaño, MA Pérez-Farrera, DW Stevenson 2012 What is *Ceratozamia brevifrons* (Zamiaceae)? Brittonia 64:35–42.

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

Vovides AP, MA Pérez-Farrera, J González-Astorga, C Iglesias 2008 A new species of *Ceratozamia* (Zamiaceae) from Oaxaca, Mexico with comments on habitat and relationships. Bot J Linn Soc 157: 169–175.

Vovides AP, MA Pérez-Farrera, C Iglesias 2001 Another new species of *Ceratozamia* (Zamiaceae) from Chiapas, Mexico. Bot J Linn Soc 137:81–85.

Vovides AP, JD Rees, M Vázquez Torres 1983 Zamiaceae. Pages 1–31 *in* A Gómez-Pompa, V Sosa, eds. Flora de Veracruz. Fasc. 26. Instituto Nacional de Investigaciones sobre Recusos Bióticos (INIREB), Xalapa.

Whitelock L 2002 The cycads. Timber, Portland.

Wright S 1943 Isolation by distance. Genetics 28:114–138.