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Original Research

Epidermal morphology and leaflet anatomy of *Dioon* (Zamiaceae) with comments on climate and environment

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ABSTRACT

Anatomical descriptions can be effective for solving systematic issues, but these studies are relatively scarce for cycads. Therefore, we present here a leaflet and cuticle anatomical study on the genus *Dioon*, to provide a set of epidermal traits that clarify species delimitation and relationships between species and their habitats. We used standard micro-technique for leaflet sectioning, and cuticular peel preparation for light microscopy. Also, we used the chromium trioxide method for scanning electron microscope observations on cuticles. Measurements were taken on 10 randomly chosen replicates of each cell or tissue type, for each of the leaflets sampled per taxon. Micromorphological variation among species was calculated for each trait. Finally, we reconstructed the ancestral states of the observed epidermal fibre-like cell and pore shapes, by tracing the characters on the species phylogenetic tree of *Dioon*. We were able to describe the leaflet anatomy, cuticles, and epidermal features for 14 *Dioon* species. The quantitative analysis was useful to reveal five geographically structured species groups. Character tracing on the phylogenetic tree of *Dioon* has amplified our current understanding on species relationships with respect to habitats. The presence/absence data suggest that the evolutionary acquisition-deletion of structural shapes is phylogenetically independent, thus climate seems to play a very important role in the variation of cuticular and stomatal traits. Many epidermal traits, especially adaxial cuticle thickness and epistomatal pore width and depth, might be adaptations resulting from a long-term influence of climate, since they appear to have correlation with climatic conditions in relation to their biogeography. We conclude that the variation of all traits are mostly sustained and intrinsic to the species, and are of promising taxonomic value. The combination of the epidermal traits with other characters has potential for taxonomic resolution at species level.

1. Introduction

Morphology has provided important insights in vascular plants systematics (Endress et al., 2000). Even with modern molecular methods, traditional anatomy has proved efficiency in uncovering complex evolutionary trends among closely related species (Zhigila et al., 2015). The systematic value of cuticular and other epidermal characters was emphasised by Stace (1965) who did extensive work on about 250 species of angiosperms and Barthlott (1981) who examined 5000 species at SEM level describing cell shape and surface sculpturing. In spite that the living cycads consist of 348 species worldwide (Calonje et al., 2017), there are remarkably few micromorphological and anatomical studies in comparison to the work done on other gymnosperms, i.e. *Auraucaria* Juss. (Stockey and Taylor, 1978a,b) and Podocarpaceae (Stockey and Ko, 1988; Stockey et al., 1992, 1995; Mill and Schilling, 2009). For cycads, Chamberlain (1935) reported that stomata are found inside crypts for most of the 117 cycad species known at that time (Calonje et al., 2017). Pant and Nautiyal (1963) described cuticular and epidermal structures of 59 species, and Greguss (1966) found similarity in the epidermis of *Dioon edule* Lindl. and the fossil *Elatocladus punctatus*. Later, Greguss (1968) describes the general stem anatomy of 40 cycad species along with epidermal and leaf structure of 83 species of

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the 139 known at that time (Calonje et al., 2017). More recently, Tang et al. (2004) described the leaflet anatomy of ten species from six genera, and concluded that his observed pinna venation and anatomy is consistent with the Stevenson (1992) classification of Zamiaceae in two subfamilies. Mickle et al. (2011) studied the cuticular micromorphology of five species of *Cycas,* and found that cuticular data contrasted with the taxonomic placement of *C. normanbyana* as synonymous to *C. media*. For Neotropical cycads, leaflet anatomy has helped to resolve species complexes in the genus *Ceratozamia* (Vovides et al., 2012; Pérez-Farrera et al., 2014, 2016). Also, Barone-Lumaga et al. (2015) characterised the leaf cuticle micromorphology of 12 *Dioon* species at SEM level. Their results showed that *Dioon* can be easily distinguished based on this micromorphology from other genera within the Cycadales, suggesting that the leaf cuticle micromorphology can be used as a tool characterising ecological specialisations in species and within subgroups.

The genus *Dioon* consists of 15 species (Calonje et al., 2017) that range from north-western Mexico in the state of Sonora to Chiapas along the Pacific slope (10spp.), and from Nuevo León to Honduras along the Gulf of Mexico slope with four species, and Caribbean slope in northern Honduras, with the endemic *D. mejiae* Standl. & L.O. Williams. The delimitation of these species has been largely based on macromorphological studies (Lindley, 1843; Rose, 1909; De Luca and Sabato, 1979; De Luca et al., 1978, 1980a, 1980b, 1981a, 1981b, 1984; Gregory et al., 2003), but anatomical characters have received little attention. Molecular phylogenetic studies of the genus (Moretti et al., 1993; González et al., 2008; Gutiérrez-Ortega et al., 2017) generally agree upon two major clades for the genus, which have diversified during successions of dispersal and vicariance events that occurred during the early and mid Cenozoic (Moretti et al., 1993), followed by recent speciation events that produced the modern taxa (Moretti et al., 1993; González et al., 2008; Nagalingum et al., 2011; Gutiérrez-Ortega et al., 2017). The two major clades in *Dioon* distinguished by these studies are: the Spinulosum/Edule clade, *sensu* Moretti et al. (1993) and González et al. (2008), which includes *D. angustifolium* Miq., *D. edule* Lindl., *D. mejiae, D. rzedowskii* De Luca, A.Moretti, Sabato & Vázq.Torres and *D. spinulosum* Dyer ex Eichler of the Gulf of Mexico and Caribbean slopes, and a major Purpusii clade, that comprises the remaining species.

1.1. Brief description of the genus and habitats

All species of *Dioon* are dioecious, pachycaulous, and arborescent with adult trunks ranging from < 50 cm to 10 m or taller, armed with persistent petiole bases that protect the trunk from bush fires, especially those species inhabiting tropical dry forests. They attain great longevity in the wild, since some specimens of *D. edule* have been estimated over 2000 years in habitat (Chamberlain, 1919; Vovides, 1990). Coriaceous pinnate leaves are borne in a terminal crown. Ovulate strobili emerge from the centre of the foliar crown, are erect, semi-pendent or pendent at maturity, and lanose. Pollen strobili are tomentose, cylindrical to conical, and emerge from the centre of the foliar crown. The leaflets can be lanceolate to linear-lanceolate, falcate to subfalcate, imbricate, and opposite to sub-opposite. The margins are entire in the species *D. angustifolium* Miq., *D. edule*, and old specimens of *D. rzedowskii* and *D. mejiae*. The leaflet margins of the remaining species; *D. argenteum* T.J. Greg, Chemnick, S. Salas-Mor. & Vovides, *D. caputoi* De Luca, Sabato & Vázq. Torres, *D. holmgrenii* De Luca, Sabato & Vázq. Torres, *D. merolae* De Luca, Sabato & Vázq. Torres, *D. purpusii* Rose, *D. sonorense* (De Luca, Sabato & Vázq. Torres) Chemnick, T.J. Greg. & S. Salas-Mor., *D. spinulosum*, *D. stevensonii* Nicolalde-Morejón & Vovides and *D. tomasellii* De Luca, Sabato & Vázq.Torres, are denticulate, varying from one to several marginal spinulose denticles. It is interesting to note that leaflets of eophylls and one to two-year old seedlings are denticulate for all species.

Habitats of *Dioon* species range from semi-deciduous tropical moist forest in the Mexican southeast and Honduras (selva mediana subperennifolia, according to Miranda and Hernández-X, 1963), through open stunted oak forest associated with thorn-brush, cacti and agaves, and pine oak forests at higher elevations and latitudes in Mexico. All species are found on rocky shallow soils on steep slopes, rocky canyons and ravines, rarely under deep forest canopies with deep soils. Also, a single *D. edule* population is known in stable coastal sand dune vegetation at sea level. Average annual precipitation ranges from 319 mm in the driest habitat to over 3200 mm in the wettest. Except for the mesic forests in southeast Mexico and Honduras, most habitats are water stressed with up to eight months or more of dry season (Vovides, 1990). The effects of these extreme climate conditions are remarkable in one deciduous forest habitat of *D. sonorense,* in the extreme northwest distribution of the genus in Sonora, where the stunted oaks are being replaced with mesquite (*Prosopis* sp.), a Sonoran Desert matorral element, probably due to climate change (Gutiérrez-Ortega et al., 2014). In this study, our goals are to describe and compare the leaflet anatomy, cuticles and epidermal features of each known species of *Dioon.* Comparisons of anatomical features with respect to the species' habitats also have been made. Our enquiry is to elucidate any consistent microanatomical differences between the different *Dioon* species. Are these traits of any taxonomic or ecological utility?

2. Materials and methods

The leaflet material was obtained from living field collected *Dioon* plants representing 14 of the 15 recognized species held in the Living National Cycad Collection of the Clavijero Botanic Garden of the Instituto de Ecología, A.C., in Xalapa, Mexico. One newly described species (Salas-Morales et al., 2016) was excluded since this was published after our sampling was done. One to four samples per species, including the same specimens used for the molecular phylogenetic study by González et al. (2008), were used for sectioning. Garden accession numbers and vouchers deposited at XAL are listed (Appendix A). The median part of fresh leaflet tissue (ca.1–2 cm length) was taken from the median part of mature leaves from each taxon for sectioning and epidermal studies. Further fresh leaf material was collected from the Montgomery Botanical Centre (Coral Gables, FL, USA), where two samples were taken per species from plants of known wild origin (Appendix B).

2.1. Transverse leaflet sectioning and fibre maceration for light microscopy

For transverse sectioning (TS), 2 cm length of leaflets pieces were fixed in formalin acetic alcohol (FAA) for 24 h then stored in glycerine alcohol (30% glycerine in 70% ethanol) until use. Prior to sectioning with a sliding microtome, the fixed leaflet material was softened in a solution of equal parts acetic acid and 30% hydrogen peroxide for a

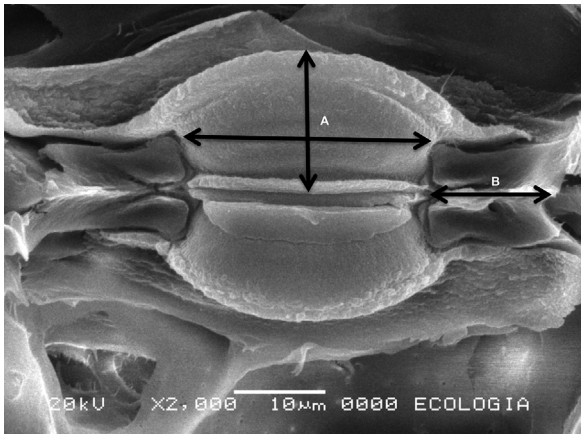

Fig 1. Dimensions taken from inner cuticle stomatal flange and polar cell flanges of *Dioon*: A, length and width of stomatal flange; B, length of polar cell flanges.