

Comparative anatomy of leaflets of *Zamia acuminata* and *Z. pseudomonticola* (Zamiaceae) in Costa Rica

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Abstract: The genus *Zamia* is morphologically and ecologically the most diverse of the order Cycadales. Throughout its history this genus has been restricted to the New World and is presently almost entirely restricted to the Neotropics. Unusual anatomical traits of the leaflets, such as the sunken stomata and thick cuticle, are common in this and related genera. The objective of this research was to study and compare the leaflet anatomy of *Zamia acuminata* and *Z. pseudomonticola* and establish possible phylogenetic relationships between the anatomical traits and the near relatives of these species. The leaf material was obtained from living plants and then processed for electron microscopy study. We found that both species are very similar to each other and to *Z. fairchildiana*, and that they share several unusual traits with other species of the genus, such as the parenchyma morphology, the spatial distribution of tissues between the veins and the stomata morphology. The main differences between these species were seen in their fiber clusters and in the abundance of trichome basal cells on the epidermis. The anatomical similarities between the three species could be the result of their close phylogenetic relationship and the divergences between them could be the result of recent speciation during the Pleistocene, resulting from geological changes in Southern Costa Rica. Rev. Biol. Trop. 61 (2): 539-546. Epub 2013 June 01.

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Cycadales Pers. ex Bercht. & J. Presl is the least derived group amongst the extant seed plant orders (Taylor *et al.* 2009), with 331 recognized species (Osborne *et al.* 2012). This order is morphologically conservative, with little anatomical variation within families, especially when compared to groups like the angiosperms (Norstog & Nicholls 1998). Nowadays, the order is restricted to tropical and subtropical areas of both hemispheres, extending into warm temperate areas only in Southern Africa and Australia. Hill *et al.* (2003) proposed a classification model that divides the order in two families supported by the divergence between *Cycas* L. and a clade that includes all the other genera of the order.

Zamia L. is the second most speciose genus of the order and it shows greater

morphological, karyological and ecological variation than other cycad genera (Norstog 1981, Vovides 1983, Caputo *et al.* 1996, Stevenson 2001, Jones 2002). Also, *Zamia* is the only genus of Cycadales which exhibits karyotypic variation both intra- and inter-specifically (Norstog 1981, Vovides 1983, Caputo *et al.* 1996). According to some researchers in other plant groups such as conifers and angiosperms, this could indicate rapid evolutionary rates (Eckenwalder 2008, Takhtajan 2009).

Cycads are the only gymnosperms which have developed compound leaves. Most extant representatives show adaptations for xerophytic environments: a thick cuticle, a well defined hypodermis made up of thick-walled cells and sunken stomata, usually only on the underside of the leaflets. These traits were possibly

inherited from an ancestor which inhabited dry and strongly irradiated environments (Norstog & Nicholls 1998).

All known species of *Zamia* have pinnate leaves (Jones 2002), but the number of leaflets per leaf is variable. The main axis of the leaf is constituted by the petiole, often with prickles, and the rachis, which holds the leaflets. The leaflets are articulated to the rachis and, unlike most other cycads, the leaflets are shed independently from the rachis. The petiole bases and cataphylls are shed completely and do not persist on the stem after senescing. The internal anatomy of the petiole-rachis axis is characterized by the dominance of parenchyma along with some mucilage ducts and relatively few and small discreet vascular strands which, in cross section, show an omega (Ω) shape arrangement. This trait is diagnostic of the order (Norstog & Nicholls 1998).

In *Zamia* the leaflets lack a well differentiated midvein (except in *Z. restrepoi* (D.W. Stev.) A. Lindstr.), while the leaflet is crossed by several vascular strands (Norstog & Nicholls 1998, Jones 2002). Greguss (1968) studied in great detail the epidermal features of most cycadalean species known at the time. In more recent works, Newell (1985, 1989) studied variation in the external morphology of the leaflets of some Caribbean species of *Zamia*. Stevenson (1981, 1990), Stevenson *et al.* (1996) and Norstog & Nicholls (1998) provide general descriptions of several aspects of the leaf anatomy of diverse cycad genera. In other genera, such as *Cycas*, the differences in the arrangement, abundance and distribution of the tissues inside the leaflets are used to clarify the taxonomy of some confusing species groups (Hill 1996). Previous studies in *Z. fairchildiana* L.D. Gómez and *Z. neurophyllidia* D.W. Stev. have demonstrated that even though the foliar anatomy follows a general pattern, there are details in which the species differ, such as the shape and distribution of fiber bundles associated with the vascular tissue, and the size and shape of the air chambers of the mesophyll (Acuña-Castillo & Marín-Méndez 2012).

The goals of this study were to analyze and compare the leaflet anatomy of *Z. acuminata* Oerst. ex Dyer and *Z. pseudomonticola* L.D. Gómez and establish possible relationships between the leaflet anatomy and the phylogeny of some *Zamia* species.

MATERIALS AND METHODS

We worked with leaf samples of *Z. acuminata* and *Z. pseudomonticola*. The first species (as currently understood, Acuña-Castillo 2010) is endemic to Costa Rica, in San José and Puntarenas Provinces from the Central Pacific region, where it is found in wet and rain forests of tropical and premontane elevations, between 100 and 1200m altitude. *Z. pseudomonticola* is known from Eastern Puntarenas Province, Costa Rica, and Western Chiriquí Province, Panama; this species grows in wet and rain forests between 1000 and 1600m altitude in both countries and it is one of the few Central American species of *Zamia* that could be considered montane (along with *Z. lindleyi* Warsz. ex A. Dietr. and *Z. gomeziana* R.H. Acuña).

The study material of *Z. acuminata* was obtained from wild growing plants from the Mastatal sector of La Cangreja National Park, in Puriscal, San José at 450m altitude, while material of *Z. pseudomonticola* was collected from cultivated plants from the José María Orozco botanical garden, University of Costa Rica, San Pedro de Montes de Oca, San José at 1200m altitude.

All samples for microscopic observation were about 0.5cm², from the center of the leaflet. The samples were first fixed in Karnovsky solution (glutaraldehyde 2.5% / paraformaldehyde 2% / sodium phosphate buffer 0.1M, pH 7.4), for 24 hours at 4°C. The material was then washed in sodium phosphate buffer, and post-fixed using 1% osmium tetroxide (OsO₄). The samples were further washed in distilled water and were then processed for scanning electron microscopy or light microscopy.

The samples for scanning electron microscopy were dehydrated through an ascending series of ethanol solutions and then the material

was left in tert-butanol for 24 hours at 4°C, to be sublimed thereafter in an Eiko ID-2 vacuum sublimizer. The material was mounted on aluminum bases and covered with a 30nm thick gold-palladium layer. Samples were observed using a Hitachi S-570 scanning electron microscope with an acceleration voltage of 15KV (Sánchez & Espinoza 2005). For light microscopy, the material was dehydrated through an ascending series of propanone (acetone) solutions, then embedded in Spurr's epoxy resin. The embedded samples were sectioned at 500nm thickness with a Power Tome PC (RMC Products) Ultramicrotome, dyed with Touluidin Blue and observed with an Olympus IX-51 inverted light microscope.

RESULTS

The leaflets of *Z. acuminata* are characterized by their entire margins and their long, narrow tip which represents about 25% of the total leaflet length. The veins are flat on both the upper and lower surfaces of the leaflets, which results in a smooth texture. This texture is also found in *Z. pseudomonticola*, but in this species the leaflets are proportionally broader and with a shorter tip. In addition, the leaflet width diminishes more abruptly towards the tip as opposed to *Z. acuminata*.

As with other species of the genus, *Z. acuminata* has hypostomatic leaflets. The stomata are restricted to the areas between the veins. The subsidiary cells show a concentric pattern surrounding the guard cells (Fig. 1A), and the latter are in turn located at the bottom of a shallow depression (Fig. 1B). The other cells of the epidermis are sigmoid shaped and wider than the epidermal cells over the veins, which are narrower and more elongated. On the veins some trichome basal cells can be seen, which are ovoid or cylindrical. However, they are very scarce in this species (Fig. 1C). The adaxial surface is more uniform, and is characterized by the absence of stomata and trichomes.

In the vein area it is possible to see, immediately under the epidermis (both adaxially and

abaxially), fiber bundles with different shapes. The adaxial fiber bundle is compact, narrow and extends from just under the epidermis to the periphery of the xylem. This bundle is four to five cells in width and up to seven cell layers in depth. The abaxial bundle is wider, but not as deep as the adaxial bundle since it only has two cell layers of depth. Towards the interior of the vein region the parenchyma becomes the dominant tissue and it is especially compact near the vascular tissues (Fig. 1D). In the area between the veins there are two to four cell layers of palisade parenchyma on top of the air chambers. The air chambers are large and their main axis is perpendicular to the main axis of the leaflets. The air chambers are formed by parenchymatic cells which are very elongated and recurved, looking like branched tubes, forming walls that are one cell thick (Fig. 1E).

As with the previous species, *Z. pseudomonticola* also has hypostomatic leaflets. As occurs in other species of the genus, the epidermal cells over the veins and between the veins are dimorphic: the former are sigmoid and relatively wide in contrast to the latter, which are narrower and straighter (Fig. 2A). The stomata are sunken and are surrounded by two pairs of subsidiary cells (Fig. 2B). In the samples of this species no basal trichome cells were observed.

Under the adaxial epidermis in the vein area it is possible to distinguish a rectangular to wedge-shaped fiber bundle, relatively narrow but deep, one to three cells in width and three to five cell layers deep. Under the abaxial epidermis, there is another fiber bundle but this is less developed than in other studied species of the genus and with only one to three cell layers of depth. Between the fibers and the vascular tissues it is possible to see dense parenchyma, with polygonal cells that decrease in diameter gradually the closer they are to the xylem or the phloem. Some of these cells have developed thick walls (Fig. 2C).

In the area between the veins there are no conspicuous differences between this species and *Z. fairchildiana*. Palisade parenchyma formed by up to three cell layers is located above the spongy parenchyma, with very large air

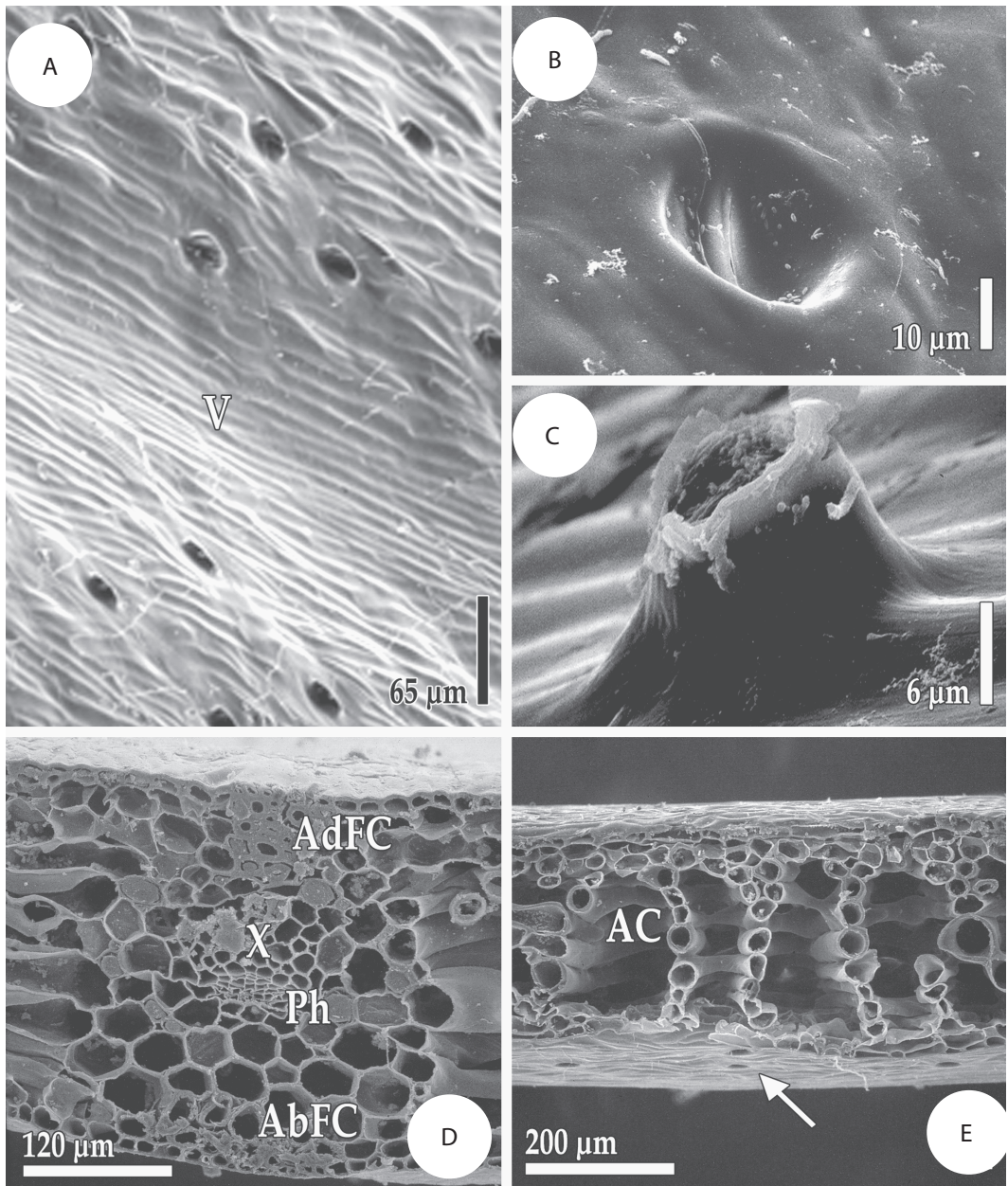


Fig. 1. *Zamia acuminata*. **A.** SEM panoramic view of the abaxial epidermis of the leaflet showing the surface of the vein with abundant stomata on both sides. **B.** SEM detail of a stoma with subsidiary and guard cells. **C.** SEM lateral view of a trichome basal cell. **D.** SEM cross section at the vein area. **E.** SEM longitudinal section of the blade, showing the air chambers and stomata (arrow) on the abaxial epidermis. Acronyms: Abaxial fiber cluster (AbFC), adaxial fibers (AdF), phloem (Ph), scanning electron microscopy (SEM), vein (V), xylem (X).

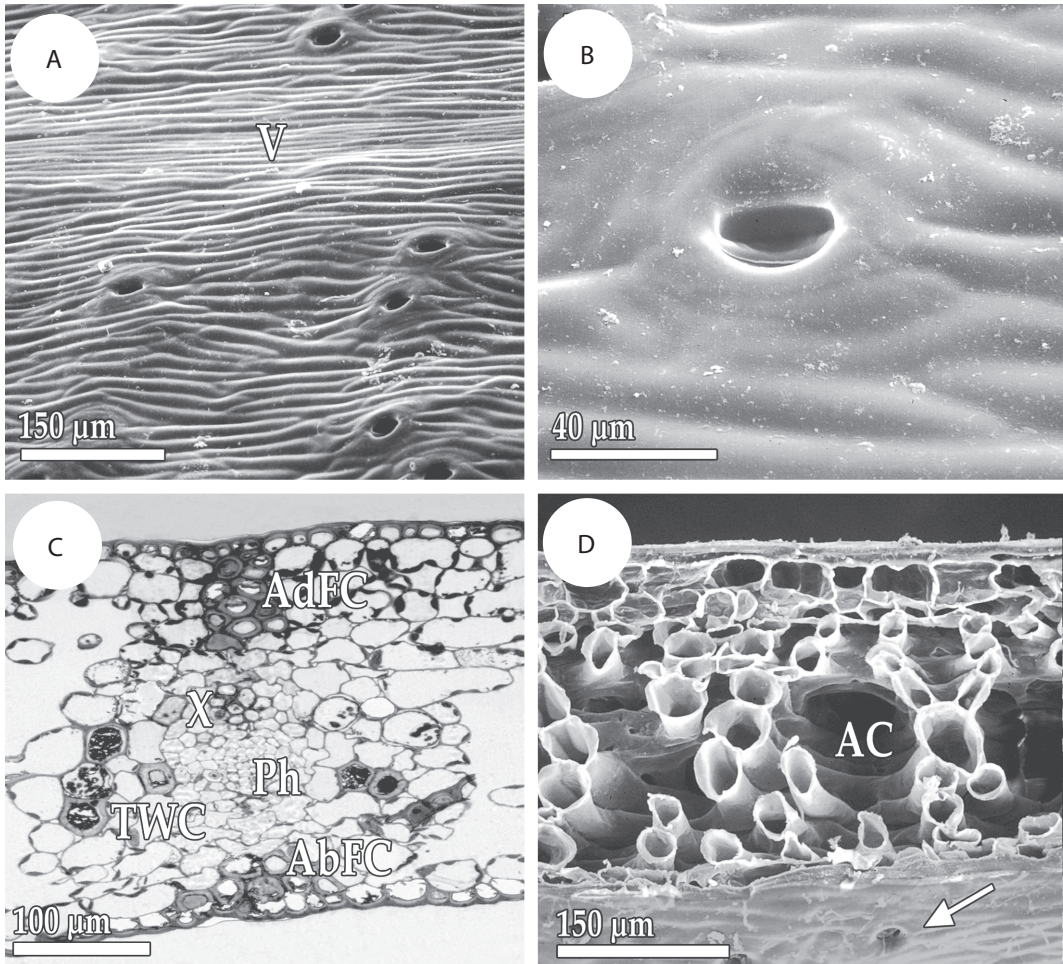


Fig. 2. *Zamia pseudomonticola*. **A.** SEM panoramic view of the abaxial epidermis of the leaflet showing the surface of the vein and stomata on both sides. **B.** SEM detail of a stoma with subsidiary and guard cells. **C.** Light microscopy cross section of the leaflet at the vein area. **D.** SEM longitudinal section of the blade, showing the air chambers and stomata (arrow) on the abaxial epidermis. Acronyms: Abaxial fiber cluster (AbFC), Air chambers (AC), adaxial fiber cluster (AdFC), phloem (Ph), scanning electron microscopy (SEM), thick walled cells (TWC), vein (V), xylem (X).

chambers, and whose axes are perpendicular to the main axis of the leaflet blade (Fig. 2D).

DISCUSSION

As with other *Zamia* species, *Z. acuminata* and *Z. pseudomonticola* leaflets show an arrangement of traits for very different environments (Greguss 1968, Acuña-Castillo & Marín-Méndez 2012). On one hand there are adaptations for xeric environments, which are most likely

evolutionary remnants from the ancestors of the genus. Such adaptations include the thick cuticle, the sunken stomata and the hypostomatic leaflets (Esau 1977). On the other hand, the leaflets also show adaptations for mesic, high humidity environments, such as the long drip tips and the large air chambers that allow the exchange of large volumes of gas.

The external macroscopic structure of the leaflets of *Z. acuminata* and *Z. pseudomonticola* is very similar to that observed in *Z.*

fairchildiana. Most *Zamia* species from the Pacific slope of Southern Costa Rica and Western Panama have been given the name *Z. fairchildiana*, due to the similarities among the three species (Merello 2004). However, this confusion is a byproduct of their externally similar vegetative morphology and the incorrect taxonomic interpretation of *Z. acuminata* and *Z. pseudomonticola*. Some aspects of the leaflet anatomy are shared by all three species, such as the scarcity of trichome basal cells on mature leaflets, the presence of a rectangular- to wedge-shaped adaxial fiber bundle, and the relatively small abaxial fiber bundle (especially when compared to plicate-leaved *Zamia* species). However, the three species differ in some aspects of their foliar anatomy as well as their ecology and distribution. Even though basal trichome cells are infrequent in the studied, smooth-leaved species of *Zamia*, none were observed in the samples of *Z. pseudomonticola*, which could be because they are absent in mature leaflets of this species. The adaxial fiber bundle of *Z. acuminata* is more developed than in any other species of *Zamia* examined so far and also shows more thick-walled parenchyma cells.

Both species of this study inhabit wet and rain forests, but in their natural habitat *Z. acuminata* inhabits forests between 100 and 1 200m, which have a more defined seasonality, due to their more northwesterly geographic position, than the forests which are inhabited by *Z. pseudomonticola*. In addition, both species show significantly different morphometrics (Acuña-Castillo 2010). The apparently similar vegetative habits of *Z. acuminata*, *Z. fairchildiana* and *Z. pseudomonticola*, and the scarcity of specimens with reproductive structures, have caused problems for some botanists who have relied on vegetative characters to differentiate these species (e.g., Jones 2002, Merello 2004). This similarity could indicate that the three species are indeed closely related to each other and that they have diverged only recently. Events that could be responsible for such a divergence include the Pleistocene orogeny of the Fila Costeña and the Pacific slope branches of the

Cordillera Talamanca (Denyer & Kussmaul 2000), as well as the fluctuation in temperature and precipitation regimes during the glacial and inter-glacial periods of the Quaternary (Haffer 1969, Colinvaux *et al.* 1996). These events are relatively recent in the geologic timescale; also, the spatial proximity of the geographical ranges of the three species suggests a relatively recent common ancestor for all three as occurs in other genera of Cycadales, such as *Ceratozamia* Brongn. (Vovides *et al.* 2004), *Cycas* (Hill 1996, 2004) and *Encephalartos* Lehm. (Vorster 2004), in which the species groups are distributed spatially close to each other, even though the individual species have allopatric distributions. The habitat changes and isolation between populations, resulting from orogeny and climatic changes, even in the lapse of just tens of thousands of years, could be responsible for the development of the observed anatomical differences of the studied species, as occurs in *Ceratozamia* in Southern Mexico, where species of the same species group, separated by just tens of kilometers, show significant and noticeable differences in their leaf architecture (Pérez-Farrera *et al.* 2004).

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RESUMEN

Zamia es morfológica y ecológicamente el género más diverso del orden Cycadales. Este género siempre ha estado restringido a América, pero en la actualidad habita principalmente en la región neotropical. Características anatómicas inusuales en los foliolos como los estomas

hundidos y las cutículas gruesas son comunes en *Zamia* y géneros afines. El objetivo de este trabajo consiste en comparar la anatomía de los foliolos de *Zamia acuminata* y *Z. pseudomonticola* y establecer posibles relaciones filogenéticas entre las características anatómicas y los parientes cercanos de esta especie. Las hojas de las especies seleccionadas fueron obtenidas de plantas vivas y luego procesadas para el estudio por microscopía electrónica. Ambas especies son muy similares entre sí y respecto a *Z. fairchildiana* y comparten varias características en común con otras especies del género como son la morfología del parénquima, de los estomas y la distribución espacial de tejidos alrededor de las haces vasculares. Las diferencias más notables entre especies se vieron a nivel de sus paquetes de fibras y en la abundancia de células basales de los tricomas en la epidermis. Las similitudes anatómicas entre estas tres especies pueden ser el resultado de su cercanía filogenética y las diferencias podrían ser el resultado de especiación durante el Pleistoceno, producto de los eventos geológicos y cambios sucedidos en el sur de Costa Rica en esa época.

Palabras clave: *Zamia*, Costa Rica, anatomía foliolos.

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