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Abstract: Premise of research: The morphology of leaves is shaped by both historical and current selection acting on constrained developmental systems. For this reason, the phylogenetic signal of these characters is usually overlooked. **Methodology:** We investigate morphology of the leaflets of all genera of the Zamiaceae using multiple microscopical techniques to test whether leaf characters present a phylogenetic signal and whether they are useful to define clades at a suprageneric level. **Pivotal results:** Our investigation shows that most genera are quite uniform in their leaflet anatomy, with the largest genera (*Zamia*, *Encephalartos*) presenting the highest degree of variation. Using both Bayesian and parsimony methods on two different molecular scaffolds, we are able to show that leaflet anatomy has a strong phylogenetic signal in the Zamiaceae and that many clades retrieved by molecular analyses present potential synapomorphies in their leaflet anatomy. Particularly, the placement of *Stangeria* in a clade with *Zamia* and *Microcycas* is supported by the presence of both an adaxial and an abaxial girder sclerenchyma and the absence of sclerified hypodermis. The placement of *Stangeria* as sister to *Bowenia*, on the other hand, is not supported by our analysis. Instead, our results put into question the homology of the similar guard cell morphology in the two genera. **Conclusions:** We show that leaflet anatomy has a substantial amount of phylogenetic signal in the Zamiaceae, supporting relationships that are not supported by general morphology. Therefore, anatomical investigation represents a promising avenue for plant systematists.

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Evolutionary signal of leaflet anatomy in the *Zamiaceae*

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Keywords: Cycadales; morphology; xeromorphy; convergence; paedomorphosis.

Abstract

Premise of the Research: The morphology of leaves is shaped by both historical and current selection acting on constrained developmental systems. For this reason, the phylogenetic signal of these characters is usually overlooked.

Methodology: We investigate morphology of the leaflets of all genera of the *Zamiaceae* using multiple microscopical techniques to test whether leaf characters present a phylogenetic signal, and whether they are useful to define clades at a suprageneric level.

Pivotal results: Our investigation shows that most genera are quite uniform in their leaflet anatomy, with the largest genera (*Zamia*, *Encephalartos*) presenting the highest degree of variation. Using both Bayesian and Parsimony methods on two different molecular scaffolds, we are able to show that leaflet anatomy has a strong phylogenetic signal in the *Zamiaceae*, and that many clades retrieved by molecular analyses present potential synapomorphies in their leaflet anatomy.

Particularly, the placement of *Stangeria* in a clade with *Zamia* and *Microcycas* is supported by the presence of both an adaxial and abaxial girder sclerenchyma and the absence of sclerified hypodermis. The placement of *Stangeria* as sister to *Bowenia*, on the other hand, is not supported by our analysis. Instead, our results put into question the homology of the similar guard cell morphology in the two genera.

Conclusions: We show that leaflet anatomy has a substantial amount of phylogenetic signal in the *Zamiaceae*, supporting relationships that are not supported by general morphology. Therefore, anatomical investigation represent a promising avenue for plant systematists.

INTRODUCTION

Cycads represent an extremely charismatic group for both scientists and laypeople. Among seed plants, they are supposed to retain many plesiomorphic characters (Brenner *et al.* 2003). This, together with the (often superficial) resemblance of leaves of extant cycads with the relatively rare fossils of presumed Mesozoic relatives, has built their reputation as “living fossils”. Our modern understanding of the group paints a more dynamic picture: if some of the extant genera seem to have a deep history (Coiro and Pott 2017), most of the species diversity within genera seems to have originated much more recently than the presumed Mesozoic peak of disparity (Harris 1961), potentially postdating the Mid-Miocene climate change (Nagalingum *et al.* 2011; Condamine *et al.* 2015).

The systematics of Cycadales, which currently includes 10 genera, has traditionally seen the genus *Cycas* L. clearly segregated from the rest of the order, and belonging to its own family, Cycadaceae. However, the relationships between some of the more morphologically distinct genera, i.e. *Bowenia* Hook. and *Stangeria* T.Moore, with respect to the more “canonical” Zamiaceae have proven to be more problematic (Fig. 1). Johnson (1959) segregated *Stangeria* into its own family Stangeriaceae, including *Bowenia* within Zamiaceae. Stevenson (1981) subsequently separated the latter genus into its own family Boweniaceae based on the presence of a unique circinate pinna ptyxis. With the introduction of a formal phylogenetic analysis based on morphology, Stevenson (1990) retrieved a sister relationship between *Bowenia* and *Stangeria*. He later included both genera within his circumscription of Stangeriaceae, which was placed as sister to the Zamiaceae (Stevenson 1992). Stevenson (1992) also introduced subfamilial groups within Zamiaceae, with the Encephalartoideae including *Dioon* Lindl., *Encephalartos* Lehm., *Lepidozamia* Regel, and *Macrozamia* Miq., and the Zamioideae including *Ceratozamia* Brogn., *Microcycas* A.DC., *Zamia* L., and *Chigua* D.W.Stev., the latter of which has now been subsumed into *Zamia* (Lindstrom 2009).

Stevenson's morphology-based familial classification has been challenged by strong molecular evidence showing that the members of Stangeriaceae (*Stangeria* and *Bowenia*) are nested within Zamiaceae (Rai *et al.* 2003; Zgurski *et al.* 2008; Salas-Leiva *et al.* 2013). Moreover, the subfamilial classification of Stevenson (1992) also appears to be at odds with the molecular evidence: *Dioon* is consistently retrieved as sister to all other Zamiaceae; *Encephalartos*, *Lepidozamia* and *Macrozamia* form a strongly supported clade, with *Lepidozamia* sister to *Encephalartos*; the Zamioideae appear to be paraphyletic, with *Stangeria* nested within them either as sister to *Ceratozamia* (Nagalingum *et al.* 2011) or to *Microcycas* plus *Zamia* (Salas-Leiva *et al.* 2013). The placement of *Bowenia* is more uncertain, placed as either sister to all Zamiaceae except *Dioon* (Salas-Leiva *et al.*, 2013), sister to the *Macrozamia-Encephalartos-Lepidozamia* clade or sister to the *Ceratozamia-Stangeria-Microcycas-Zamia* clade (PHYP matrix of Nagalingum *et al.*, 2011). The inconsistent topologies revealed in different phylogenetic studies have led to the re-inclusion of *Bowenia* and *Stangeria* in Zamiaceae (Christenhusz *et al.* 2011).

The results from the molecular studies conflict with the phylogenies suggested by morphology, and open the possibility of convergent patterns masking the phylogenetic signal of morphology. In plants, both vegetative and reproductive traits can present high degrees of convergence: reproductive structures tend to converge on similar morphologies because of selection on pollination mode (Fenster *et al.* 2004), while leaves and other vegetative organs tend to vary in a similar, predictable way to the environment of the species (Wright *et al.* 2004, Gutiérrez-Ortega *et al.* 2018 for the cycad genus *Dioon*) In spite of these well-known patterns, morphology can still present a surprising degree of phylogenetic signal in plants (Coiro *et al.* 2018).

Many of the first anatomical studies of cycad leaflets were undertaken in order to advance the understanding of the relationship between fossil and extant taxa, with many authors focusing on

cuticular characters which can be retrieved in fossils (Thomas and Bancroft 1913; Florin 1933; Greguss 1968). Only a few authors stressed the importance of other aspects of the anatomy of the leaflets (e.g. Lamb 1923, Stevenson *et al.* 1996). Recently, a revival of the studies on leaflet anatomy has shown it to be a good source of characters to distinguish between species (Pérez-Farrera *et al.* 2014), as well as recovering a high level of uniformity in leaflet anatomy within genera, i.e. in *Cycas* (Griffith *et al.* 2014), and in *Dioon* (Barone Lumaga *et al.* 2015; Magellan *et al.* 2018; Vovides *et al.* 2018). However, little attention has been paid to the systematic value of leaflet anatomy at the suprageneric level.

Here, we conduct an investigation of leaflet anatomical characters across Zamiaceae, including all nine genera, employing traditional anatomical techniques as well as new techniques such as confocal microscopy. This allowed us to test the presence of variation in leaflet anatomy at both generic and suprageneric level. We then summarize the information present in the literature, and combine these with our observations to test hypotheses of congruence between the relationships supported by molecular data and the distribution of leaflet anatomical characters. In particular, we test whether leaflet anatomy presents synapomorphies for the major groups in the Zamiaceae, and whether it supports the placement of *Stangeria* in the *Ceratozamia-Microcycas-Zamia* clade.

MATERIALS AND METHODS

Sampling

Leaflet samples were collected from plants grown at the Botanical Garden of the University of Naples, Naples, Italy (NAP), the Montgomery Botanical Center in Coral Gables, Florida, USA (MBC), the Botanical Garden of the University of Zurich, Zurich, Switzerland (UZH), the Nong-Nooch Botanical Garden, Thailand (NN), the Fairchild Tropical Botanical Garden, Florida, USA (FTBG), the Jardín Botánico Francisco Javier Clavijero (JBC), and from private collections. We

sampled either whole leaflets when possible or sections of the median part of the leaflets. All observations were conducted on the median part of the leaflets. We sampled all extant genera of the Zamiaceae, including both species of *Bowenia*, and the monotypic *Microcycas* and *Stangeria*, as well as species of *Ceratozamia* (5 species sampled), *Dioon* (9 species sampled), *Encephalartos* (11 species sampled), *Lepidozamia* (1 species sampled), *Macrozamia* (5 species sampled), and *Zamia* (21 species sampled). A full list of the samples is given in Table S1. The literature was also searched for other treatments on cycad leaflet anatomy, and characters were scored based on observation of illustrated sections.

Material preparation

Samples of leaflets for embedding were cut to ~1 cm² pieces using a razor blade and fixed in formalin acetic acid alcohol (10:5:50) (FAA) overnight, then transferred to 70% ethanol for preservation. For observation of external surfaces using SEM, samples were fixed in FAA or in ethanol 50% (the fixation with ethanol reduces costs and toxicity of the reagents used, producing, at the same time, excellent samples for scanning electron microscopy observation), then dehydrated in a graded ethanol series and critical-point dried in liquid CO₂. Cuticles for SEM observation were prepared by treating samples with 20% Cr₂O₃ (Alvin and Boulter 1974) for up to 72 h. Isolated cuticles were then washed several times in distilled water, mounted on aluminium stubs and air dried.

Sectioning

Hand sections were cut from leaf samples using a hand microtome. Unstained sections were then transferred into glycerol for imaging, and then transferred into Hoyer's medium (prepared according to Coiro and Truernit (2017)) for permanent mounting. Sections for staining were mounted directly in Hoyer's or mounted in water for imaging (Phloroglucinol).

Staining and histochemistry

Hand sections were stained using Pseudo-Schiff Propidium Iodide (PS-PI) staining according to the protocol of Coiro and Truernit (2017) to identify mucilage canals.

Freshly made hand sections were stained using a solution of 0.1 g Phloroglucinol (Sigma) in 100 ml 95% Ethanol, where 12 ml of concentrated HCl were added. Sections were left to incubate for 15—30 minutes and then quickly transferred to glycerol for observation.

Light and Fluorescence microscopy

All hand-cut sections were observed with a Zeiss Axioscope and photographed with a Zeiss AxioCam HRc with a Zeiss HF wide-spectrum fluorescence filter.

Confocal microscopy

Processing for confocal microscopy was conducted as previously described in Barone Lumaga et al. (2015). PS-PI stained samples of *Stangeria eriopus* (Kunze) Baill. and *Bowenia spectabilis* Hook. ex Hook.f. were observed using a Leica TCS SP8 microscope. Excitation was obtained using a 488 nm and 405 nm diode laser.

Scanning electron microscopy

All samples for SEM were coated to ca. 25 nm with gold. Specimens were observed under a FEI Quantas 200 ESEM at an accelerating voltage of 25 kV.

Image analysis

Raw images were analysed and measured using the software FiJi (Schindelin *et al.* 2012), with brightness and contrast adjusted using the “auto” option in the software.

Phylogenetic analysis

To reconstruct the evolution of characters on the phylogeny of the Zamiaceae, we estimated a dated phylogeny of the order Cycadales. We used the molecular matrix of (Condamine *et al.* 2015) (including two plastid loci (*matK* and *rbcL*) and one nuclear locus (*PHYC*)). This matrix was trimmed to only include crown-group Cycadales, then polished by removing synonymous taxa (as per the World List of Cycads), and new sequences for *matK* and *rbcL* were integrated using data from (Clugston *et al.* 2016) (listed in Table S2) and aligned with the rest of the sequences by hand. This resulted in an alignment of 228 taxa.

This matrix was then analysed using BEAST2 as implemented on the Cipres science Gateway (Drummond and Rambaut 2007; Suchard and Rambaut 2009; Miller *et al.* 2010). The input XML

file was generated using BEAUTi for BEAST2. The three markers were analysed under separate site model partitions, while the clock model was linked between the two plastid markers. Site models were averaged for each gene partition using bModelTest (Bouckaert and Drummond 2017). The uncorrelated lognormal relaxed clock was implemented for both clock partitions. A birth-death prior was used for the tree, as it has been demonstrated to be the best prior for the parent dataset (Condamine *et al.* 2015). Four node calibrations were employed, all implemented using uniform distributions. The root of the Cycadales was calibrated with the age of the fossil sporophyll *Crossozamia* (Gao and Thomas 1989) as the minimum age constraint (265.1 mya, middle Permian) and the oldest age for the Carboniferous (358.9 mya) as a maximum age constraint, representing the oldest crown group fossils of the seed plants. The age of *Crossozamia* was also used as a conservative maximum on all other calibrations. The stem group of *Bowenia* was calibrated using the Eocene fossils *Bowenia eocenica* Hill and *Bowenia papillosa* Hill (Hill 1978), with a minimum age of 33.9 mya. The split between *Encephalartos* and *Lepidozamia* was calibrated using the Eocene fossil *Lepidozamia foveolata* Hill (Hill 1980) (minimum age 33.9 mya). The split between the *Encephalartos-Lepidozamia* clade and *Macrozamia* was calibrated using the fossil *Austrozamia stockei* from the Early Eocene Laguna del Hunco locality (Wilf *et al.* 2016) (minimum age 52 mya). We used this fossil to calibrate this node instead of the stem of Encephalartinae (as suggested by Wilf *et al.* 2016) because the extreme cuticular similarity between *Encephalartos* and *Lepidozamia* and the divergent morphology of *Macrozamia* (see Coiro & Pott 2017) seem to suggest that *Austrozamia* is closer to the *Encephalartos-Lepidozamia* clade than to *Macrozamia*. The split between *Zamia* and *Microcycas* was calibrated using *Zamia nelliae* from the Eocene of Panama (Erdei *et al.* 2018). All nodes corresponding to dating calibrations were constrained to be monophyletic. For the topology of the tree, two different analyses were run: one with a mostly unconstrained topology (with the exception of the calibrated nodes) and another with *Stangeria* constrained as sister to *Microcycas* and *Zamia* (as per (Salas-Leiva *et al.* 2013)), the Caribbean

Zamia as sister to the mainland *Zamia* (as per (Calonje *et al.* 2019)), and *Dioon* as sister to the other *Zamiaceae*.

Two separate chains were run for 10000000 generations sampling every 5000th generation for each of the two analyses. Convergence was checked by looking at the effective sample size (ESS) of the parameters of the mcmc using Tracer (Rambaut *et al.* 2018), waiting for ESS higher than 200. Tree posterior were then combined by removing the first 10% as burn-in and subsampling to the 100000th generation using LogCombiner. Maximum Clade Credibility (MCC) trees were obtained using TreeAnnotator as implemented on the Cipres Science Gateway.

Ancestral state reconstruction

To reconstruct the evolutionary history of the characters identified here we employed stochastic character mapping (Bollback 2006). First, we converted the summary table of the traits (Table 1) to a csv format. We then modified character scoring by coding discontinuous adaxial sclerified hypodermis as absent, coding parenchymatous abaxial girder sclerenchyma as simply present, and splitting the palisade parenchyma character into a lobation character (lobed, unlobed, undifferentiated) and a character representing the number of layers (one, two, many). To test the potential homologies of the multiple canals of *Ceratozamia*, we employed alternative coding schemes: in the first, we coded the presence of one large canal between the vascular bundles and multiple smaller canals as separate states (coding 1), while in the second they were merged in a single state (canals between vascular bundles) (coding 2). We then trimmed the trees from the unconstrained and constrained analyses to only include the species with the coded characters. These trees were then used to reconstruct the evolutionary history of all the characters identified using the function `make.simmap` from the package `phytools` (Revell 2012). First, the best model for the substitution matrix was selected for each character by obtaining the corrected Akaike Information Criterion (AICc) using the `fitDiscrete` function from the package `geiger` (Harmon *et al.* 2008). 100 replicates were run using the best model (i.e. with the lowest AICc or, in case of

identical AICc, the lowest number of parameters, AICc are found in Table S3). The two trimmed trees were also used to reconstruct trait evolution using parsimony and compute both Consistency indices and Retention indices in Mesquite (Maddison and Maddison 2005). Lambda values for all characters were inferred using the function `fitDiscrete` from the package `geiger` (Harmon *et al.* 2008), implementing the best model found for the substitution matrix. We also tested whether the number of parsimony steps observed differed significantly from a random null obtained by randomizing the character state at the tips (Maddison and Slatkin 1991) using a custom script from Bush *et al.* (2016). The randomization was run for 999 replications.

RESULTS

Anatomical descriptions

Epidermal pavement cells

The epidermis of the leaflets is usually composed of sclerified, thick-walled pavement cells (Fig. 2).

All genera present cells with thinner walls interspersed with the thick-walled cells. These thin-walled cells present a thicker cuticle, which is clearly visible both in SEM images and fluorescence pictures when the cuticle is isolated. In *Stangeria*, sclerified thick-walled pavement cells are as common as thin-walled cells in the adaxial epidermis (Fig. 3I). In *Ceratozamia*, the abaxial epidermis also presents larger thick-walled cells with wider lumina interspersed between the other pavement cells ('macrolumen cells') (Fig. 3F).

Hypodermis

A continuous sclerified hypodermis on the adaxial side of the leaflets is present in *Dioon*, *Encephalartos*, *Macrozamia* and *Lepidozamia* (Fig. 2A, Fig.3A, C-E), as well as in some members of *Zamia* such as *Zamia meermanii* Calonje, *Zamia pseudoparasitica* J.Yates, and *Zamia furfuracea* L.f., while is absent in *Ceratozamia*, *Stangeria*, *Bowenia*, *Microcycas*, *Macrozamia heteromera* C.Moore and all the other species of *Zamia* for which data are available (Fig 3B,F-I). The thickness of the adaxial hypodermis varies from one cell thick (as in *Lepidozamia hopei* Regel, *Encephalartos manikensis* (Gilliland) Gilliland, and *Zamia meermanii*) to several cells thick (as in

Encephalartos horridus (Jacq.) Lehm.). On the abaxial side, a continuous or semi-continuous sclerified hypodermis in the intercostal regions is present in *Encephalartos*, *Lepidozamia* and *Macrozamia* sect. *Macrozamia*.

Stomatal apparatus

The guard cells of all genera present an almost constant structure: the dorsal wall presents a substantial thickening in the medial section, and the cytoplasm is constricted to an oblique band towards the ventral side. Most of the cytoplasm of the guard cell is concentrated in the poles, that are distally raised with respect to the aperture (Fig. 4A,B,D). Thin-walled subsidiary cells with ample vacuoles are present in all genera. These cells are adjacent to the guard cells on the dorsal side. Encircling cells (mostly thick-walled) are usually placed distally to the subsidiary cells and form an anticlinally-oriented pile (Fig.4A-C). The number of encircling cells is variable within the same leaflet, with *Dioon* consistently having three layers, *Encephalartos* and *Macrozamia* having a variable number between one and two layers, with one layer being more common, and *Lepidozamia*, *Ceratozamia*, *Zamia*, *Microcycas* and *Stangeria* having one layer. Both subsidiaries and encircling cells are arranged laterally to the guard cells. Polar cells of the stomatal apparatus tend to be slightly differentiated from normal pavement cells, and in some genera (*Dioon*, *Encephalartos*, *Ceratozamia*) extend over the poles of the guard cells. Guard cells are sunken in a stomatal pit in all genera except *Bowenia* and *Stangeria* (Fig. 5A,B,E,F), where the guard cells are at the same level as the other pavement cells. In *Stangeria*, the encircling cells are not arranged distally to the subsidiaries, but they surround them laterally (Fig. 5C,D). The stomatal apparatus of *Bowenia* lacks encircling cells (Fig. 5C,E), but presents specialized subsidiary cells with lateral cuticular intrusion on the dorsal side.

Mesophyll

All species examined present a clear differentiation between an adaxial palisade and spongy parenchyma in the mesophyll, with the exception of the two species of *Bowenia*, where the mesophyll is composed of undifferentiated, isodiametrical cells, and species of *Macrozamia* sect. *Parazamia*, where a slightly differentiated palisade-like layer is present both adaxially and abaxially, and surrounds more or less undifferentiated parenchyma. Some species of *Encephalartos* and *Macrozamia* also present an abaxial palisade layer (Fig. 3E). In *Dioon*, *Encephalartos*, *Lepidozamia* and *Macrozamia* sect. *Macrozamia*, the palisade is composed of one layer of elongated cells (I-cells). In *Ceratozamia*, the palisade is composed of one layer of isodiametric cells, with relatively thick walls. In *Zamia* and *Stangeria*, the palisade is composed of one or two layers of isodiametrical, lobed cells (H-cells). Fibers are interspersed in the mesophyll of most species, being absent only in *Encephalartos humilis*, *Microcycas*, *Stangeria*, and some species of *Zamia*.

Mucilage canals

The distribution and morphology of mucilage canals presents interesting variation at both the infra- and suprageneric level. Species of *Dioon* present large mucilage canals associated with the adaxial side of the vascular bundles (Fig. 3A). Species of *Lepidozamia*, *Encephalartos* (with the exceptions of *E. villosus* Lem. and the closely related *E. umbeluziensis* R.A.Dyer, as well as *E. humilis* I. Verd., *E. laevifolius* Stapf & Burtt Davy and *E. lanatus* Stapf & Burtt Davy,), and *Macrozamia* sect. *Macrozamia* present a single large mucilage canal placed between the vascular bundles, in a median to adaxial position. In *Bowenia*, the mucilage canals are only present in the contracted base of the leaflets, and are placed adaxially but not associated with the vascular bundles; the lamina part of the leaflets does not present mucilage canals. In *Ceratozamia*, small mucilage canals are present between the vascular bundles (Fig. 6A,B); these are distributed more or less randomly in the spongy parenchyma. *Stangeria* presents mucilage canals in the midrib portion of the leaflet, distributed both

adaxially and abaxially and interspersed in the ground tissue (Fig. 6C,D); the lamina is however devoid of mucilage canals (Fig. 3I). *Zamia*, *Microcycas* and members of *Macrozamia* sect. *Parazamia* do not present any mucilage canals in the leaflets.

Vascular bundles

The vascular bundles are rather uniform in all genera, presenting exarch xylem and a bundle sheath with interspersed fibers. In *Stangeria*, some species of *Zamia*, and *Microcycas*, sclerified adaxial bundle sheath extensions (i.e. adaxial girder sclerenchyma) are present (Fig. 3G-I). In *Zamia*, this can be found in all *Zamia* from the Caribbean clade (*sensu* Calonje et al. 2019), as well as species from the Mesoamerica and South America clade, but seems to be absent from all species from the Isthmus clade with the exclusion of the acuminata clade. Abaxial girder sclerenchyma is present in all genera with the exception of *Bowenia*. In *Ceratozamia* it is only present in *C. norstogii* D.W.Stev., *C. zoquorum* Pérez-Farr., Vovides & Iglesias and *C. mirandae* Vovides, Pérez-Farr. & Iglesias, incipient in *C. mexicana* Brongn. (one layer) and is absent in the other species investigated. It is also absent in *Zamia vazquezii* D.W.Stev., Sabato & De Luca. In some species of *Zamia*, especially those with plicate leaflets, the abaxial girder tissue is of collenchymatous nature. Tissue interpretable as transfusion tissue is present around the bundles of all genera. In *Dioon*, *Encephalartos*, *Lepidozamia*, *Macrozamia*, *Bowenia* and some species of *Ceratozamia*, this transfusion-like tissue is clearly differentiated, and tracheid-like cells are present also on the phloem pole of the bundles (Fig. 7).

Phylogenetic signal and ancestral state reconstruction

Both phylogenetic trees inferred are of comparable age, though with older splits compared to Condamine *et al.* (2015). Full trees are shown in Figure S1.

All characters considered here present a strong phylogenetic signal on both topologies, as indicated both by the estimates of Pagel's Lambda (Table 2) and the parsimony-based shuffling test (Table 3). Both Consistency index and Retention index indicate a low level of homoplasy, with the constrained tree explaining the evolution of the abaxial girder sclerenchyma, the fibers in the mesophyll and the abaxial transfusion tissue better than the unconstrained tree (Table 4). The character history reconstruction shows that most of the transitions happened at the internal nodes (Fig. 8).

The evolutionary history of the adaxial hypodermis, the abaxial hypodermis, the abaxial girder sclerenchyma, the lobation of the parenchyma, the number of parenchyma layer and the abaxial transfusion tissue is reconstructed consistently between the different methods and topology (Fig. 8, Fig. S2-4). The history of the adaxial girder sclerenchyma is reconstructed very differently between stochastic mapping and parsimony, with the former reconstructing the presence of this character at all the stem nodes of the Zamiaceae (Fig. 8, Fig S2 B) while the latter reconstructs its gain either somewhere at the base of the *Ceratozamia-Zamia* clade (unconstrained topology) or at the most recent common ancestor (MRCA) of *Stangeria* and *Zamia* (constrained topology). This is mostly due to the maximum-likelihood estimation of the Q-matrix for this character having a transition rate of 0 from absent to present, i.e. the model does not allow for any gain of the character. The remaining characters are mostly consistent between methods, with only minor inconsistencies (Fig. 8).

The ancestral node of the Zamiaceae and the MRCA of *Encephalartos* and *Zamia* are reconstructed as having the same character states, namely the presence of an adaxial sclerified hypodermis, an abaxial girder sclerenchyma, fibers in the mesophyll, and an abaxial transfusion tissue, an unlobed palisade with a single layer of cells, and a non-sclerified abaxial hypodermis. The other characters are reconstructed with a high degree of ambiguity, with the exception of the adaxial girder

sclerenchyma that is reconstructed as present by stochastic mapping and absent by parsimony (Fig. 8A).

The origin of the Encephalarteae (*Macrozamia*, *Lepidozamia*, and *Encephalartos*) correspond with the origin of an abaxial sclerified hypodermis and (but less clearly) a single mucilage canal between veins. An adaxial girder sclerenchyma was highly probably absent, while most other characters remained unchanged with respect to the parent node (Fig. 8B). The MRCA of the *Ceratozamia*-*Zamia*-*Stangeria* clade underwent the loss of two characters; mucilage canals (though the condition is more uncertain with the assumption of primary homology between the multiple small canals of *Ceratozamia* and the single canals of the Encephalarteae) and the adaxial hypodermis. While stochastic mapping favours the retention of most ancestral states at this node, parsimony is more ambiguous (Fig. 8B).

In the constrained topology, the *Stangeria*-*Zamia* clade is subtended by the gain of an adaxial girder sclerenchyma (according to parsimony) and the loss of fibers in the mesophyll (regained by a few clades within *Zamia*, Fig. S4) and abaxial transfusion tissue (though the stochastic mapping is more ambiguous than parsimony). Whether the loss of mucilage canals in this clade is a genuine synapomorphy or an independent loss depends on both coding and method, with the stochastic mapping reconstruction of coding scheme 2 preferring the independent loss scenario (Fig. 8C).

DISCUSSION

Our results indicate that leaflet anatomy presents a degree of variation in the Zamiaceae. All genera present a rather uniform leaflet anatomy, in agreement with previous studies (Barone Lumaga *et al.* 2015; Magellan *et al.* 2018; Vovides *et al.* 2018). However, the genera *Zamia*, *Encephalartos*, and *Macrozamia* present a higher degree of variation, with the first presenting the highest variability.

Unfortunately, given the radically different architecture of the leaflets of *Cycas*, it is hard to identify homologies between the characters identified here and the ones described for that genus (Griffith *et al.* 2014). The presence of both thin and thick walled epidermal cells seems to represent a genuine synapomorphy of the Zamiaceae as a whole, as previously suggested based on cuticular evidence (Coiro and Pott 2017). Some of the characters of the mesophyll of *Cycas*, such as the absence of both an adaxial and abaxial sclerified hypodermis, could be homologous to the conditions present in some genera of the Zamiaceae. However, the different structure of the cycadacean leaflet, with its midrib acting as the main source of structural support, could also explain the absence of such structural tissue in the lamina.

Congruence between leaflet anatomy and molecular phylogenies

Some of the characters identified in this study are variable between genera and present some potential to delimit subfamilial clades. The members of the tribe Encephalarteae *sensu* Stevenson (1992) (i.e. *Encephalartos*, *Macrozamia*, and *Lepidozamia*) share some potentially synapomorphic characters such as the presence of large mucilage canals in the areas between the vascular bundles and the presence of an adaxial sclerified hypodermis. These are reconstructed as ancestral in the clade by both Parsimony and stochastic mapping approaches on both topologies (Fig. 8A). Moreover, only among members of this clade is it possible to find fully bifacial leaflets, with an adaxial palisade parenchyma and adaxial stomata. These are quite common in *Macrozamia* sect. *Macrozamia* (*M. moorei* F.Muell., *M. macdonnellii* (F.Muell. ex Miq.) A.DC., *M. riedlei* (Gaudich.) C.A.Gardner etc.). The main deviation from the “bauplan” of the Encephalarteae is present in the members of *Macrozamia* sect. *Parazamia*: these species lack clear mucilage canals in the lamina as well as continuous hypodermis, and their mesophyll lacks differentiation between palisade and spongy parenchyma (Johnson 1959, Carpenter 1991). These species are thought to be neotenous derivatives (Carpenter 1991), and the derived condition of their leaflets is supported by our analyses (Fig. S2-4). Within the Encephalarteae, the genus *Lepidozamia* and some members of

Encephalartos are extremely hard to distinguish based on their leaflet anatomy, both sharing a single-layered hypodermis, short palisade cells, few fibers between the bundles and relatively shallow guard cells. This supports the close relationship between these two genera suggested by molecular phylogenies.

Our investigation also supports the separation between *Dioon* and the Encephalarteae. The leaflet characters that are shared between these two groups (presence of an adaxial sclerified hypodermis, adaxial girder sclerenchyma, fibers in the mesophyll, and adaxial transfusion tissue) are reconstructed as plesiomorphic for the Zamiaceae as a whole. On the other hand, the presence of mucilage canals associated with the bundles, non-lignified fibers (Magellan *et al.* 2018), and three layers of encircling cells in their stomatal apparatuses (Barone Lumaga *et al.* 2015; Vovides *et al.* 2018) clearly sets *Dioon* apart from the other genera, consistent with its isolated phylogenetic placement and its long independent history (Condamine *et al.* 2015).

Leaflet anatomy supports a close relationship between Stangeria and Zamia-Microcycas
Leaflet anatomy also shows unexpected similarities between *Zamia*, *Microcycas* and the morphologically diverging *Stangeria*. These three genera share the presence of both abaxial and adaxial girder sclerenchyma (even if the latter is variable within *Zamia*), the absence of mucilage canals in the leaflet lamina, abaxial transfusion tissue, and fibers in the mesophyll (present in some species of *Zamia*). Moreover, *Stangeria* and *Zamia* share the presence of lobed, H-type palisade parenchyma, while *Microcycas* present a I-type palisade. Given the uncertainty in the placement of *Stangeria* and the old age of the split between the genera in this group, the history of trait evolution is more ambiguous than in the Encephalarteae, with methods and topologies giving different answers (Fig. 8B,C). However, the constrained topology with *Stangeria* as sister to *Microcycas* and *Zamia* seems to better explain the evolution of leaflet characters than the unconstrained topology with *Stangeria* as sister to *Ceratozamia* (Table 4). Our results suggest that a reinvestigation of other

anatomical aspects of *Stangeria* might still reveal characters validating its placement within Zamiaceae.

Surprisingly, *Ceratozamia* seems to share very few anatomical characters with *Zamia*, *Microcycas*, and *Stangeria*. This runs contrary to the results obtained using general leaf morphology, with *Ceratozamia* sharing articulated leaflets and the presence of stipules with *Zamia* and *Microcycas* (but not *Stangeria*, where leaflets are decurrent and the base of the leaves is surrounded by a cowl-like structure interpreted as vascularized stipules). However, it would be coherent with a long, independent history of *Ceratozamia* as retrieved by molecular dating analyses (Salas-Leiva *et al.* 2013; Condamine *et al.* 2015).

Leaflet anatomy does not support Stangeriaceae

Our reinvestigation identifies some shared characters between *Bowenia* and *Stangeria*, that is the absence of mucilage canals in the lamina and the presence of stomata with guard cells at the same level as the epidermal pavement cells (‘flush’ guard cells). The latter character state separates these two genera from all other genera of the Cycadales, which present stomata more or less sunken with respect to the epidermis (Fig. 4). It is also quite rare in fossil leaves assigned to the Cycadales, being limited to *Eobowenia* (*Almargemia*) *incrassata* M.Coiro & C.Pott and *Mesodescolea plicata* S.Archang. from the Early Cretaceous of Argentina. The former has been hypothesized to be a stem-relative of *Bowenia* (Coiro and Pott 2017), and the latter has been linked by its authors with *Stangeria* (Archangelsky 1963; Archangelsky and Petriella 1971; Artabe and Archangelsky 1992). Coiro and Pott (2017) suggested on the basis of cuticular anatomy that the stomatal apparatus in *Bowenia* was monocyclic, i.e. lacked encircling cells, while the stomatal apparatus of *Stangeria* more closely resembled that found in other cycads in being dicyclic. Our observations confirm this inference, clearly showing that the stomatal apparatuses of *Bowenia* and *Stangeria* are constructed in a radically different way (Fig. 5C-F). Another stomatal character which was thought to link *Stangeria* and *Bowenia* as well as *Mesodescolea* was the presence of “circular” stomata. However,

our investigation reveals that the morphology of the guard cells in *Stangeria* and *Bowenia* conform to the elongated morphology of all other members of the Cycadales (Fig. 5C,D), and cannot really be homologized with the “circular” stomata present in many angiosperms or ferns. Besides the stomatal apparatus, the leaflets of *Bowenia* and *Stangeria* are extremely divergent. *Bowenia* presents leaflets with an undifferentiated mesophyll of densely packed parenchyma cells and fibers, while *Stangeria* has a clearly differentiated palisade and spongy parenchyma without fibers. Moreover, the bundles in *Stangeria* are associated with bundle sheath extensions, while in *Bowenia* the bundles are free, and neither adaxial nor abaxial girder sclerenchyma is present. Such divergence in leaflet anatomy seems to be in stark contrast with the characters identified from morphological and anatomical studies of the whole plants, with many synapomorphies identified for the Stangeriaceae (Stevenson 1992). However, if some of these characters are harder to interpret as independent gains, such as the presence of root buds and vascularized stipules, others are more easily explained as parallelisms or convergence. For example, the absence of an omega pattern in the rachis, which has been recently connected to the hydraulic need of a simple pinnate leaf (Tomlinson *et al.* 2018), is expected given the different functional needs of a bipinnate leaf (such as in *Bowenia*) and a leaf with multiple-veined midribs (such as in *Stangeria*). A re-evaluation of these characters, perhaps in a developmental framework, could help to disentangle the selective or developmental causes of these striking apparent convergences.

Paedomorphic shifts and convergence

Some characters shared by *Bowenia*, *Ceratozamia*, and *Macrozamia* sect. *Parazamia* are mainly related to reduction, such as the lack of mucilage canals in the lamina, the lack of abaxial girder sclerenchyma, and the absence of a sclerified hypodermis. In *Macrozamia* sect. *Parazamia*, these losses were probably a result of neoteny (Johnson 1959; Carpenter 1991). Recently, paedomorphosis has been suggested as an important mechanism acting in the evolution of *Ceratozamia* (Medina-Villarreal *et al.* 2019). In the case of *Bowenia*, a paedomorphic origin could

also explain some of the other peculiar traits of this genus, such as the production of a single leaf per growing season, the irregular production of cataphylls, and the subterranean tuberous habit. Progenesis (i.e. an early onset of mature characteristics during development) seems to fit the pattern of wood development in *Bowenia* better than neoteny, given that *Bowenia* completely lacks the scalariform tracheids that characterize the xylem of juvenile cycads (Chrysler 1937). However, the absence of close living relatives and the old divergence of *Bowenia* from the other Zamiaceae (Coiro and Pott 2017) makes testing this hypothesis rather difficult. The small size of the leaflets in the close relative *Eobowenia* might represent another indication of progenetic reduction, but a more detailed understanding of the morphology of other fossil relatives as well as a deeper understanding of the development of extant *Bowenia* is required to solve this puzzle.

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Figure Captions

Figure 1 Comparison of some of the relationships between cycads inferred using molecular and morphological data. The tree on the left represents the relationships found in Salas-Leiva et al. (2013), while the tree on the right represents the relationships found in Stevenson (1990).

Figure 2: details of the adaxial epidermis, indicating the presence of two kinds of epidermal pavement cells. A) *Lepidozamia hopei*, B) *Microcycas calocoma*, C) *Ceratozamia mexicana*. th: thin-walled cells; tk: thick-walled cells; pal: palisade parenchyma; hyd: hypodermis. Scale bars: 50 μm .

Figure 3: Autofluorescence pictures illustrating the anatomy of the leaflets for representatives of all genera of the Zamiaceae. Sections are shown with adaxial side up. A) *Dioon rzedowskii*, B) *Bowenia serrulata*, C) *Lepidozamia hopei*, D) *Encephalartos horridus*, E) *Macrozamia moorei*, F) *Ceratozamia robusta*, G) *Microcycas calocoma*, H) *Zamia erosa*, I) *Stangeria eriopus*. hyd: sclerified hypodermis; xy: xylem; ph: phloem; mc: mucilage canal; bse: bundle sheath extension. White arrows indicate stomata. Scale Bars = 100 μm .

Figure 4: Details of the stomatal apparatus of the Zamiaceae in section. A,B) *Macrozamia communis*, C,D) *Encephalartos villosus*. All three stomata shown present thin-celled, vacuolate subsidiary cells (sc) contacting the thick-walled guard cells (gc) and the encircling cells (ec). D) Paradermal sectioning shows the cytoplasm of the guard cells concentrated at the poles (gccy). A,B and D represent optical slices from confocal stacks, with A and B coming from the same stomatal apparatus. Images A and B are shown at the same scale. Scale Bar = 50 μm .

Figure 5: Comparative illustrations of the stomatal apparatus in *Bowenia* and *Stangeria*. A,C and E) *Bowenia spectabilis*; B, D and F) *Stangeria eriopus*. *Bowenia* has monocyclic stomatal apparatuses with only subsidiary cells (sc), while *Stangeria* presents lateral encircling cells (ec). A and B are SEM images of outer leaf surfaces, while C, D, E, and F) are confocal images of PS-PI stained samples. Scale Bar = 100 μm .

Figure 6: Identification of mucilage canals illustrated using PS-PI stained sections. A and B show the small mucilage canals in the lamina of *Ceratozamia robusta*, C and D show the canals scattered in the midrib of *Stangeria eriopus*. A and C are transmitted light image, B and D are fluorescent images. Scale Bar = 100 μ m.

Figure 7: Phloroglucinol stained hand sections of leaflets illustrating the differentiation of the transfusion tissue associated with the vascular bundle at the phloem pole. A) *Macrozamia macdonnellii*; B) *Bowenia serrulata*; C) *Lepidozamia hopei*. Arrows indicate transfusion tracheids associated with the phloem pole of the vascular bundles. Transfusion tracheids are further highlighted by the dashed line. Scale Bar = 100 μ m.

Figure 8: Summary of the ancestral state reconstruction analyses on A) the ancestral nodes of the Zamiaceae, B) the ancestral node of Encephalarteae and the *Ceratozamia-Zamia-Stangeria* clade, and C) the *Stangeria-Zamia* clade. Circles represent results of the stochastic mapping analysis while squares represent parsimony reconstruction on the unconstrained tree (A,B) or the constrained tree (C). Colour legend: for characters 1-4 and 9-10, cyan= present and yellow=absent; character 5, red=one large, blue=multiple, cyan=associated with veins, yellow=absent; character 6, blue=between veins, cyan=associated with veins, yellow=absent; character 7, red=unlobed, yellow=lobed, cyan=undifferentiated; character 8, blue=zero, cyan=one, red=two, yellow=multiple.

Table 1

	Adaxial Sclerified Hypodermis	Abaxial Sclerified Hypodermis	Adaxial Girder Schlerenchyma	Abaxial Girder Schlerenchyma	Mucilage canals in the lamina	Palisade parenchyma	Fibers in the mesophyll	Tranfusion tracheids in the phloem pole
<i>Bowenia serrulata</i>	Absent	Absent	Absent	Absent	Absent	Undifferentiated	Present	Present
<i>Bowenia spectabilis</i>	Absent	Absent	Absent	Absent	Absent	Undifferentiated	Present	Present
<i>Ceratozamia alvarezii</i> *	Absent	Absent	Absent	Absent	Multiple	One layer	Present	?
<i>Ceratozamia becerrae</i>	Absent	Absent	Absent	Absent	Multiple	One layer	Present	?
<i>Ceratozamia brevifrons</i> *	Absent	Absent	Absent	Absent	Multiple	One layer	Present	Present
<i>Ceratozamia chimalapensis</i> *	Absent	Absent	Absent	Absent	Multiple	One layer	Present	Present
<i>Ceratozamia hildae</i>	Absent	Absent	Absent	Absent	Multiple	One layer	Present	Present
<i>Ceratozamia mexicana</i>	Absent	Absent	Absent	Absent	Multiple	One layer	Present	Present
<i>Ceratozamia mirandae</i> *	Absent	Absent	Absent	Present	Multiple	One layer	Present	Present
<i>Ceratozamia norstogii</i>	Discontinuous	Absent	Absent	Present	Multiple	One layer	Present	Present
<i>Ceratozamia robusta</i>	Absent	Absent	Absent	Absent	Multiple	One layer	Present	Present
<i>Ceratozamia zoquorum</i>	Absent	Absent	Absent	Present	Multiple	One layer	Present	?
<i>Dioon angustifolium</i> *	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon argenteum</i> *	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon califanoi</i>	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon caputoi</i>	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon edule</i>	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon holmgrenii</i>	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon mejiae</i>	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon merolae</i>	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present

<i>Dioon purpusii</i>	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon rzedowskii</i>	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon sonorensis</i> *	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon spinulosum</i>	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon stevensonii</i> *	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Dioon tommasellii</i> *	Present	Absent	Absent	Present	Associated with the bundle	One layer	Present	Present
<i>Encephalartos altensteinii</i>	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos caffer</i> *	Present	Present	Absent	Present	One large	?	?	?
<i>Encephalartos cupidus</i> *	Present	Present	Absent	Present	One large	One layer	Absent	Present
<i>Encephalartos cycadifolius</i> *	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos eugene-maraisii</i> *	Present	Present	Absent	Present	One large	One layer	Absent	Present
<i>Encephalartos ferox</i>	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos friderici-guilielmi</i> *	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos ghellinckii</i> *	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos heenani</i> *	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos horridus</i>	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos humulis</i> *	Present	Present	Absent	Present	Absent	One layer	Absent	Present
<i>Encephalartos inopinus</i> *	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos laevifolius</i> *	Present	Present	Absent	Present	Absent	One layer	Present	Present
<i>Encephalartos lanatus</i> *	Present	Present	Absent	Present	Absent	One layer	Present	Present
<i>Encephalartos latifrons</i> *	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos laurentianus</i>	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos lebomboensis</i> *	Present	Present	Absent	Present	One large	?	?	?

<i>Encephalartos lehmanii</i>	Present	Present	Absent	Present	One large	One layer abaxial, one adaxial	Present	Present
<i>Encephalartos longifolius</i> *	Present	Present	Absent	Present	One large	?	?	?
<i>Encephalartos macrostrobilus</i>	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos manikensis</i>	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos princeps</i> *	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos trispinosus</i> *	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos sclavoi</i>	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos senticosus</i>	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos transvenosus</i>	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Encephalartos umbeluziensis</i>	Present	Present	Absent	Present	Absent	One layer	Present	Present
<i>Encephalartos villosus</i>	Present	Present	Absent	Present	Absent	One layer	Present	Present
<i>Lepidozamia hopei</i>	Present	Present	Absent	Present	One large	One layer	Present	Present
<i>Macrozamia communis</i>	Present	Present	Absent	Present	One large	One layer abaxial, one adaxial	Present	Present
<i>Macrozamia heteromera</i>	Absent	Absent	Absent	Absent	Absent	One layer abaxial, one adaxial	Present	Present
<i>Macrozamia macdonnellii</i>	Present	Present	Absent	Present	One large	One layer abaxial, one adaxial	Present	Present
<i>Macrozamia moorei</i>	Present	Present	Absent	Present	One large	One layer abaxial, one adaxial	Present	Present
<i>Macrozamia pauli-guilielmi</i>	Discontinuous	Absent	Absent	Present	Absent	One layer abaxial, one adaxial	Present	Present
<i>Microcycas calocoma</i>	Absent	Absent	Present	Present	Absent	Two layers, non lobed	Absent	Absent
<i>Stangeria eriopus</i>	Absent	Absent	Present	Present	Absent	Two layers, lobed	Absent	Absent
<i>Zamia acuminata</i> *	Absent	Absent	Present	Present (collenchymatous)	Absent	Multiple layers, lobed	Present	?
<i>Zamia amplifolia</i>	Absent	Absent	Present	Present	Absent	Two layers, lobed	Present	Absent
<i>Zamia angustifolia</i>	Discontinuous	Absent	Present	Present	Absent	Two layers, lobed	Absent	Absent
<i>Zamia disodon</i>	Absent	Absent	Present	Present (collenchymatous)	Absent	Two layers, lobed	Absent	Absent

<i>Zamia dressleri</i>	Absent	Absent	Absent	Present (collenchymatous)	Absent	Two layers, lobed	Absent	Absent
<i>Zamia erosa</i>	Discontinuous	Absent	Present	Present	Absent	Multiple layers, lobed	Absent	Absent
<i>Zamia gentryi</i>	Absent	Absent	Present	Present (collenchymatous)	Absent	Two layers, lobed	Present	Absent
<i>Zamia hamannii</i>	Absent	Absent	Absent	Present (collenchymatous)	Absent	Two layers, lobed	Present	Absent
<i>Zamia lindenii</i>	Absent	Absent	Absent	Present (collenchymatous)	Absent	Two layers, lobed	Present	Absent
<i>Zamia loddigesii</i> *	Absent	Absent	Present	Present	Absent	Two layers, lobed	Present	?
<i>Zamia meermanii</i>	Present	Absent	Absent	Present	Absent	Multiple layers, lobed	Present	Absent
<i>Zamia nesophila</i>	Absent	Absent	Absent	Present (collenchymatous)	Absent	Two layers, lobed	Present	Absent
<i>Zamia neurophyllidia</i>	Absent	Absent	Absent	Present (collenchymatous)	Absent	Two layers, lobed	Present	Absent
<i>Zamia oligodonta</i>	Absent	Absent	Present	Present	Absent	Two layers, lobed	Present	Absent
<i>Zamia portoricensis</i>	Absent	Absent	Present	Present	Absent	Multiple layers, lobed	Absent	Absent
<i>Zamia pseudomonticola</i> *	Absent	Absent	Present	Present	Absent	Multiple layers, lobed	Absent	?
<i>Zamia pseudoparasitica</i>	Present	Absent	Absent	Present	Absent	Two layers, lobed	Present	Absent
<i>Zamia pumila</i> *	Absent	Absent	Present	Present	Absent	?	?	?
<i>Zamia purpurea</i>	Absent	Absent	Present	Present (collenchymatous)	Absent	Two layers, lobed	Absent	Absent
<i>Zamia pyrophylla</i>	Present	Absent	Absent	Present	Absent	Two layers, lobed	Present	Absent
<i>Zamia roezlii</i>	Absent	Absent	Present	Present (collenchymatous)	Absent	Two layers, lobed	Present	Absent
<i>Zamia skinneri</i>	Absent	Absent	Absent	Present (collenchymatous)	Absent	Two layers, lobed	Present	Absent
<i>Zamia splendens</i> *	Absent	Absent	Absent	Present (collenchymatous)	Absent	Two layers, lobed	?	?
<i>Zamia urep</i>	Absent	Absent	Present	Present (collenchymatous)	Absent	Two layers, lobed	Present	Absent
<i>Zamia wallisii</i>	Absent	Absent	Present	Present (collenchymatous)	Absent	Two layers, lobed	Present	Absent
<i>Zamia vazquezii</i>	Absent	Absent	Absent	Absent	Absent	Two layers, lobed	Absent	Absent

Table 1: Characters present in different species of Zamiaceae. Species scored from the literature are indicated using an asterisk. References: (Lamb 1923; Koeleman *et al.* 1981; Acuña-Castillo and Marín-Méndez 2013; Pérez-Farrera *et al.* 2014, 2016; Magellan *et al.* 2018; Vovides *et al.* 2018)

Table 2: Pagel’s Lambda values for the characters inferred using the two different topologies.

	Unconstrained	Constrained
Adaxial sclerified hypodermis	0.972	0.969
Abaxial sclerified hypodermis	1.000	0.99
Adaxial girder sclerenchyma	0.942	0.821
Abaxial girder sclerenchyma	1.000	1.000
Mucilage canals (coding 1)	0.972	0.978
Mucilage canals (coding 2)	0.948	0.96
Palisade lobation	1.000	1.000
Palisade layers number	1.000	0.987
Fibers in the mesophyll	0.749	0.89
Tracheid-like transfusion tissue	1.000	1.000

Table 3: results of the parsimony randomization test for each character on the two different topologies.

	Tree	p-value	Steps observed	Steps expected
Adaxial sclerified hypodermis	unconstrained	<0.001	2	20 (14-26)
Abaxial sclerified hypodermis	unconstrained	<0.001	2	21 (13-27)
Adaxial girder sclerenchyma	unconstrained	<0.001	5	10 (7-11)
Abaxial girder sclerenchyma	unconstrained	<0.001	4	9 (6-9)
Mucilage canals (coding 1)	unconstrained	<0.001	8	29 (23-35)
Mucilage canals (coding 2)	unconstrained	<0.001	8	25(19-31)
Palisade lobation	unconstrained	<0.001	3	16 (12-18)
Palisade layers number	unconstrained	<0.001	6	18 (13-19)
Fibers in the mesophyll	unconstrained	0.015	7	10 (6-10)
Abaxial transfusion tissue	unconstrained	<0.001	2	11 (7-12)
Adaxial sclerified hypodermis	constrained	<0.001	2	21 (13-25)
Abaxial sclerified hypodermis	constrained	<0.001	2	21 (14-26)
Adaxial girder sclerenchyma	constrained	<0.001	5	11 (7-11)
Abaxial girder sclerenchyma	constrained	<0.001	4	9 (5-9)
Mucilage canals (coding 1)	constrained	<0.001	8	29 (23-35)
Mucilage canals (coding 2)	constrained	<0.001	8	25 (19-31)
Palisade lobation	constrained	<0.001	3	16 (11-18)
Palisade layers number	constrained	<0.001	6	18 (14-19)
Fibers in the mesophyll	constrained	0.001	6	10 (6-10)
Abaxial transfusion tissue	constrained	<0.001	1	11 (7-12)

Table 4: Consistency index and Retention index for all characters over the two different topologies.

	Unconstrained		Constrained	
	Consistency	Retention	Consistency	Retention
	index	index	index	index
Adaxial sclerified hypodermis	0.5	0.96	0.5	0.96
Abaxial sclerified hypodermis	0.5	0.96	0.5	0.96
Adaxial girder sclerenchyma	0.2	0.6	0.2	0.6
Abaxial girder sclerenchyma	0.25	0.62	0.33	0.75
Mucilage canals (coding 1)	0.37	0.85	0.37	0.85
Mucilage canals (coding 2)	0.25	0.8	0.25	0.8
Palisade lobation	0.67	0.94	0.67	0.94
Palisade layers number	0.5	0.81	0.5	0.81
Fibers in the mesophyll	0.14	0.33	0.17	0.44
Abaxial transfusion tissue	0.5	0.91	1	1

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Table S1: Specimens investigated in this study.

Species	Garden Code	Voucher	Origin
<i>Bowenia serrulata</i>	2004865*R		MBC
<i>Bowenia serrulata</i>	2004086*Q		MBC
<i>Bowenia serrulata</i>	2004865*P		MBC
<i>Bowenia spectabilis</i>	9367*S		MBC
<i>Bowenia spectabilis</i>	9367*Q		MBC
<i>Bowenia spectabilis</i>	9367*G		MBC
<i>Ceratozamia becerrae</i>	20010249*A		MBC
<i>Ceratozamia hildae</i>	20010207*A		MBC
<i>Ceratozamia hildae</i>	20010216*A		MBC
<i>Ceratozamia hildae</i>	8839*E		MBC
<i>Ceratozamia mexicana</i>	520-0-1		NAP
<i>Ceratozamia norstogii</i>	93944*G		MBC
<i>Ceratozamia robusta</i>	20020827*B		MBC
<i>Ceratozamia robusta</i>	20010920*A		MBC
<i>Ceratozamia zoquorum</i>	2000720		JBC
<i>Dioon califanoi</i>	BLetal001		NAP
<i>Dioon caputoi</i>	BLetal002		NAP
<i>Dioon edule</i>	457-1		NAP
<i>Dioon holmgrenii</i>	BLetal004		NAP
<i>Dioon mejiae</i>	CAL01		NAP
<i>Dioon merolae</i>	464-0-1		NAP
<i>Dioon purpusii</i>	468-0-1		NAP
<i>Dioon rzedowskii</i>	BLetal008		NAP
<i>Dioon spinulosum</i>	463-3-1		NAP
<i>Encephalartos altensteinii</i>	382-3-1		NAP
<i>Encephalartos ferox</i>	6145*N		MBC
<i>Encephalartos hildebrandtii</i>	CAL65		NAP
<i>Encephalartos horridus</i>	9838*A		MBC
<i>Encephalartos horridus</i>	9838*B		MBC
<i>Encephalartos horridus</i>	9838*D		MBC
<i>Encephalartos horridus</i>	9837*F		MBC
<i>Encephalartos laurentianus</i>	383-0-1		NAP

<i>Encephalartos lehmannii</i>	97983*A		MBC
<i>Encephalartos lehmannii</i>	98502*A		MBC
<i>Encephalartos lehmannii</i>	981501*A		MBC
<i>Encephalartos macrostrobilus</i>	981917*A	FLAS 267668 Nagalingum 17-80 (UF)	MBC
<i>Encephalartos macrostrobilus</i>	981917*B		MBC
<i>Encephalartos macrostrobilus</i>	981917*E		MBC
<i>Encephalartos manikensis</i>	2006198*A		MBC
<i>Encephalartos manikensis</i>	2006199*A		MBC
<i>Encephalartos manikensis</i>	385-0-1		NAP
<i>Encephalartos sclavoi</i>	479-0-1		NAP
<i>Encephalartos senticosus</i>	9722*A	Little & Stevenson 1039(FTG)	MBC
<i>Encephalartos senticosus</i>	9861*D		MBC
<i>Encephalartos umbeluzensis</i>	401-1-1		NAP
<i>Lepidozamia hopei</i>	99967*A		MBC
<i>Lepidozamia hopei</i>	2000116*A		MBC
<i>Lepidozamia hopei</i>	99970*A		MBC
<i>Macrozamia heteromera</i>	438-14-1		NAP
<i>Macrozamia heteromera</i>	438-13-1		NAP
<i>Macrozamia macdonnellii</i>	96275*C	Little & Stevenson 1057 (FTG)	MBC
<i>Macrozamia macdonnellii</i>	95926*C		MBC
<i>Macrozamia macdonnellii</i>	96275*D		MBC
<i>Macrozamia moorei</i>	20041199*K		MBC
<i>Macrozamia moorei</i>	20041199*Q		MBC
<i>Macrozamia moorei</i>	20030060*L		MBC
<i>Macrozamia paulii-guilielmii</i>	444-4-1		NAP
<i>Macrozamia plurinervia</i>	437-8-1		NAP
<i>Microcycas calocoma</i>	77404*K	Little & Stevenson 1063 (FTG)	MBC
<i>Microcycas calocoma</i>	20010812*D		MBC
<i>Stangeria eriopus</i>	651325*H		MBC

<i>Stangeria eriopus</i>	771002*A		MBC
<i>Stangeria eriopus</i>	80727*E		MBC
<i>Stangeria eriopus</i>	STA01		NAP
<i>Zamia amplifolia</i>	FTBG 91596	Bogler 1223 (FTG)	FTBG
<i>Zamia disodon</i>		Lindstrom s.n. (FTG, L, V)	NN
<i>Zamia dressleri</i>	2000345*A	Stevenson et al. 1145 (FTG)	MBC
<i>Zamia elegantissima</i>	2000775*G	Little & Stevenson 1149 (FTG)	MBC
<i>Zamia erosa</i>	20030290*K		MBC
<i>Zamia erosa</i>	20030290*F		MBC
<i>Zamia erosa</i>	20030290*G		MBC
<i>Zamia furfuracea</i>	99798*K		MBC
<i>Zamia furfuracea</i>	2001131*A		MBC
<i>Zamia furfuracea</i>	20011306*A		MBC
			Private
<i>Zamia gentryi</i>		Luther et al. 1235 (SEL)	Collection
		Taylor et al. ASTB04-ZE1	
<i>Zamia hamannii</i>	20040874*A	(PMA)	MBC
<i>Zamia imperialis</i>	20010765*A		MBC
<i>Zamia imperialis</i>	20010766*A		MBC
<i>Zamia imperialis</i>	20010766*A		MBC
<i>Zamia imperialis</i>	20010435*B		MBC
<i>Zamia lindenii</i>	20001000*F	Espinosa 2011-007 (FTG)	MBC
		Calonje et al BZ08-152 (BRH,	
<i>Zamia mermannii</i>	20080671*A	FTG, MO, NY, XAL),	MBC
			Private
<i>Zamia oligodonta</i>		Calderón-Sáenz 183 (NY)	Collection
<i>Zamia nesophila</i>	20010123*C	Espinosa 2011-008 (FTG)	MBC
<i>Zamia neurophyllidia</i>	86567*JJ		MBC
<i>Zamia neurophyllidia</i>	86567*H		MBC
<i>Zamia neurophyllidia</i>	86567*GGG		MBC
<i>Zamia portoricensis</i>	20030469*A		MBC
<i>Zamia portoricensis</i>	20030298*P		MBC
<i>Zamia portoricensis</i>	20030297*D		MBC
<i>Zamia portoricensis</i>	528-0-1		NAP

<i>Zamia pseudoparasitica</i>	2000319*A	Stevenson DWS1206A (FTG, PMA, NY),	MBC
<i>Zamia pyrophylla</i>	20100027*A		MBC
<i>Zamia roezlii</i>	94635*B	Little & Stevenson 1082 (FTG)	MBC
<i>Zamia urep</i>	NN 15223A	Lindstrom s.n. (FTG, L, V)	NN
<i>Zamia vazquezii</i>	229301 ZV01		NAP
<i>Zamia wallisii</i>	20010301*A	Little & Stevenson 1165 (FTG)	MBC

Table S2: Sequences added to the matrix of Condamine *et al.* (2015) to generate the new molecular matrix.

Species	matK	rbcL
<i>Ceratozamia robusta</i>	KX503752	KX503803
<i>Zamia acuminata</i>	-	KX503804
<i>Zamia integrifolia</i>	KX503755	KX503799
<i>Zamia lecointei</i>	KX503760	KX503795
<i>Zamia manicata</i>	KX503758	KX503797
<i>Zamia muricata</i>	KX503759	KX503794
<i>Zamia neurophyllidia</i>	KX503753	KX503798
<i>Zamia pumila</i>	KX503757	KX503801
<i>Zamia pygmaea</i>	KX503754	KX503802
<i>Zamia roezlii</i>	KX503761	KX503802
<i>Zamia erosa</i>	KX503756	KX503800

Table S3: AICc for the different models on the traits examined. Bold typeface indicates the model selected for the trait reconstruction analysis.

Character	Tree	ER	SYM	ARD
Adaxial sclerified hypodermis	unconstrained	22.1852	22.1852	21.349
Abaxial sclerified hypodermis	unconstrained	19.648	19.6477	20.4098
Adaxial girder sclerenchyma	unconstrained	37.7574	37.7574	32.099
Abaxial girder sclerenchyma	unconstrained	28.49	28.4903	29.2901
Mucilage canals (coding 1)	unconstrained	71.7137	71.44	80.2305
Mucilage canals (coding 2)	unconstrained	64.5818	60.41	63.7628
Palisade lobation	unconstrained	22.287	25.8684	31.4206
Palisade layers number	unconstrained	54.603	54.045	64.5659
Fibers in the mesophyll	unconstrained	50.794	50.7936	47.8522
Abaxial transfusion tissue	unconstrained	14.195	14.1946	15.3349
Adaxial sclerified hypodermis	constrained	22.2876	22.2876	21.402
Abaxial sclerified hypodermis	constrained	19.751	19.7508	20.5516
Adaxial girder sclerenchyma	constrained	44.0231	44.0231	37.887
Abaxial girder sclerenchyma	constrained	28.065	28.0647	29.1594
Mucilage canals (coding 1)	constrained	70.3713	70.282	79.1725
Mucilage canals (coding 2)	constrained	63.3676	59.552	62.6993
Palisade lobation	constrained	22.083	25.6805	31.1126
Palisade layers number	constrained	59.9938	54.0054	63.481
Fibers in the mesophyll	constrained	52.65	52.6496	48.4393
Abaxial transfusion tissue	constrained	13.188	13.1876	14.8008

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Figure S2: results of the stochastic mapping reconstruction of the characters A) Adaxial sclerophyllous hypodermis, B) Abaxial sclerophyllous hypodermis, C) Adaxial girder sclerenchyma, and D) Abaxial girder sclerenchyma. Results from the unconstrained topology are shown on the left, while results from the constrained topology are shown on the right.

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Figure S3: results of the stochastic mapping reconstruction of the characters A) Mucilage canals (coding 1), B) Mucilage canals (coding 2), C) Parenchyma lobation, and D) Palisade layers number. Results from the unconstrained topology are shown on the left, while results from the constrained topology are shown on the right.

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