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ORIGINAL ARTICLE

Leaflet Anatomical Diversity in *Zamia* (Cycadales: Zamiaceae) Shows Little Correlation with Phylogeny and Climate

Rosemary A. E. Glos[1] · Shayla Salzman[1] · Michael Calonje[2] · Andrew P. Vovides[3] · Mario Coiro[4,5] · Maria Alejandra Gandolfo[1] · Chelsea D. Specht[1]

[1] Section of Plant Biology and the L.H. Bailey Hortorium, School of Integrative Plant Science, Cornell University, Ithaca, NY, USA
[2] Montgomery Botanical Center, Coral Gables, FL, USA
[3] Instituto de Ecología, Xalapa, Veracruz, Mexico
[4] Ronin Institute for Independent Scholarship, Montclair, NJ, USA
[5] Department of Biology, University of Fribourg, Fribourg, Switzerland
[6] Author for Correspondence; e-mailrag295@cornell.edu 2021

Abstract

Cycads (Cycadales) are among the most ancient lineages of extant seed-bearing plants and are the most threatened plant order on Earth, with circa 75% of the 356 accepted species endangered or threatened with extinction. *Zamia* is the most species-rich (81 spp.) and widely distributed cycad genus in the Americas, notable for its morphological and ecological diversity. Across the genus, there appears to be a high degree of convergence among macromorphological traits, with many characters that are useful for species identification proving uninformative for elucidating relationships among species. However, it remains unknown whether anatomical variation in leaflet structure corresponds with phylogenetic or geographic patterns, as has been investigated in *Dioon* and *Cycas.* Here, we present a broad comparative survey of leaflet anatomy across *Zamia* species with the goals of describing anatomical diversity and uncovering diagnostic characters for resolved clades. Anatomical characters were scored based on the literature and newly prepared sections of leaflets from 20 *Zamia* species plus the outgroup species *Microcycas calocoma.* The resulting matrix covers 39 *Zamia* species representing all five major clades and spanning the geographic distribution of the genus. Anatomical characters scored from leaflet sections were mapped onto a previously published phylogeny and evaluated for their phylogenetic signal. Most anatomical characters examined are not diagnostic for clades, but newly reported mesophyll sclereids may be unique to one large lineage. Given the widespread incongruence between phylogenetic relationships and the distribution of anatomical traits, we tested the relationship between anatomical characters and environmental signals but did not uncover significant correlations between anatomy and ecology. While further work is required to elucidate the evolutionary history of anatomical characters in this genus, this research improves our understanding of micromorphological character evolution, anatomical diversity, and phylogenetic relationships within this highly threatened lineage of plants.

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Keywords Cycads · Neotropics · Gymnosperms · Sclereids · Biogeography · Hypodermis

Introduction

Zamia L. is the second-largest genus in the Cycadales, and the most morphologically, ecologically, and karyologically diverse in the order (Caputo et al., 1996; Jones, 2002; Norstog, 1981; Stevenson, 1990; Vovides, 1983). The genus is primarily restricted to the neotropical region, with the exception of *Zamia integrifolia,* the northernmost populations of which extend into the Nearctic region (Calonje et al., 2019). Extant *Zamia* species occupy three geographic areas (sensu Calonje et al., 2019), including a Caribbean group (specifically the Bahamas and the Greater Antilles), a Mesoamerican group (Tamaulipas, Mexico to northern El Salvador), and a Central and South American group (southern Nicaragua to Bolivia). The last decade has seen considerable advances in *Zamia* taxonomy, with significant progress being made in the less-understood populations of the Amazon basin (see e.g. Segalla & Calonje, 2019). *Zamia* species are typically distinguished from each other on the basis of macromorphology, including leaflet shape, margin dentation, petiole armature, stem habit (epigeous or hypogeous), and a range of characters associated with the pollen- and ovule-bearing strobili (see e.g. Pérez-Farrera et al., 2016; Segalla & Calonje, 2019). However, many macromorphological characters show high degree of convergence in the genus rendering them unsuitable for use in elucidating fine-scale species relationships (Calonje et al., 2019; Caputo et al., 2004; Meerow et al., 2018). As such, species-level phylogenies have only recently been resolved through analyses of DNA-based character data (Calonje et al., 2019). Nevertheless, it remains unknown whether fine-scale variation in leaflet structure corresponds with evolutionary (phylogenetic) or ecological (geographic/habitat) patterns, as has been investigated in the cycad genera *Dioon* Lindl. and *Cycas* L. (Griffith et al., 2014; Vovides et al., 2018). An understanding of potential variation in leaflet anatomy is essential to a robust characterization of evolutionary and ecological diversity in *Zamia*.

Several previous studies have examined leaflet anatomy across the order Cycadales, however the majority were focused on descriptions of anatomical differences at the generic level (Stevenson, 1990; Stevenson et al., 1996) or have been limited to epidermal characters alone (Greguss, 1968). More recent studies focusing on a few *Zamia* species showed that anatomical details can be taxonomically informative among groups of species with highly similar morphology (Acuña-Castillo and Marín-Méndez, 2012, 2013a,b; Pérez-Farrera et al., 2014, 2016). Furthermore, anatomical traits have also proven useful in the delimitation of closely related species even when DNA sequence data cannot (Vovides et al., 2012, 2020). A recent paper (Coiro et al., 2020) reveals that leaflet anatomy has a strong phylogenetic signal across the Zamiaceae, paving the way for additional studies that examine the systematic value of anatomical traits in the family.

Here, we conduct a broad comparative survey of *Zamia* leaflet anatomy to determine how anatomical information complements the most recent phylogenetic

hypothesis for the genus. We focus on anatomical characters observable in transverse and longitudinal sections, with the acknowledgment that additional work is required to explore potential epidermal and stomatal diversity across the genus. The objectives of this research are to examine leaflet anatomical diversity across the major clades of *Zamia* and to determine whether any of the major clades can be defined by anatomical synapomorphies. The information obtained through this investigation helps to evaluate if leaflet anatomical data can provide further insight into diversity and phylogenetic relationships within the genus and improves our understanding of the evolution and diversification of ecologically relevant anatomical traits.

Material and Methods

Sampling

All material was obtained from living collections at Montgomery Botanical Center (MBC) and Fairchild Tropical Botanical Garden (FTBG), both located in Coral Gables, FL, USA. Collection took place between January 11th and 16th, 2020. Leaflets from a total of 54 *Zamia* species and *Microcycas calocoma* (Miq.) A.DC. were collected and photographed. For this initial survey of leaflet anatomy, twenty *Zamia* species including all major clades as defined by Calonje et al. (2019) and covering the geographic distribution of the genus were chosen for sectioning. *Microcycas calocoma* from the monotypic sister genus was included as the outgroup. Table 1 lists the provenance and accession numbers for the plants from which leaflets were sectioned, imaged, and studied. Accession information of imaged whole leaflets for morphology is included in Online Resource 1. Whenever possible, samples were collected from the same individuals used in the phylogeny published by Calonje et al. (2019), but in some cases where high quality (unblemished) leaflets were in short supply, leaflets were collected from other plants derived from the same wild source population. Leaflets were collected from one representative individual with a single leaflet most often collected, or up to three if the leaflets were damaged or very small. Leaflets were sampled at the midpoint of a mature leaf and sectioned in the midportion of the leaflet to facilitate comparison among the species studied. Samples were fixed for at least 12 h in formalin-acetic acid–ethanol (FAA, Thermo Fischer Scientific, Waltham, MA, USA) and stored in 50% ethanol.

Sectioning

Transverse and longitudinal sections were prepared with the sample embedded in a block of carrot on a Spencer 820 rotary microtome (American Optical, Buffalo, NY, USA) at a thickness of approx. 30–50 µm. Sections were immediately transferred to water using a fine paintbrush and stored in 70% ethanol. Three transverse and three longitudinal sections from each species were bleached with 6% sodium hypochlorite and stained with toluidine blue (0.01% in water) as a general metachromatic stain. Additional stains on bleached sections from select species include safranin (2% in

Table 1 Living accession, herbarium voucher and provenance information of taxa sectioned in this study

Taxon	Accession #	Representative voucher	Origin
Microcycas calocoma (Miq.) A.DC	**MBC 59875*B**	Calonje MBC12-006 (FTG)	Cuba
Zamia angustifolia Jacq	MBC 20110152*G	Calonje et al. BS10-200 (FTG)	Bahamas
Z. boliviana (Brongn.) A.DC	**MBC 981682*J**	Little & Stevenson 1067 (FTG)	Bolivia
Z. decumbens Calonje, Meerman, M.P.Griff. & Hoese	**MBC 20080715*V**	Calonje et al. BZ08-201 (BRH, FTG, MO, NY, XAL)	Belize
Z. encephalartoides D.W.Stev	**MBC 94910*A**	Espinosa 2011–003 (FTG)	Colombia
Zamia furfuracea L.f	**MBC 20010214*L**	Little & Stevenson 1098 (FTG)	Mexico
Z. imperialis A.S.Taylor, J.L.Haynes & Holzman	MBC 20010755*A	Stevenson DWS1220 (NY, PMA)	Panama
Z. inermis Vovides, J.D.Rees & Vázq.Torres	**MBC 92143*D**	Espinosa 2011–006 (FTG)	Mexico
Z. integrifolia L.f	MBC 20050872*E	Jody Haynes JLH05-263 (FTG)	Florida
Z. ipetiensis D.W.Stev	MBC 2000278*A	Damon P. Little & Dennis Wm. Stevenson No. 1071 (FTG, NY)	Panama
Z. nana A.Lindstr., Calonje, D.W.Stev. & A.S.Taylor	**MBC 20020234*B**	Stevenson & Valdespinos 1147 (FTG, PMA, MO)	Panama
Z. obliqua A.Braun	**FTBG 89162-B**	Bogler 1240 (FTG)	Panama?
Z. pseudoparasitica J.Yates	**MBC 2000319*A**	Stevenson DWS1206A (FTG, PMA, NY)	Panama
Z. purpurea Vovides, J.D.Rees & Vázq.Torres	MBC 93928	Walters TW10-1 (FTG, XAL)	Mexico
Z. pyrophylla Calonje, D.W.Stev. & A.Lindstr	**MBC 20100027*A**	Kress & Echeverry 89–2571 (CHOCO, HUA, MEDEL, SEL, US)	Colombia
Z. restrepoi (D.W.Stev.) A.Lindstr	**MBC 20100026*A**	Calonje MBC18-03 (FTG)	Colombia
Z. roezlii Linden	MBC 20030797*A	Calonje US21-01 (FTG)	Colombia
Z. soconuscensis Schutzman, Vovides & Dehgan	MBC 20011293*A	Walters TW2001-30 (XAL)	Mexico
Z. variegata Warsz	**MBC 20080675*A**	Calonje et al. BZ08-175 (FTG)	Belize
Z. vazquezii D.W.Stev., Sabato & De Luca	MBC 941486*Z	Damon P. Little & Dennis Wm. Stevenson No. 1088 (FTG, NY)	(garden)
Z. wallisii Braun	**MBC 20010301*A**	Little & Stevenson 1165 (FTG)	Colombia

Montgomery Botanical Center (MBC) and Fairchild Tropical Botanical Garden (FTBG) accessions in bold match those individuals used in the molecular phylogeny of Calonje et al. (2019)

water) and safrablau (1:9, 1% Astra blue in 50% ethanol: 1% safranin in 50% ethanol) for lignified cell walls and Sudan IV (saturated solution in 70% isopropyl alcohol) for lipids and fatty acids. Sections were rinsed in tap water and semi-permanently mounted in glycerol: water (1:1 by volume), the coverslip sealed with clear nail polish. The phloroglucinol and hydrochloric acid test for lignin was performed by immersing sections in a 1% solution of phloroglucinol in absolute ethanol for 3 to 5 min, then placing them in concentrated HCl until the red lignin reaction occurred. The sections were then rinsed in 70% ethanol and temporary preparations made with 30% glycerol in distilled water for mapping of lignified tissues.

Imaging

Images were prepared using an Olympus BX60 compound microscope with cellSens imaging software at the Cornell University Plant Anatomy Collection, Ithaca, NY, USA. All figures were created in Adobe Illustrator Creative Cloud 2020.

Character Scoring

Phylogenetic trees were viewed and edited in Mesquite version 3.61 (Maddison and Maddison 2019). Slides and images were initially reviewed for characters that displayed variation within the genus. Selected characters were scored during microscopic survey under an M-series compound microscope (AmScope, Los Angeles, CA, USA) and compared to published relationships (Calonje et al., 2019). At least four sections (three stained and one unstained) were considered when scoring characters. Twenty additional species were scored based on the literature, data reported in Coiro et al. (2020), and re-examination of sections generated in Coiro et al. (2020). A character matrix was completed for the following observed variable character states, all scored as present/absent unless otherwise noted: (1) adaxial hypodermis (present/discontinuous/absent), (2) adaxial girder sclerenchyma, (3) lignified fibers in the mesophyll, (4) secretory idioblasts in the mesophyll and/or bundle sheath, and (5) sclereids in the mesophyll.

Environmental correlation analysis: To test the correlation between anatomical traits and macroclimatic factors, we extracted bioclim variables for a list of occurrences of species of *Zamia* from Calonje et al. (2019) using the dismo package in R (R Core Team, 2020). We then followed the strategy of Ehmig et al. (2018) to obtain a climatic envelope for *Zamia*. This allows to take into account the correlation between bioclim variables and remove the effect of 'climatic availability' in the native range of *Zamia.* We obtained the bioclim variables for each 2.5 arc cell between 106.58 W and 55.43 W longitude and 17.91S and 30.67 N latitude, representing the whole geographical span of the genus *Zamia,* and used these scores to run a principal component analysis (PCAenv). We calculated the PCA scores of the species occurrences within the PCAenv and obtained an average for each species (PCAsp). The resulting 17 PCAsp loadings were used as response variables in a phylogenetically corrected MANOVA (using the aov.phylo function in the R package geiger) with five anatomical traits (adaxial hypodermis, adaxial girder

sclerenchyma, mesophyll fibers, secretory idioblasts, and sclereids) used as explanatory variables.

Results

Leaflet Morphology

Zamia leaves consist of a petiole and rachis upon which a variable number of leaflets are born (Jones, 2002). The petiole and rachis may be smooth or prickled, a character that is commonly used to distinguish or identify species (see e.g. Segalla and Calonje, 2019). *Zamia* leaflets are quite variable with respect to size, shape, thickness, and texture (Fig. 1). All species but one, *Zamia restrepoi* (formerly *Chigua restrepoi*), lack a conspicuous midrib (Stevenson et al., 1996). Plicate (corrugated) leaflets are found in several Central and South American species, but this character

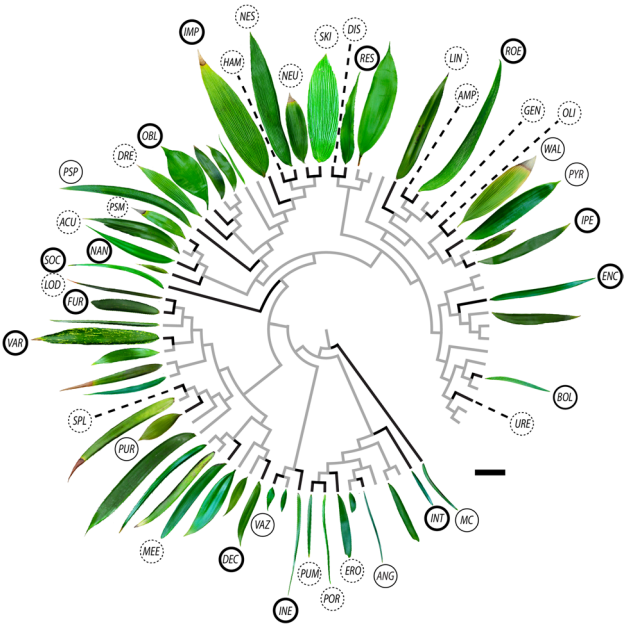


Fig. 1 Leaflet morphological variation across *Zamia*. Leaflets from 52 *Zamia* species plus *Microcycas calocoma* (MC) shown alongside the published cladogram of Calonje et al. (2019). Bold circles = spp. sectioned in this study only, dotted circles = spp. analyzed in Coiro et al. (2020), plain circles = spp. sectioned in both studies. *INT* (*Zamia*) *integrifolia*, *ANG angustifolia*, *ERO erosa*, *POR portoricensis*, *PUM pumila*, *INE inermis*, *VAZ vazquezii*, *DEC decumbens*, *MEE meermanii*, *PUR purpurea*, *SPL splendens*, *VAR variegata*, *FUR furfuracea*, *LOD loddigesii*, *SOC soconuscensis*, *NAN nana*, *ACU acuminata*, *PSM pseudomonticola*, *PSE pseudoparasitica*, *DRE dressleri*, *OBL obliqua*, *IMP imperialis*, *HAM hamannii*, *NES nesophila*, *NEU neurophyllidia*, *SKI skinneri*, *DIS disodon*, *RES restrepoi*, *LIN lindenii*, *AMP amplifolia*, *ROE roezlii, GEN gentryi*, *OLI oligodonta*, *WAL wallisii, PYR pyrophylla, IPE ipetiensis*, *ENC encephalartoides*, *BOL boliviana*, *URE urep.* Scale bar = 5 cm. See Online Resource 1 for accession information associated with all leaflets and Online Resource 2 for cladogram with taxon names

does not define a monophyletic group (Calonje et al., 2019). Additional characters of interest include, among others, variegation in *Zamia variegata*, a petiolule with flared "collar" in *Zamia manicata*, and unusually colored immature leaflets (e.g. fiery-orange in *Zamia pyrophylla* and purple-green in *Zamia purpurea*).

Anatomical Organization and Variation

All *Zamia* species examined present a fairly uniform anatomical organization (Fig. 2). Deviations can largely be considered "variations on a theme," with some species displaying notable extremes. Species with plicate leaflets follow the same overall *bauplan*, albeit with modifications that reflect the longitudinal folds in the leaflet (Fig. 3c). All species have pinnae with longitudinally parallel, dichotomously branching veins that are uniformly spaced in cross section (Fig. 3i). Vascular bundles are roughly isodiametric in cross section, except in *Z. restrepoi,* which has a conspicuous midrib consisting of a single large vascular bundle (Stevenson et al., 1996). They are characterized by the thick cuticle typical of cycads (Norstog and Nicholls, 1997, Fig. 3f herein) and both epidermal layers consist of roughly isodiametric lignified or non-lignified cells that are circular in cross section (Fig. 3a). Stomatal complexes with the sunken guard cells typical of *Zamia* and most other genera in the Zamiaceae (Coiro et al. 2020) are visible in transverse section (Fig. 2). In some species, the epidermal cells contain secondary metabolites that appear yellow, brown or red in unstained sections.

In many species, the epidermis is followed by one to two continuous or discontinuous layers of sclerified hypodermal cells (Figs. 2, 3a and 4). The margin is typically reinforced with one to three layers of hypodermis (Fig. 3a). Most of the examined taxa have an undifferentiated palisade parenchyma, although some species (see

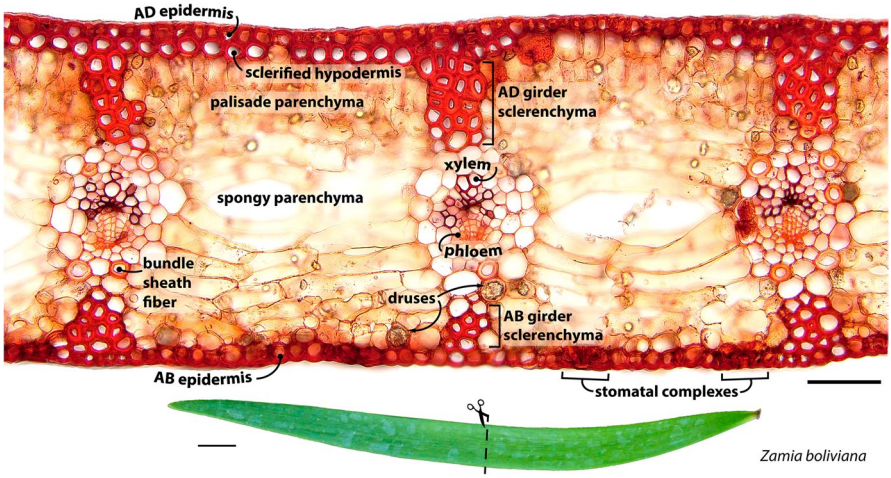


Fig. 2 General anatomical features of a *Zamia* leaflet, transverse section of *Z. boliviana* stained with safranin. *AD* adaxial, *AB* abaxial. Single mature leaflet shown below with dotted line indicating approximate location of sectioning. Section scale bar = 100 µm, leaflet scale bar = 1 cm

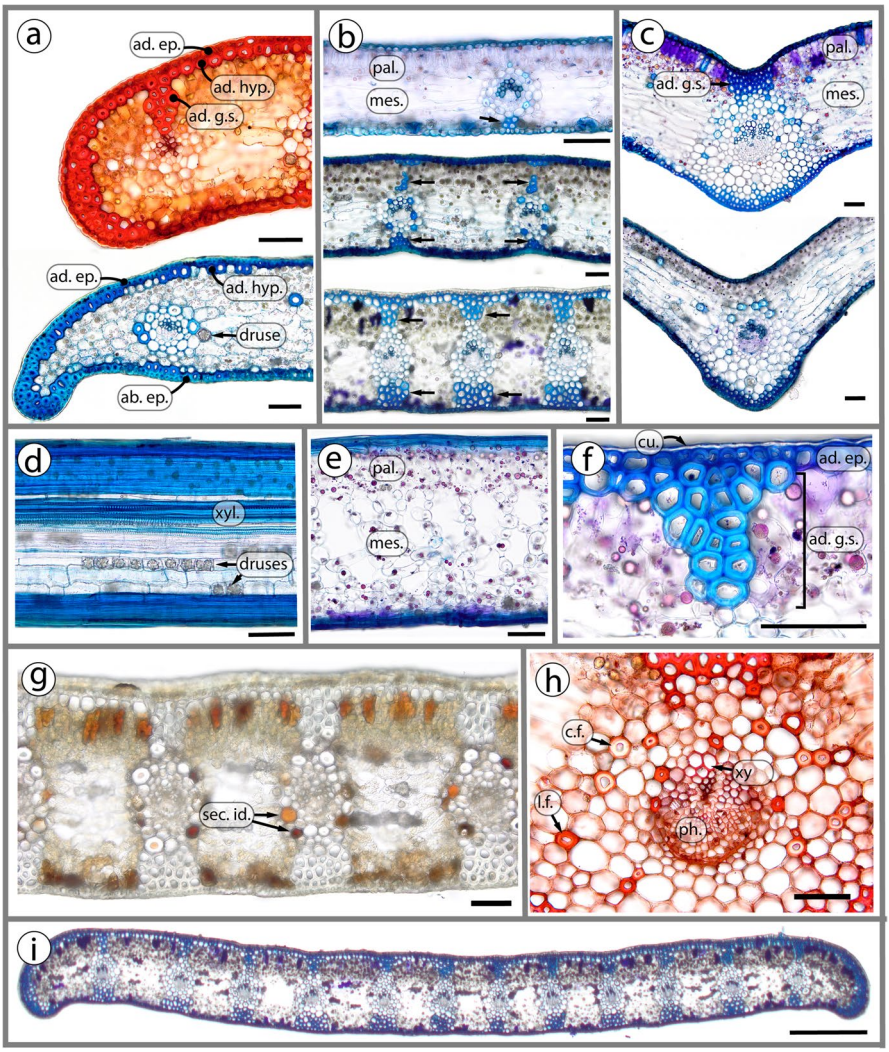


Fig. 3 Various leaflet anatomical features of *Zamia* spp. All sections are transverse except for d and e, which are longitudinal sections. **a** *Z. boliviana* (top) and *Z. purpurea* (bottom). **b** *Z. vazquezii* (top), *Z. purpurea* (middle), *Z. angustifolia* (bottom), adaxial and abaxial girder sclerenchyma indicated by arrows. Note variation in presence/absence and thickness of girder sclerenchyma. **c** *Z. wallisii* (top) and *Z. imperialis* (bottom). Note the large parenchymatous cells that form the bulk of the abaxial bundle sheath in these plicate-leafleted taxa. **d** *Z. decumbens*. **e f** *Z. boliviana*. **g** *Z. angustifolia* (unstained). **h** *Z. wallisii*. **i** *Z. angustifolia*. *ad. ep*. adaxial epidermis, *ad. hyp* adaxial hypodermis, *druse* cluster crystal, *ab. ep.* abaxial epidermis, *pal* palisade parenchyma, *mes* mesophyll parenchyma, *xyl* xylem, *cu* cuticle, *sec. id.* secretory idioblast, *c.f.* cellulosic fiber, *l.f.* lignified fiber, *xy.* xylem, *ph.* phloem. Scale bars: A-H = 100 µm, I = 500 µm

e.g. *Zamia wallisii* in Fig. 3c) have a clearly visible layer of cells that are elongated (sometimes lobed) and vertically oriented in transverse section. The remainder of the intervascular mesophyll consists of spongy parenchyma with large air spaces

and, in some taxa, scattered fibers and densely staining idioblasts with thick or thin walls. Some fibers are fully lignified while others seem to be cellulosic fibers, in which only the outer layers react with safranin (Vovides and Galicia, 2016; Fig. 3h).

Clusters of lignified fibers form an incipient girder sclerenchyma above and below the vascular bundles in most species (Figs. 2 and 3b). Its depth, width, and shape varies greatly according to the distance from the leaflet margin and across species. Whereas all species examined have some form of abaxial girder sclerenchyma, adaxial girder sclerenchyma is absent in 13 of the examined species (Fig. 4). Species with plicate leaflets have a preponderance of large diameter, thin-walled cells in the abaxial portion of the bundle sheath (Fig. 3c). In all species, the vasculature is surrounded by a bundle sheath of parenchymatous (sometimes collenchymatous) cells interspersed with lignified fibers, cellulosic fibers and/or idioblasts (Fig. 3g, h). Thin walled (secretory) idioblasts are commonly associated with the vascular bundle (Fig. 3g). In unstained sections, idioblast contents range from golden yellow to dark brown and, in some taxa (e.g. *Z. variegata*), bright red. Druses are found in the mesophyll and/or vascular bundle of all examined species, although they are very rare in *Microcycas* (Figs. 2 and 3d).

Sclereids are present in the mesophyll of seven species: *Zamia soconuscensis, Zamia nana, Zamia pseudoparasitica, Zamia obliqua, Zamia neurophyllidia, Zamia pyrophylla,* and *Z. wallisii* These cells are isodiametric, oblong, or angular in transverse section and circular in longitudinal section (Fig. 5b) with thick, striated walls and conspicuous pitting. They stain bright blue with toluidine, red with safranin, and pink with phloroglucinol and HCL (Fig. 5). Sclereids are restricted to the spongy parenchyma in all the species except *Z. wallisii,* where they also appear to be part of the palisade.

Climatic Correlations

Our MANOVA analyses does not retrieve significant differences for any of the traits (Table 2). Though species with an adaxial girder sclerenchyma seem to have different environmental preferences, this effect disappears when phylogenetic correlation is considered.

Discussion

Anatomical Consistency Across Species

Although whole-leaflet characters are variable and diagnostic for certain species in combination, most do not appear to be phylogenetically informative or conserved across *Zamia*. Obvious characters like leaflet plication and shape are scattered across the phylogeny (Fig. 1). All species studied show a fairly uniform anatomy which is consistent with the relatively recent radiation of *Zamia* and cycads as a whole (Calonje et al., 2019; Nagalingum et al., 2011; Salas-Leiva et al., 2013; Online Resource 3). All species are characterized by evenly spaced, longitudinally parallel

Fig. 4 Distribution of five variable anatomical characters compared to phylogenetic relationships from ▶ Calonje et al. (2019) for 39 *Zamia* species plus *Microcycas calocoma.* Species in bold font were newly sectioned in this study, species in pale font were analyzed in Coiro et al. (2020), and species in regular font were sectioned in both studies. Characters: *AD Hyp.* adaxial hypodermis, *AD G.S.* adaxial girder sclerenchyma, *Mes. Fibers* mesophyll fibers, *Sec. Id.* secretory idioblasts, *Sclereids* sclereids in the mesophyll. *Scored from the literature (Acuña-Castillo and Marín-Méndez, 2013a,b; Lamb, 1923; Pérez-Farrera et al, 2016)

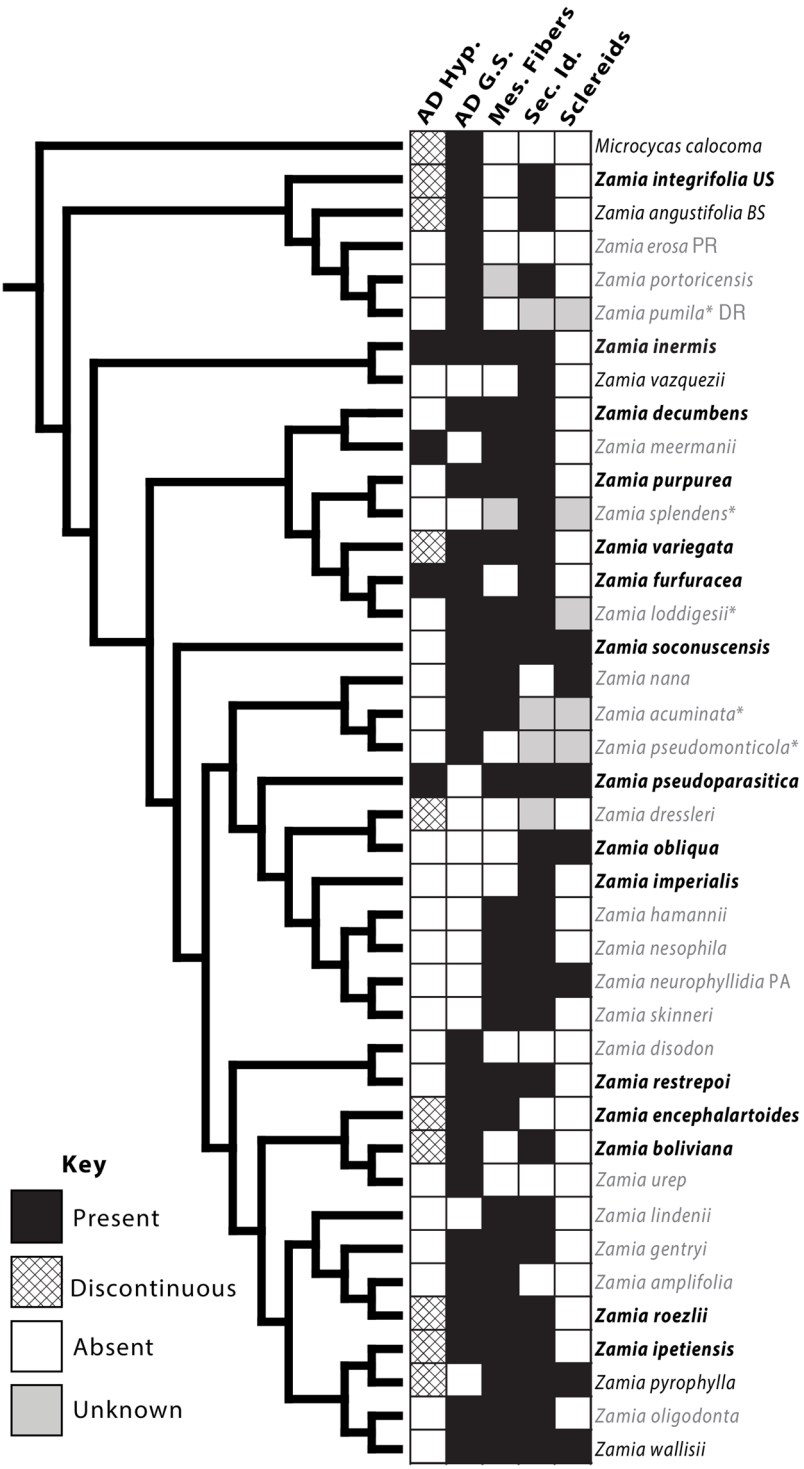
veins with a surrounding bundle sheath and incipient abaxial girder sclerenchyma while other characters, such as the presence of abaxial girder sclerenchyma, mesophyll fibers, secretory idioblasts, and sclereids, vary across the genus.

Variation in the color of secretory idioblast contents suggests that they contain multiple secondary metabolites. Some resemble the "gold cells" identified in cycad cones (Fig. 3g). These are thought to contain toxic substances, including the neurotoxin BMAA and the toxic glycoside macrozamin, that are associated with defense against herbivores (Vovides et al., 1993). Others contain brown or red substances that may be tannins, as reported in other cone idioblasts (Vovides, 1991). Although the presence of secretory idioblasts was not phylogenetically informative or broadly correlated with climatic variables, more detailed analysis of idioblast contents and scoring based on idioblast type may reveal patterns not apparent in our survey.

Sclereids

Of special interest are the sclereids observed in Z. *soconuscensis, Z. nana, Z. pseudoparasitica, Z. obliqua, Z. neurophyllidia, Z. pyrophylla,* and *Z. wallisii.* Sclereids are morphologically diverse, highly lignified cells found in a wide variety of plant species and tissues (Esau, 1960). In *Zamia,* sclereids were restricted to the mesophyll and varied in shape and size across taxa. They typically occurred singly, less often in clusters of two to three. All *Zamia* sclereids had thick, striated walls, often with a narrow, irregular lumen. Some resembled osteosclereids as described in Rao and Bhupal (1973), in that they had an elongated bone-like shape with rounded ends. However, while the aforementioned authors defined osteosclereids as running perpendicular to the epidermis, the sclereids we identified ran parallel to the leaflet surface. Some sclereids, such as those found in *Z. nana* (Fig. 5c) were isodiametric and more closely resembled spheroidal sclereids or brachysclereids. In *Z. wallisii,* sclereids were found in both the palisade and spongy mesophyll. Sclereids in the palisade parenchyma conformed to the description of palosclereids in Rao and Bhupal (1973). They were roughly the same size and shape as the surrounding palisade cells, except with noticeably thickened walls.

Sclereids have been identified in a variety of cycad tissues, but our observations constitute the first published example of sclereids in *Zamia* leaflets. Vovides (1991) identified idioblastic sclereids in the micro- and/or megasporophylls of *Cycas rumphii* and several species of *Encephalartos.* Sclereids have also been reported in cataphylls of the fossil taxon *Antarcticycas schopfii* (Hermsen et al., 2006). Like the sclereids identified herein, the idioblastic sclereids in *Encephalartos* were typically oblong and angular to isodiametric with thick, pitted walls (Vovides, 1991). Foliar


AD Hyp.
AD G.S.
Mes. Fibers
Sec. Id.
Sclereids
Microcycas calocoma
Zamia integrifolia US
Zamia angustifolia BS
Zamia erosa PR
Zamia portoricensis
Zamia pumila* DR
Zamia inermis
Zamia vazquezii
Zamia decumbens
Zamia meermanii
Zamia purpurea
Zamia splendens*
Zamia variegata
Zamia furfuracea
Zamia loddigesii*
Zamia soconuscensis
Zamia nana
Zamia acuminata*
Zamia pseudomonticola*
Zamia pseudoparasitica
Zamia dressleri
Zamia obliqua
Zamia imperialis
Zamia hamannii
Zamia nesophila
Zamia neurophyllidia PA
Zamia skinneri
Zamia disodon
Zamia restrepoi
Zamia encephalartoides
Zamia boliviana
Zamia urep
Zamia lindenii
Zamia gentryi
Zamia amplifolia
Zamia roezlii
Zamia ipetiensis
Zamia pyrophylla
Zamia oligodonta
Zamia wallisii
Key
Present
Discontinuous
Absent
Unknown

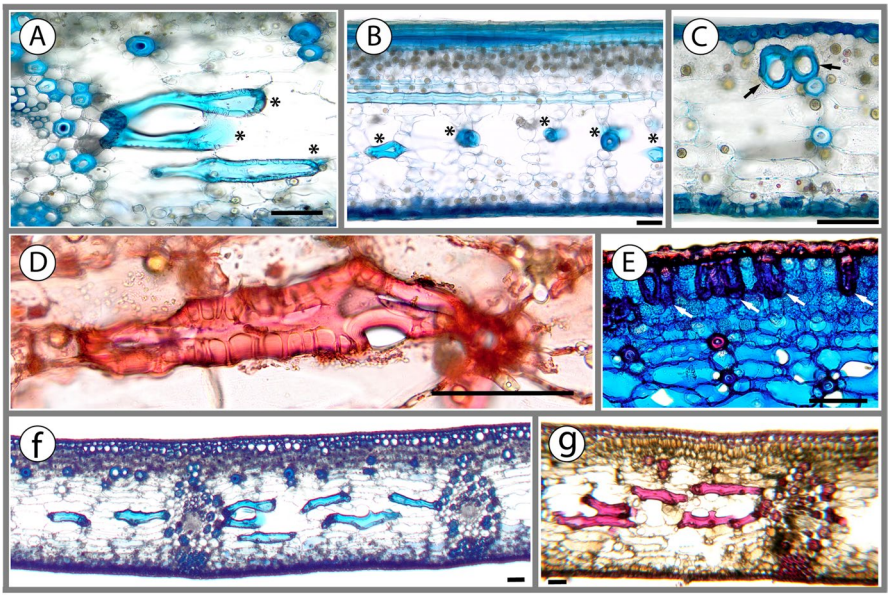


Fig. 5 Sclereids in transverse (**a**, **c**–**f**), and longitudinal sections (**b**) of *Zamia* leaflets. **a**, **b** *Z. pseudoparasitica,* sclereids marked with asterisks. **c** *Z. nana,* arrows indicate isodiametic sclereids. **d** *Z. wallisii,* single sclereid. **e** *Z. wallisii,* palosclereids indicated with arrows **f** *Z. pseudoparasitica,* sclereids staining bright blue. **g** *Z. pseudoparasitica,* sclereids, hypodermis, and fibers staining for lignin. Scale bars: 100 µm

Table 2 Results of a phylogenetically corrected MANOVA with the anatomical traits as predictors and the scores for 17 PCA axes for the Zamia climatic envelope for each species (PCAsp) as dependent variables

Anatomical trait	DF	Wilks	approx-F	Non-corrected p-value	P-value given phylogeny
Adaxial hypodermis	2	0.32389	0.75711	0.7893	0.957
Adaxial girder sclerenchyma	1	0.32133	2.2363	0.049658	0.7023
Mesophyll fibers	1	0.54207	0.7951	0.67851	0.8951
Secretory idioblasts	1	0.43994	1.0484	0.47034	0.4735
Sclereids	1	0.30932	1.7075	0.16623	0.5135

The p-values for a non-corrected analysis are also included

sclereids have been described from plants across the seed plant phylogeny (Esau, 1960; Rao and Bhattacharya, 1978). They are typically implicated in mechanical support, but their exact function remains unknown in many taxa. Sclereids have also been hypothesized to perform a light-guiding role (Karabourniotis, 1998), aid in water conduction (Heide-Jorgensen, 1990), and provide defense against herbivory (Nyffeler et al., 1997). Little is known about their role in cycads. The clearly visible pittting in the sclereids found in *Zamia* leaflets suggest a potential role in water

conduction or sensing. It is also possible that they are providing mechanical support to the tough, coriaceous leaves or causing herbivore-deterring grittiness. Further targeted investigations are required to elucidate the role(s) that these sclereids play in *Zamia* leaflets.

Phylogenetic Utility of Anatomical Traits

Comparison of anatomical characters with the published phylogeny suggests limited correlation between phylogenetic grouping and the gain/loss of anatomical traits. Most traits examined herein are scattered across the phylogeny and are not diagnostic for any clade (Fig. 4). Adaxial lignified hypodermis is present in thirteen of the 39 *Zamia* species examined plus *Microcycas,* with no discernable pattern formed by the occurrences. Likewise, the presence of an adaxial girder sclerenchyma, mesophyll fibers, and secretory idioblasts did not identify any specific group within the genus. The only character that shows some utility as a diagnostic trait is the presence of sclereids in the leaflet mesophyll. Of the species examined, sclereids were only found in those belonging to the large clade that consists of species from South America and the isthmus of Panama plus *Z. soconuscensis* (Calonje et al., 2019). However, sclereids were only identified in seven of the 25 examined species from that clade. Their presence in the Mexican species *Z. soconuscensis* lends some support to its surprising placement as sister to all species from the isthmus of Panama and South America rather than among other Mesoamerican species (see Calonje et al., 2019).

Detailed ancestral state reconstructions such as those conducted in *Dioon* (Vovides et al., 2018) and *Cycas* (Griffith et al., 2014) would further elucidate potential homoplasy in *Zamia*. Such a study would also benefit from additional preparations (e.g. epidermal peels and more targeted staining) and broader taxon sampling, which were beyond the scope of the present investigation.

Correlations Between Anatomy and Ecology

The incongruence between anatomy and phylogeny indicates that we must look deeper to enhance our understanding of the phylogenetic, ecological, and geographic distribution of these anatomical traits in *Zamia*. The wide geographic and ecological range of the genus suggests that some traits may primarily reflect adaptations in response to the long-term or historic influence of climate. Indeed, variation in anatomical characters has been shown to correlate with changes in habitat (specifically aridification) in *Dioon* (Gutiérrez-Ortega et al., 2018). Many extant cycad species present adaptations to both moist and xeric environments. In species currently found in mesic habitats, the latter may be a possible evolutionary remnant present in the ancestors of living species (Norstog and Nicholls, 1997). Previous works have established connections between anatomy and ecology in other cycad genera (Vovides et al., 2018) and in several *Zamia* species (Acuña-Castillo and Marín-Méndez, 2012, 2013a,b; Stevenson et al., 1996). For example, it has been suggested that the membranous pinnae found in *Z. restrepoi*

(South America clade) and *Z. vazquezii* (Mexico clade) represent a convergent adaptation to the low-light conditions prevailing under the dense forest canopies that characterize their native habitats (Stevenson et al., 1996). However, this hypothesis is complicated by the fact that highly coriaceous leaflets are also found in species that occur in shaded habitats of dense rainforest (e.g. *Z. skinneri* in Costa Rica).

A MANOVA analysis was performed to determine whether correlations exist between the anatomical characters scored in this study and a range of environmental variables. Our analysis did not retrieve significant differences using large-scale climate variables from the bioclim model. However, this does not preclude the possibility that other factors, such as soil, microclimate, herbivore or pollinator associations, or other complex ecological associations not captured by the bioclim variables might influence leaflet anatomy. Further studies using population-level sampling and local measurements could shed more light on this issue.

Conclusions

The present study describes leaflet anatomical diversity in *Zamia* and identifies possible traits of further taxonomic and histological interest. It builds on previous anatomical investigations in specific *Zamia* species (Acuña-Castillo and Marín-Méndez, 2012, 2013a,b; Pérez-Farrera et al., 2016) and complements recent work that examines the phylogenetic utility of anatomical traits in the Zamiaceae and Cycadales as a whole (Coiro et al., 2020; Griffith et al., 2014; Vovides et al., 2018).

Our observations demonstrate that *Zamia* leaflet anatomy shows widespread anatomical variation within a consistent core organization. Most anatomical characters are highly homoplasious, which is consistent with the variation seen in gross leaflet morphology. Newly reported mesophyll sclereids appear to be restricted to the clade consisting of species from South America and the isthmus of Panama plus *Z. soconuscensis*. Additional work is required to determine the diversity, distribution, and function of these cells across the entire genus. An initial analysis comparing anatomical characters to environmental variables did not uncover any significant correlations, indicating that further investigation is required to elucidate the evolutionary history of leaflet anatomy in *Zamia.*

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Data Availability All data necessary to evaluate this manuscript are included. Slide images are housed at the Cornell University Plant Anatomy Collection (CUPAC), Ithaca, New York and living specimens are maintained at Montgomery Botanical Center and Fairchild Tropical Garden in Coral Gables, Florida.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Acuña-Castillo, R., and Marín-Méndez, W. 2012. Some aspects of the foliar anatomy of two *Zamia* species from Costa Rica: *Z. fairchildiana* L. D. Gómez and *Z. neurophyllidia* D. W. Stev. Proceedings of Cycad 2008. The 8th International Conference on Cycad Biology, Panama City, Panama, 13–15 January 2008: 192–203.

Acuña-Castillo, R., and Marín-Méndez, W. 2013a. Comparative anatomy of leaflets of *Zamia acuminata* and *Z. pseudomonticola* (Zamiaceae) in Costa Rica. Revista de Biología Tropical *61*, 539–546.

Acuña-Castillo, R., and Marín-Méndez, W. (2013b). Leaflet anatomy of *Zamia disodon* D.W. Stev. & sabato and *Z. restrepoi* (D.W. Stev.) A. Lindstr. Caldasia *35*, 1–9.

Calonje, M., Meerow, A.W., Griffith, M.P., Salas-Leiva, D., Vovides, A.P., Coiro, M., and Francisco-Ortega, J. (2019). A Time-Calibrated Species Tree Phylogeny of the New World Cycad Genus *Zamia* L. (Zamiaceae, Cycadales). International Journal of Plant Sciences *180*, 286–314.

Caputo, P., Cozzolino, S., Gaudio, L., Moretti, A., and Stevenson, D.W. (1996). Karyology and Phylogeny of Some Mesoamerican Species of *Zamia* (Zamiaceae). American Journal of Botany *83*, 1513–1520.

Caputo, P., Cozzolino, S., De Luca, P., Moretti, A., and Stevenson, D. (2004). Molecular phylogeny of *Zamia* (Zamiaceae). Cycad Classification: Concepts and Recommendations 149–158.

Coiro, M., Jelmini, N., Neuenschwander, H., Calonje, M.A., Vovides, A.P., Mickle, J.E., and Lumaga, M.R.B. (2020). Evolutionary signal of leaflet anatomy in the Zamiaceae. American Journal of Botany *181:7*, 697–1715.

Ehmig, M., Coiro, M., and Linder, H. (2018). Ecophysiological strategy switch through development in heteroblastic species of mediterranean ecosystems - An example in the African Restionaceae. Annals of Botany *123*.

Esau, K. (1960). Anatomy of Seed Plants (Wiley).

Greguss, P. (1968). Xylotomy of the living cycads, with a description of their leaves and epidermis (Budapest: Akadémiai Kiadó).

Griffith, M.P., Magellan, T.M., and Tomlinson, P.B. (2014). Variation in Leaflet Structure in *Cycas* (Cycadales: Cycadaceae): Does Anatomy Follow Phylogeny and Geography? International Journal of Plant Sciences *175*, 241–255.

Gutiérrez-Ortega, J.S., Yamamoto, T., Vovides, A.P., Farrera, M.A., Martínez, J., Molina-Freaner, F., Watano, Y., and Kajita, T. (2018). Aridification as a driver of biodiversity: A case study for the cycad genus *Dioon* (Zamiaceae). Annals of Botany *121*, 47–60.

Hermsen, E.J., Taylor, T.N., Taylor, E.L., and Stevenson, D.W. (2006). Cataphylls of the Middle Triassic cycad *Antarcticycas schopfii* and new insights into cycad evolution. American Journal of Botany *93*, 724–738.

Heide-Jorgensen, H. S. (1990). Xeromorphic leaves of Hakea suaveolens R. Br. IV. Ontogeny, structure and function of the sclereids. *Australian journal of Botany*, *38*(1), 25–43.

Jones, D.L. (2002). Cycads of the World (Washington, D.C.: Smithsonian Institution Press).

Karabourniotis, G. (1998). Light-guiding function of foliar sclereids in the evergreen sclerophyll *Phillyrea latifolia*: a quantitative approach. Journal of Experimental Botany *49*, 739–746.

Lamb, M.A. (1923). Leaflets of Cycadaceae. Botanical Gazette *76*, 185–202.

Maddison, W. P. and D.R. Maddison. (2019). Mesquite: a modular system for evolutionary analysis. Version 3.61 http://www.mesquiteproject.org

Meerow, A.W., Salas-Leiva, D.E., Calonje, M., Francisco-Ortega, J., Griffith, M.P., Nakamura, K., Jiménez-Rodríguez, F., Lawrus, J., and Oberli, A. (2018). Contrasting Demographic History and Population Structure of *Zamia* (Cycadales: Zamiaceae) on Six Islands of the Greater Antilles Suggests a Model for Population Diversification in the Caribbean Clade of the Genus. International Journal of Plant Sciences *179*, 730–757.

Nagalingum, N.S., Marshall, C.R., Quental, T.B., Rai, H.S., Little, D.P., and Mathews, S. (2011). Recent Synchronous Radiation of a Living Fossil. Science *334*, 796–799.

Norstog, K.J. (1981). Karyotypes of *Zamia* (Cycadales). Caryologia *34*, 255–260.

Norstog, K., and Nicholls, T.J. (1997). The Biology of the Cycads (Ithaca, N.Y: Comstock Publishing Associates).

Nyffeler, R., Eggli, U., and Leuenberger, B.E. (1997). Noteworthy idioblastic sclereids in the stems of Eulychnia (Cactaceae). American Journal of Botany *84*, 1192–1197.

Pérez-Farrera, M.A., Vovides, A.P., Avendaño, S., and Herendeen, E.P.S. (2014). Morphology and Leaflet Anatomy of the *Ceratozamia norstogii* (Zamiaceae, Cycadales) Species Complex in Mexico with Comments on Relationships and Speciation. International Journal of Plant Sciences *175*, 110–121.

Pérez-Farrera, M.A., Vovides, A.P., Ruiz Castillejos, C., Galicia, S., Cibrian, A., and López, S. (2016). Anatomy and morphology suggest a hybrid origin of *Zamia katzeriana* (Zamiaceae). Phytotaxa *270*, 161-181.

Rao, T.A., and Bhattacharya, J. (1978). A Review on Foliar Sclereids in Angiosperms. Nelumbo *20*, 91–99.

Rao, T.A., and Bhupal, O. (1973). Typology of sclereids. Proceedings of the Indian Academy of Sciences - Section B *77,* 41–55.

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Salas-Leiva, D.E., Meerow, A.W., Calonje, M., Griffith, M.P., Francisco-Ortega, J., Nakamura, K., Stevenson, D.W., Lewis, C.E., and Namoff, S. (2013). Phylogeny of the cycads based on multiple single-copy nuclear genes: congruence of concatenated parsimony, likelihood and species tree inference methods. Annals of Botany *112*, 1263–1278.

Segalla, R., and Calonje, M. (2019). *Zamia brasiliensis*, a new species of *Zamia* (Zamiaceae, Cycadales) from Mato Grosso and Rondônia, Brazil. Phytotaxa *404*, 1–11.

Stevenson, D.Wm. (1990). Morphology and systematics of the Cycadales. Memoirs of the New York Botanical Garden *57*, 8–55.

Stevenson, D.Wm., Norstog, K.J., and Molsen, D.V. (1996). Midribs of Cycad Pinnae. Brittonia *48*, 67–74.

Vovides, A.P. (1983). Systematic studies on the Mexican Zamiaceae. I. Chromosome numbers and karyotypes. American Journal of Botany *70*, 1002–1006.

Vovides, A.P. (1991). Cone Idioblasts of Eleven Cycad Genera: Morphology, Distribution, and Significance. Botanical Gazette *152*(1): 91–99.

Vovides, A.P., and Galicia, S. (2016). G-fibers and Florin ring-like structures in *Dioon* (Zamiaceae). Botanical Sciences *94*(2)**:** 263–268.

Vovides, A.P., Avendaño, S., Pérez-Farrera, M.A., and Stevenson, D.W.M. (2012). What is *Ceratozamia brevifrons* (Zamiaceae)? Brittonia *64*, 35–42.

Vovides, A.P., Clugston, J., Gutiérrez-Ortega, J.S., Farrera, M.A., Tinoco, M.Y., and Galicia, S. (2018). Epidermal morphology and leaflet anatomy of *Dioon* (Zamiaceae) with comments on climate and environment. Flora - Morphology Distribution Functional Ecology of Plants *239*, 20–44.

Vovides, A.P., Norstog, K., Fawcett, P.K.S., Duncan, M., Nash, R., and Molsen, D. (1993). Histological changes during maturation in male and female cones of the cycad Zamia furfuracea and their significance in relation to pollination biology. Botanical Journal of the Linnean Society *111*, 241–252.

Vovides, A.P., Pérez-Farrera, M.A., Gutiérrez-Ortega, J.S., Avendaño, S., Medina-Villarreal, A., González-Astorga, J., and Galicia, S. (2020). A revision of the *Ceratozamia miqueliana* (Zamiaceae) species complex based on analyses of leaflet anatomical characters. Flora *270*, 151649.