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Reciprocal illumination of morphological characters upon a molecular hypothesis supports the proposal of a new species of cycad from Mexico

Abstract The new species *Dioon stevensonii*, from the Rio Balsas basin spanning the states of Michoacán and Guerrero, Mexico, is described and illustrated. The description of this species implies a recircumscription of the populations of *Dioon* that constitute the previously characterised *D. tomasellii*, which also includes populations located in Durango, Nayarit and Jalisco. *Dioon stevensonii* differs from its congeners in characters of both vegetative and reproductive structures – namely, leaflet contour shape, leaflet curvature and reflection of the megasporophyll tips. Despite its morphological affinities with *D. tomasellii*, complementary cladistic analyses of molecular matrices indicate that *D. stevensonii* has closer phylogenetic affinities with the *D. edule* and *D. spinulosum* species groups, which are distributed along the Gulf of Mexico and Caribbean seaboards. The proposal of *D. stevensonii* rests on the re-evaluation of overlooked differences in morphological character states in herbarium material, corresponding to populations previously identified as *D. tomasellii*, which were confirmed after *ex profeso* field collections. The fact that such re-evaluation was prompted by a hypothesis of relationships based on molecular data for the entire genus *Dioon*, establishes the proposal of *D. stevensonii* as an unusual case of reciprocal illumination, where the morphological evidence provided confirmation of a molecular hypothesis, and not vice versa.

Key words cycads, *Dioon stevensonii*, Mexico, reciprocal illumination, DNA barcoding

Introduction

Dioon is a genus of cycad described by Lindley in 1843, with *D. edule* being the generic type species. The centre of diversity for this genus is undoubtedly Mexico, with 12 currently recognised species distributed along the Sierra Madre Oriental, the Sierra Madre Occidental, and the Sierra Madre del Sur. *D. mejiae* Standl. & L.O. Williams from Honduras, which represents the southernmost distribution of the genus, constitutes the only disjunction to the endemic pattern of distribution in Mexico. Endemism for the genus *Dioon* is particularly marked within the Sierra de Juárez of Oaxaca.

Taxonomically, *Dioon* has experienced substantial changes after the classical work of Chamberlain (1919) and Schuster (1932), in which the existence of only three species, namely *Dioon edule* Lindley, *D. purpusii* Rose and *D. spinu-*

losum Dyer & Eichler, was recognised. However, during the last 30 years this cycad genus has received special attention, resulting in the description of approximately two-thirds of the number of currently valid species (De Luca & Sabato, 1979; De Luca *et al.*, 1980a, b; De Luca *et al.*, 1981a, b; De Luca *et al.*, 1984; Chemnick *et al.*, 1997; Gregory *et al.*, 2003). Besides this noticeable increase in taxonomic knowledge, systematic studies based on morphological and molecular characters, as well as population genetic analyses have advanced our perspectives on possible evolutionary patterns and processes within the genus (De Luca *et al.*, 1982; Sabato & De Luca, 1985; Moretti *et al.*, 1993; González-Astorga *et al.*, 2003a, b, 2005, 2008; Vovides *et al.*, 2007).

Historically, the first reports of the presence of *Dioon* in Sierra Madre Occidental territories (mainly Nayarit and Sonora) were presented by Standley (1920) and Gentry (1942); however, these original collections were erroneously determined as *D. purpusii*. The issue of the taxonomic identity of these specimens was confronted by De Luca *et al.* (1984), leading

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to a nomenclatural decision that defined the new species *D. tomasellii*, with two varieties: (a) *var. tomasellii*, distributed in Guerrero, Michoacán, Jalisco, Nayarit and Durango; and (b) *var. sonorensis*, located in Sonora and Northern Sinaloa. In a subsequent study on *D. tomasellii* based on the same materials evaluated by De Luca and collaborators, Chemnick *et al.* (1997) proposed that the two varieties of *D. tomasellii* deserved recognition as separate species, based on discontinuous patterns of morphological variation not previously observed by De Luca *et al.* (1984). This was especially patent in the Sonora and Northern Sinaloa populations, providing support to the combination *D. sonorensis* (De Luca, Sabato & Vázq. Torres) Chemnick, T.J. Greg. & S. Salas-Mor).

Unexpectedly, through reanalyses of nucleotidic variation in matrices of nuclear loci that had been prepared for a separate study (Vovides *et al.*, 2007), it was confirmed that sequences from a single specimen identified as *D. tomasellii* from the Rio Balsas basin zone (located in Guerrero) were not retrieved in the same clade as the other *D. tomasellii* specimens from the states of Jalisco, Nayarit and Sinaloa, and that these differences were not sequencing artefacts. These findings prompted us to review herbarium specimens corresponding to collections from these locations. In a complementary manner, we have visited populations currently recognised as *D. tomasellii* from the states of Jalisco, Nayarit, Sinaloa and Michoacán, and gathered new materials of *Dioon* from the field in the Guerrero localities, in order to refine the available information on morphological character states and habitat. Comparative evaluation of states for vegetative and reproductive characters in specimens from the aforementioned locations led to us to segregate two sets. The first set corresponds to the Guerrero populations, from which the anomalous sequence had been obtained, and the other set includes the type specimen for *D. tomasellii* (which corresponds to a population from Nayarit). Given that the morphological character states of the Michoacán populations were the same as those scored for the Guerrero specimens, we therefore consider that the populations from these two sites, currently ascribed to *D. tomasellii*, actually correspond to a species new to science. The variation found in the complete set of morphological characters will complement a future systematic analysis for the genus, which will also integrate the available molecular and biogeographical information. In this paper, we further comment on how 'reciprocal illumination' (Hennig, 1966) is established between molecular and morphological sources of characters in current taxonomic work, as molecular evidence takes an increasingly prominent role in such practice.

Taxonomic description

Dioon stevensonii Nicolalde-Morejón & Vovides (Figs 1, 2)

DIAGNOSIS. Truncus cylindricus, erectus, usque ad 1.20 m alto, usque ad 26 cm diam. Cataphylla coriacea, lanata, 6–8.5 cm longa, basi 3–4.5 cm lata. Folia 2–25, 63–125 cm longa, 20–30 cm lata, in statu juvenili tomentosa, deinde glabra. Petiolus

semiterete, 8–15 cm longus, 0.7–1.2 cm diam. Foliola 50–85, opposita-subopposita, lineari-lanceolata, plana, recta angulo inserta, foliola media 7–14 cm longa, 0.6–1.1 cm lata. Strobilus ♂ solitarius, cylindricus, 20 cm longa, 7.5 cm diam. Strobilus ♀ solitarius, ovoideus, 30–35 cm longa, 10–15 cm diam.

TYPE. MEXICO, GUERRERO, Coyuca de Catalán, 13-mar-2007, F. Nicolalde-Morejón, J. González-Astorga & F. Vergara-Silva 1551 ♀ (Holotype: XAL Isotype: MEXU)

PARATYPES. F. Nicolalde-Morejón *et al.* 1552 (NY), 1553 (HEM), 1554 (CIB), 1555♂, 1556, 1557, 1558, 1559, 1560 (XAL)

DESCRIPTION. Trunks aerial, cylindrical, up to 120 cm long, up to 26 cm diameter, sometimes bifurcate in mature plants, protected by an armour of persistent leaf bases. Cataphylls coriaceous, triangular, 3–4.5 cm wide at base, 6–8.5 cm long, acuminate, yellowish tomentose. Leaves, 2–25; 63–125 cm long, 20–30 cm wide, erect to descending, linear-elliptical, tomentose throughout all growth stages, tomentum golden-brown when emerging, yellowish at maturity; petiole 8–15 cm long, 0.7–1.2 cm diameter, subterete, unarmed; rachis 55–110 cm long, subterete; petiole and rachis densely whitish tomentose when young, green-yellowish during subsequent stages, glabrous at senescence. Leaflets opposite to subopposite, 50–85 pairs, linear-lanceolate, flat, sometimes subfalcate, 7–14 cm long, 0.6–1.1 cm wide at middle portion of leaf, base attenuate, apex rarely deflexed, acuminate, inserted at right angles on the rachis; margins scarcely denticulate, with up to 3 fine spinulose denticles on the distal margin, and generally 1 on the proximal margin, up to 3 mm long; inter-leaflet distance ≈ 0.5 cm, slightly imbricate at distal portion of leaf in juvenile stages, imbrication lost when mature. Pollen strobili solitary, densely white tomentulose, ovoid when emerging, cylindrical when mature, 20 cm long, 7.5 cm diameter, appearing sessile; Microsporophylls numerous, inserted spirally along cone axis, cuneiform, fertile portion covering $\frac{2}{3}$ of the abaxial surface, 1.6–2.2 cm long, sterile portion, triangular, reflex distally ending with slight pungent apex, 1.3–1.6 cm long; synagia with 4–5 sporangia, ovoid, longitudinally dehiscent, simple hyaline trichomes present solely on the base of the male sporangio-phores. Cataphyll long-triangular, stipulate, tomentose, 8.5–10 cm long, 0.7–1.2 cm wide at base. Ovulate strobili solitary, ovoid, 30–35 cm long, 10–15 cm diameter; peduncle short, up to 5 cm long, densely whitish tomentose; Megasporephylls strongly imbricate, triangular, apex non-reflexed, green, base tomentose, very coriaceous, 7–8.3 cm long, 4.5–6 cm wide. Seed near spherical to ovoid 2.9–3.3 long, 2.1–2.4 cm diameter, sarcotesta yellow at maturity, sclerotesta smooth with 13 to 15 radial ridge-like markings extending from micropylar to chalazal end.

ETYMOLOGY. The specific epithet has been chosen to honour Dr Dennis William Stevenson, in recognition of his outstanding contributions to knowledge of the morphology, taxonomy, systematics and genomics of the cycads.



Figure 1 *Dioon stevensonii* A, Mature plant with female strobilus; B, mature female strobilus; C, mature plant with male strobilus; D, immature male strobilus; E, sequence of maturation of female cones; F, adult megasporophyll.

OTHER VOUCHERS EXAMINED. Guerrero; Arcelia, *M. Vázquez-Torres* 2305 (XALU), *M. Vázquez-Torres* 2335 (XALU); Coyuca de Catalán, A.P. *Vovides et al.* 1411, 1412, 1414, 1415 (XAL); Zirandaro, *J. Calónico-Soto* 12162 (FCME), *J. Calónico-Soto & R. Mayorga-Saucedo* 15241, 15242 (FCME), *J.L. Contreras-Jiménez* 2397, 2398, 2399, 2400 (FCME), *F. Nicolalde-Morejón et al.* 1561, 1562 (XAL), A.P. *Vovides et al.* 1398, 1399, 1400, 1401, 1404, 1405, 1406, 1407 (XAL). Michoacán; Arteaga, *F. Nicolalde-Morejón et al.*

1530, 1531, 1532, 1533, 1534, 1540, 1541, 1542, 1543 (XAL), *T. Walters et al.* TW 2004–09A, TW 2004–15A, TW 2004–18A (MEXU).

Habitat description

The vegetation types associated with *Dioon stevensonii* are *Quercus* and Tropical Deciduous Forests or ‘Bosque de



Figure 2 *Dioon stevensonii* A, Emergent leaves with brown-golden tomentum; B, young leaves with white tomentum; C and D, adult leaves showing to form and disposition of leaflets, and rachis green-yellowish; E, adult plants; F, the species in its habitat (Guayameo locality, state of Guerrero).

Encino' and 'Bosque Tropical Caducifolio' respectively (*sensu* Rzedowski 1978). The dominant tree species in the habitat of this cycad species are *Quercus peduncularis* Née and *Q. laurina* Humb. & Bonpl., with a few scattered individuals of *Pinus oocarpa* Schiede ex Schtdl., *Bursera submoniliformis* Engl. and *Inga spuria* Humb. ex Bonpl. ex Willd. *Brahea edulis* H. Wendl. ex S. Watson, a palm species that grows in patches along streams, also occurs.

Geologically, the Rio Balsas basin was formed by rocks dating from the Mesozoic and shaped by Cenozoic volcanic activity. The primary rocks are a mixture of igneous, metamorphic and sedimentary types, distributed diversely along the Balsas depression (Nava *et al.*, 1998). According to García (2004), the climate type that corresponds to the Balsas basin is tropical with summer rains in February to April, which marks the dry season (3.5–0.5 mm precipitation), and a wet season

that runs from June to September (213.5–216.9 mm precipitation). Average annual temperature varies between 24.7 °C and 22.7 °C. The habitat of the new species occupies an altitudinal range between 500 and 1200 metres.

Diagnostic key to species of *Dioon*

- 1a. Leaflets at middle portion of leaves elongated, lanceolate, falcate, ≥ 1.2 cm wide
- 2a. Proximal leaflets not reducing to pinnacanth; short petiole present *D. rzedowskii*
- 2b. Proximal leaflets reducing to pinnacanth; petiole almost absent
- 3a. Leaflet margin entire. Plants of Honduras *D. mejiae*
- 3b. Leaflet margin spinescent. Plants of Mexico *D. spinulosum*
- 1b. Leaflets at middle portion of leaves linear, lanceolate, ≤ 1.1 cm wide
- 4a. Leaves keeled; leaflets inserted at acute angle on the rachis
- 5a. Leaflets strongly imbricate up to two thirds of leaf length *D. califanoi*
- 5b. Leaflets slightly imbricate up to one third of leaf length
- 6a. Leaflets linear-lanceolate with 1–5 spinulose denticles on distal margin, with silvery pubescence present at emergence and persistent *D. argenteum*
- 6b. Leaflets lanceolate with 2–3 spinulose denticles on distal margin; glabrous *D. purpusii*
- 4b. Leaves flat; leaflets inserted at right or slightly acute angles along rachis
- 7a. Leaflets imbricate
- 8a. Leaflets strongly imbricate (up to two thirds of leaf length); inserted at acute angle on the rachis *D. merolae*
- 8b. Leaflets slightly imbricate (up to one third of leaf length); inserted at right angle along rachis
- 9a. Leaflets lanceolate, falcate, spinulose on distal margin; apex of the megasporophylls reflexed *D. tomasellii*
- 9b. Leaflets not falcate, linear-lanceolate, with one or two denticles on distal margin; apex of the megasporophylls non-reflexed *D. stevensonii*
- 7b. Leaflets not imbricate
- 10a. Leaflets linear, narrow, ≤ 0.6 cm wide
- 11a. Inter-leaflet distance greater than leaflet width *D. caputoi*
- 11b. Inter-leaflet distance less than leaflet width
- 12a. Leaflets glabrous on both surfaces, falcate to sub-falcate, entire or rarely with 1–2 denticles on distal margin. Plants of Sonora *D. sonorensis*
- 12b. Leaflets glabrous on adaxial side, straight. Plants of Nuevo Leon and Tamaulipas *D. angustifolium*
- 10b. Leaflets linear-lanceolate, > 0.6 cm wide
- 13a. Leaflets with 3–4 denticles on distal margin *D. holmgrenii*
- 13b. Leaflet margin entire *D. edule*

Discussion

The proposal of the new species of *Dioon* described here is related to results from a separate molecular systematics study of the genus, performed under cladistic assumptions (Vovides *et al.*, 2007; see also González, Bárcenas and Vovides, 2008), in which multiple replicate specimens of all of the species were sampled. In cladograms obtained after reanalysis of matrices composed by the sequences generated in those studies (these sequences were retrieved from GenBank; data not shown), the sequence corresponding to one of the five terminals identified as *D. tomasellii* was found not to belong to the same clade as the other *D. tomasellii* terminals. This sequence corresponds to specimens from the Guerrero populations. The cladistic arrangement obtained in such analyses suggested that we were dealing with (i) a sequencing artefact; (ii) a misidentified specimen; or (iii) an individual sample that effectively is a member of a previously unrecognised taxonomic entity. After careful inspection of the corresponding electropherograms, and further estimation of pairwise distance parameters between the anomalous terminal and the remaining *D. tomasellii* sequences (data not shown; available upon request from the corresponding author) we discarded the first alternative. To distinguish between the second and third possibilities, we critically reviewed vegetative morphological characters in herbarium specimens housed in XAL and XALU that covered the geographic range of all the *D. tomasellii* populations sampled in the molecular study. This comparison of characters led us to differentiate between two groups of herbarium sheets. The first group was composed of specimens from Compostela (Nayarit; this is the type locality of *D. tomasellii*), Pánuco (Sinaloa), and El Tuito (Jalisco). The second group included specimens from El Higueral (Michoacán), and from Arcelia, Guayameo and El Manchón; the latter three localities are located in the state of Guerrero. The main morphological character state differences distinguishing between these two groups of herbarium specimens corresponded to (a) leaflet curvature, being lanceolate and falcate in the first group [*D. tomasellii* var. *tomasellii* *sensu* De Luca *et al.* (1984) and *D. tomasellii* Chemnick *et al.* (1997)], in contrast to not falcate and linear-lanceolate in the second group, and (b) leaflet contour shape, being spinulose on the distal margins in the first group, in contrast to having one or two denticles on the same region in the second group. Additional work, carried out to investigate if these character state differences persisted in the field, involved observations of multiple living individuals at each of the populations visited in Nayarit, Jalisco, Michoacán and Guerrero. These observations confirmed the morphological differences observed in the herbarium, as well as in living specimens held at the Jardín Botánico ‘Francisco Javier Clavijero’ (Xalapa). The latter plants have been cultivated under uniform conditions over seven years, a condition which should eliminate plasticity, a potentially complicating variable in our assessment of character state variation. The field collections carried out *ex profeso* for this work led to the additional identification of a megasporangiate cone character state –namely, non-reflexed megasporophyll apices– that also distinguished *D. stevensonii* from *D. tomasellii sensu stricto*. In the latter species, the apices

of each megasporophyll are reflexed (De Luca *et al.*, 1984). With respect to the conceptualisation of the entire set of morphological characters investigated here, it should be mentioned that our inclusion of ‘absences’ as character states follows a long-standing tradition in the cycad literature. We are aware of the potential problems inherent to delimiting taxa in terms of negatively defined character states – namely, paraphyly (Ebach & Williams, 2004). We consider, however, that the future conversion of such character states into ‘positive’ ones is possible, although it will depend to a large extent on the available amount of information about their ‘causal grounding’ (*sensu* Kearney & Rieppel 2006). Developmental-genetic studies on the constraints underlying different morphogenetic outcomes in both vegetative and reproductive organs in cycads might be particularly helpful in this regard.

We are in agreement with the opinion of Moretti *et al.* (1993), who stated that speciation mechanisms in *Dioon* probably occurred through vicariance, though González-Astorga *et al.* (2005) have argued that *D. angustifolium* might have arisen through a combination of processes involving dispersal, isolation and genetic bottlenecks. In this context, we hypothesise that the historical events behind the origin of *D. stevensonii* probably involved isolation and subsequent expansion of a small group of populations during post-Pleistocene times, after the occurrence of the orogenic processes responsible for the formation of the Mexican Transverse Mountain Range (cf. González-Astorga *et al.*, 2003a, 2005). Hypotheses of this sort could be tested by comparative evaluation of additional morphological character state differences, genetic diversity in allozymes, and nucleotidic variation in selected loci both in *D. stevensonii* and the *Dioon* species that inhabit close geographical locations. In any event, though, it remains clear that evolutionary ‘process explanations’ of the origin of the species described here are subsidiary to a detailed, critical evaluation of the evidence for systematic pattern, which in turn has rested on the ability to discriminate taxonomic entities on the basis of differentially distributed suites of character states (Vovides *et al.*, 2007).

In standard taxonomic practice, extensive morphological studies of herbarium materials, conducted as part of monographic work or similar purposes, are usually the primary basis for the circumscription of species, or the proposal of new taxa. The secondary corroboration of such hypotheses on the basis of molecular information – usually from selected DNA regions – has become increasingly common (for a recent example in plant species from Mexico, see Zomlefer and Judd, 2008). For the proposal of *D. stevensonii*, the direction of the discovery process has been inverted: we have found that the distribution of certain morphological character states confirms a previously noticed disjunction between the molecular character states for two groups of populations that had been traditionally recognised as a single species, *D. tomasellii*. We consider that the ‘reciprocal illumination’ (Hennig, 1966) operated between separate sources of biological evidence, which is illustrated by our discovery of morphological character state combinations after a molecule-based suggestion of taxonomic circumscription, further exemplifies some of the issues currently discussed regarding the interplay between DNA data

and traditional taxonomic procedure – e.g. in the context of the ‘DNA taxonomy versus DNA barcoding debate’ (DeSalle, 2007). Although in our case the molecular evidence played a prominent role, our description of the species is not an instance of ‘DNA taxonomy’; instead, the DNA-based result on which our primary hypothesis was erected can be construed as a ‘red flag’ (*sensu* DeSalle, 2006: 1546), which in turn justified closer inspection of morphological characters. Moreover, we think that a DNA barcoding study based on a well-documented reference library of *Dioon* sequences could provide further reciprocal illumination of the species hypothesis proposed here, and support the ‘DNA diagnostics’ already detected for it (two in the ITS1 and one in the ITS2 gene regions; alignment available upon request).

We further suggest that, as more cases comparable to ours accumulate in the literature, the ‘active’ role of DNA barcoding in ‘species discovery’, and not only ‘species identification’ (DeSalle, 2006) could also be discussed in more detail, at a global (i.e. including as many taxonomic groups as possible) level. From our description of *D. stevensonii*, our stance in that aspect of the debate is clear: as long as the molecular information is not used in isolation as a basis of nomenclatural decisions, but in concert with other sources of evidence, DNA-based indications of the existence of previously unrecognised biological entities at the species level will be increasingly useful in fundamental taxonomic work. We also recognise, though, that using DNA barcodes in this way is conceptually related to the establishment of artificial methods of classification as opposed to ‘natural’ methods; simple logic indicates that the distribution of DNA diagnostics in species will not, in most cases, have a hierarchical distribution that can be mapped straightforwardly to, for instance, the synapomorphies that support monophyletic groups in a cladogram. In this sense, we finally claim that the need for analysis of character distributions in matrices of characters – morphological, molecular, developmental, etc. – is secured, in as much as they ultimately subtend our best corroborated hypotheses on the relationships of any given supraspecific taxonomic assemblage.

The precise coordinates of the localities where *Dioon stevensonii* populations are found have been deliberately omitted from this paper, in order to discourage indiscriminate collecting of this endangered species for commercial purposes. We recommend that *D. stevensonii* should be listed as CR C2a (ii) in the IUCN Red List (IUCN, 2005), given that the species is not found in any currently protected areas.

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