

Original Article

Young stem and leaf anatomy of 15 Malagasy-endemic *Diospyros* species (Ebenaceae): taxonomic implications

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ABSTRACT

Eighty-eight of the 255 *Diospyros* species from Madagascar are potential sources of ebony wood. Unfortunately, several species are currently threatened with extinction due in part to illegal and unsustainable logging. Reliable identification of living *Diospyros* species is necessary prior to any sustainable exploitation, so as not to compromise those species threatened with extinction. This study aims to identify features that might contribute to supporting taxonomic studies and to provide a complementary tool for the identification of standing trees. Fifteen species of *Diospyros* endemic to Madagascar were studied using standard anatomical methods. Each species showed a different shape of the leaf midrib and some have distinctive leaf anatomical characteristics, such as the presence of extrafloral nectaries in the distal part of the petiole in *Diospyros baronii* and filiform sclerenchyma in the mesophyll in *Diospyros lewisiae*. Statistical analysis showed that petiole and midrib vascular patterns, spongy parenchyma structure, the presence of papillae, thickness of epidermis, presence of extrafloral nectaries, stratification of epidermis, and presence of a cuticle are useful to distinguish species groups and even some species. The anatomy of young stems and leaves of *Diospyros* species from Madagascar is examined for the first time; the study will be extended to other potentially exploitable species.

Keywords: young stem; leaf anatomy; taxonomy; *Diospyros*; Madagascar

INTRODUCTION

Diospyros is a genus of Ebenaceae that produces ebony wood. Madagascar has long been a source of high-quality ebony (Perrier de la Bathie 1950, Normand *et al.* 1960, Schatz *et al.* 2021a). Africa and Madagascar are morphological centres of diversity of Ebenaceae (White 1983, Wallnöfer 2001, Duangjai *et al.* 2006), which includes the genus *Diospyros*. According to a recent evaluation, an estimated 255 *Diospyros* species exist in Madagascar and 252 of them are endemics to the island (Madagascar Catalogue 2022), 88 of which are large trees that can reach 20 m tall and/or 20 cm in diameter at breast height (DBH), suggesting that they may become large enough to be potential sources of ebony wood (Lowry *et al.* 2022a).

Illegal exploitation and international trade of Madagascar's precious woods, including *Dalbergia* and *Diospyros*, has increased significantly in recent decades (Randriamalala and Liu 2010,

Mason *et al.* 2016, Ratsimbazafy *et al.* 2016). More than 50% of the species that have been considered as large trees are currently threatened with extinction (Lowry *et al.* 2022b) and according to the evaluation of 152 *Diospyros* species from Madagascar, published in the IUCN Red List, 91 species are considered to be threatened with extinction (IUCN 2022).

Taxonomy and conservation should not be separated (Mace 2004). To support the sustainable management of Malagasy *Diospyros*, many studies on their taxonomy have been carried out, coupled with the evaluation of extinction risk (Schatz and Lowry 2018, 2020, Schatz *et al.* 2020, 2021a, b, Linan *et al.* 2021). All of them have been based mainly on herbarium specimens and field observations, and have defined species using morphological features such as flowers, fruits, and leaf morphology. Most Malagasy *Diospyros* species can easily be placed in one of the dozen informal morphological groups recognized on the basis of vegetative and

reproductive features (Schatz and Lowry 2020, Schatz *et al.* 2020). Flowers and fruits are seasonal, so they are not always available, but *Diospyros* species are evergreen and so their leaves are always available. According to Dickison (2000), numerous anatomical characters within the leaf have proven to be of systematic value in different plant lineages. Anatomical features play an important role in plant taxonomy (Metcalf and Chalk 1957) and have been shown to be of great value in the genera *Banistereopsis* (Malpighiaceae) (Araújo *et al.* 2020), *Campyloneurum* (Polypodiaceae) (Jaimez *et al.* 2021) and *Manilkara* (Sapotaceae) (Almeida-Jr *et al.* 2013). There are no previous studies related to the anatomy of leaves in Malagasy *Diospyros*, apart from the general descriptions by Metcalfe and Chalk (1957) of the anatomical structure of leaf, petiole, and stem of Ebenaceae.

As part of an ongoing investigation of the anatomy of Malagasy *Diospyros*, the objective of this study was to identify young stem and leaf anatomical features that might contribute to species delimitation in order to support taxonomic studies of the genus

and to provide a complementary tool to the taxonomy based on external morphology for the identification of standing trees.

MATERIALS AND METHODS

Plant material

Anatomical studies were carried out on young stems and leaves of 15 *Diospyros* species: *D. analamerensis* H.Perrier, *D. bardotiae* H.N.Rakouth, G.E.Schatz & Lowry, *D. baronii* (H.Perrier) H.N. Rakouth *et al.*, ined., *D. chitoniophora* Capuron ex G.E.Schatz & Lowry, *D. cupulifera* H.Perrier, *D. gracilipes* Hiern, *D. humbertiana* H.Perrier, *D. lewisiae* Mas, G.E.Schatz & Lowry, ined., *D. littoralis* Capuron ex G.E.Schatz & Lowry, *D. malandy* H.N.Rakouth *et al.*, *D. randrianasoloi* G.E.Schatz, Lowry & Mas, ined., *D. rubripetiolata* G.E.Schatz & Lowry, *D. squamosa* Bojer ex A. DC., *D. toxicaria* Hiern, and *D. tropophylla* (H.Perrier) G.E. Schatz & Lowry. Material was collected in the field throughout Madagascar (Table 1). Each specimen collected is accompanied by herbarium vouchers. Initial identifications were made by taxonomists of the Missouri Botanical Garden's Madagascar

Table 1. Information on *Diospyros* samples analysed.

Studied species	Collector and collection number
<i>D. analamerensis</i> H.Perrier	Ravaoherinaivalona 0151; Randrianaivo 3262; Randrianaivo 3267; Randrianaivo 3287; Randrianaivo 3289
<i>D. bardotiae</i> H.N.Rakouth, G.E. Schatz & Lowry	Ravaoherinaivalona 0113; Ravaoherinaivalona 0114; Ravaoherinaivalona 147; Randrianaivo 3272; Randrianaivo 3274; Randrianaivo 3279
<i>D. baronii</i> (H.Perrier) H.N.Rakouth <i>et al.</i> , ined.	Karatra 0085; Nombanjanahary 0047; Ramanantsialonina 0059; Ramanantsialonina 0060; Ramanantsialonina 0061; Andrianarivelo 0209; Andrianarivelo 0210
<i>D. chitoniophora</i> Capuron ex G.E.Schatz & Lowry	Randrianaivo 3222; Randrianaivo 3224; Randrianaivo 3227; Randrianaivo 3241; Randrianaivo 3242
<i>D. cupulifera</i> H.Perrier	Ravaomanalina 0046; Ravaomanalina 0047; Karatra 0177; Karatra 0178; Karatra 0179; Karatra 0180
<i>D. gracilipes</i> Hiern	Rakotova0 7375; Ramanitriniza 0015; Ramanitriniza 0038; Randrianaivo 3200; Razakamalala 7697
<i>D. humbertiana</i> H.Perrier	Ravaomanalina 0023; Ravaomanalina 0029; Ravaomanalina 0033; Ravaomanalina 0034; Ramanitriniza 0103
<i>D. lewisiae</i> Mas; G.E.Schatz & Lowry, ined.	Karatra 0115; Ramanitriniza 0019; Ramanitriniza 0020; Ramanitriniza 0023; Sandratriniaina 0020; Sandratriniaina 0021
<i>D. littoralis</i> Capuron ex G.E.Schatz & Lowry	Bernard 2713; Razakamalala 8378; Razakamalala 8383; Razakamalala 8391; Razakamalala 8442
<i>D. malandy</i> H.N.Rakouth; Randrianaivo, G.E.Schatz & Lowry	Randrianaivo 3240; Randrianaivo 3263; Randrianaivo 3264; Randrianaivo 3275; Randrianaivo 3276
<i>D. randrianasoloi</i> G.E.Schatz, Lowry & Mas, ined.	Ramanitriniza 0003; Ramanitriniza 0010; Rakotonirina 1186; Razakamalala 8467; Sandratriniaina 0003; Sandratriniaina 0004
<i>D. rubripetiolata</i> G.E.Schatz & Lowry	Ramanitriniza 0017; Rakotonirina 1185; Razakamalala 7715; Sandratriniaina 0035; Sandratriniaina 0041
<i>D. squamosa</i> Bojer ex A. DC.	Ramanitriniza 0049; Ramanitriniza 0054; Ramanitriniza 0058; Ramanitriniza 0062; Ramanantsialonina 0038; Ramanantsialonina 0039; Ramanantsialonina 0040; Ramanantsialonina 0041
<i>D. toxicaria</i> Hiern	Bernard 2616; Bernard 2644; Andrianarivelo 0190; Andrianarivelo 0192; Sandratriniaina 0018; Sandratriniaina 0022; Sandratriniaina 0039
<i>D. tropophylla</i> (H.Perrier) G.E.Schatz & Lowry	Ramanitriniza 0102; Ramanitriniza 0104; Ramanitriniza 0105; Ramanitriniza 0106; Randrianaivo 3339; Randrianaivo 3345; Randrianaivo 3414; Randimbison 0031

Table 2. Codes for anatomical characters used in statistical analysis.

Character code	Description	Character states	Character state code
C	Cuticle	Present	a
		Absent	b
DVP	Distal vascularization pattern	Open arc	a
		Open arc with lateral accessories vascular bundles	b
		Open arc with 1 or 2 adaxial vascular bundles	c
		Closed vascular cylinder with lateral slightly flattened on the adaxial size	d
EFN	Extrafloral nectaries in the distal part of the petiole	Present	a
		Absent	b
ES	Epidermal cell size	Adaxial epidermis cells = abaxial epidermis cells	a
		Adaxial epidermis cells > abaxial epidermis cells	b
EST	Epidermis stratification	Epidermis unstratified	a
		Epidermis bi- or pluristratified	b
FS	Filiform sclereides	Present	a
		Absent	b
MVP	Midrib vascularization pattern	Open arc	a
		An arc with 2 adaxial vascular bundles	b
		An arc with strongly incurved ends	c
P	Papillae	Present	a
		Absent	b
PVP	Vascularization pattern at the proximal part of the petiole	Open arc	a
		Open arc with invaginated ends	b
		Closed arc	c
		Closed sinuous arc	d
SI	Stem indumentum	Present	a
		Absent	b
SPS	Spongy parenchyma structure	Compact	a
		Moderately compact	b
		Lacunar	c

Program, and were confirmed by specialists working on the genus (P. Lowry, G. Schatz and H. Rakouth). Voucher specimens are deposited in the herbaria of the Parc Botanique et Zoologique de Tsimbazaza, Antananarivo, Madagascar (TAN), the Museum National d'Histoire Naturelle, Paris, France (P) and the Missouri Botanical Garden, Saint Louis, Missouri, USA (MO). All collection data are available online in TROPICOS (www.tropicos.org) and the Catalogue of Vascular Plants of Madagascar (Madagascar Catalogue 2021) except geo-coordinates and detailed locality data; public access to this information is limited for all large tree species that are potential sources of commercially valuable wood (Linan *et al.* 2021). At least five specimens per species were examined.

Sample preparation

Young stems and leaves from herbarium vouchers were rehydrated by boiling in water for a few minutes until complete submersion to make them more flexible, and were then stored in

50°C ethanol. Cross-sections were made using a Reichert sledge microtome for young stems, at the middle part of the leaf lamina, and at the proximal, median, and distal regions of the petiole. Standard techniques of double staining with carmino-vert de Mirande (N'Guessan *et al.* 2012) were used to highlight the different anatomical structures. Slides were prepared by mounting samples in glycerin and were examined using Olympus BX43 or CX33 microscopes; the results were recorded by photomicrography using an attached camera (Canon 700D and 800D).

Data analysis

Eleven qualitative anatomical characters that appeared in more than one state were coded as shown in Table 2. Multiple correspondence analysis (MCA) and cluster hierarchical analysis (CHA) based on Euclidean distance were performed to assess taxonomic differentiation, based on the anatomical characters. All analyses of data and visualizations were conducted with R software (R v.64 4.0.3).

RESULTS

Young stem

The epidermis usually comprises a single layer of cells (Fig. 1A) except in *Diospyros cupulifera*, *D. littoralis*, and *D. randrianasoloi*, which have two or three layers (Fig. 1B). Young stems of *D. bardotiae*, *D. baronii*, *D. humbertiana*, *D. lewisiae*, *D. littoralis*, *D. malandy*, *D. randrianasoloi*, *D. squamosa*, and *D. tropophylla* are densely covered with simple unicellular non-glandular trichomes (Fig. 1C). In addition to these non-glandular trichomes, *D. baronii*, *D. humbertiana*, *D. lewisiae*, and *D. malandy* have unicellular annular (doughnut-shaped), glandular trichomes (Fig. 1D). Most of the studied species have epidermal papillae (Fig. 2A) on the stem and the leaf midrib. They are absent only in *D. anamerensis*, *D. chitoniophora*, and *D. cupulifera*. *Diospyros*

chitoniophora, *D. cupulifera*, and *D. toxicaria* present stone cells in the cortical parenchyma (Fig. 2B). Pericycle sclerenchyma always forms a continuous ring of fibres or fibres and stone cells.

Leaf

Indumentum

The indumentum is much more abundant on the petioles of some species. Non-glandular indumentum consists of three types of hairs (Fig. 3A–D): simple unicellular trichomes (*D. anamerensis*, *D. baronii*, *D. humbertiana*, *D. lewisiae*, *D. littoralis*, *D. malandy*, *D. randrianasoloi*, and *D. tropophylla*), rarely bicellular trichomes (*D. randrianasoloi*), and unicellular trichome with two unequal arms and short stalks (*D. malandy*).

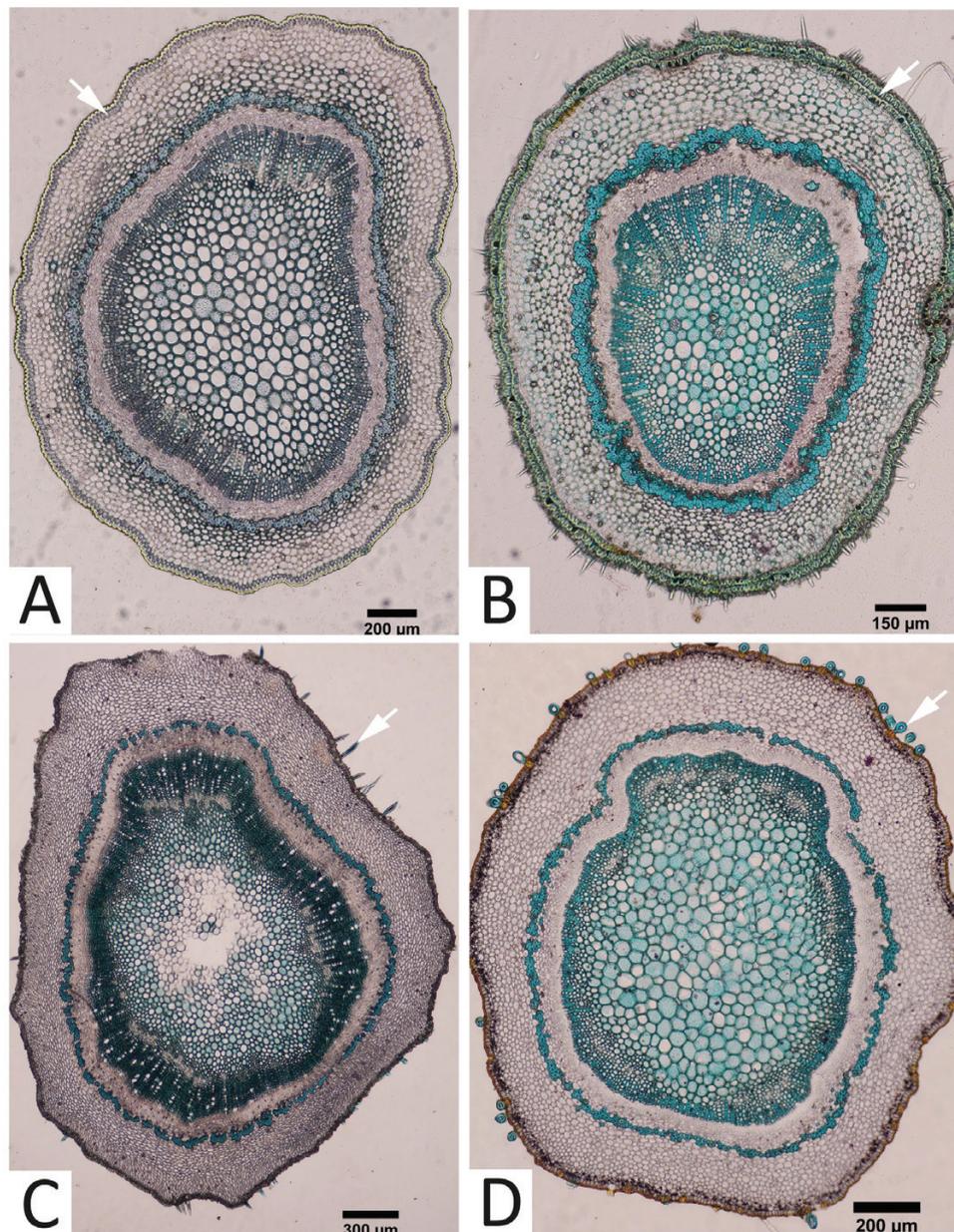


Figure 1. Cross-sections of young stem. A, *D. rubripetiolata*, uniseriate epidermis. B, *D. littoralis*, multiseriate epidermis; C, *D. baronii*, simple unicellular non-glandular hairs (arrow). D, *D. malandy*, unicellular doughnut-shaped glandular trichomes (arrow).

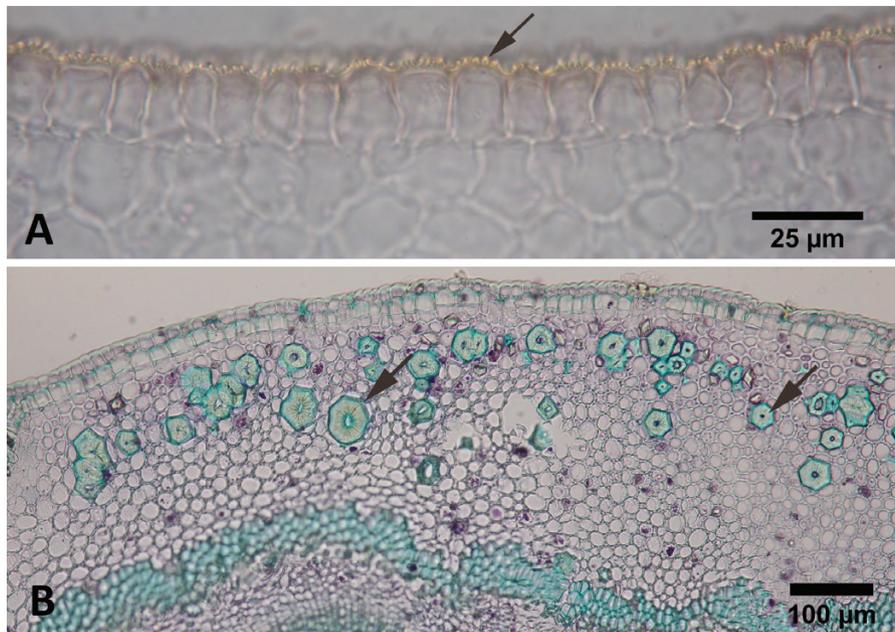


Figure 2. Cross-sections of young stem. A, *D. rubripetiolata*, epidermal papillae (arrow). B, *D. cupulifera*, stone cells in the cortical parenchyma (arrows).

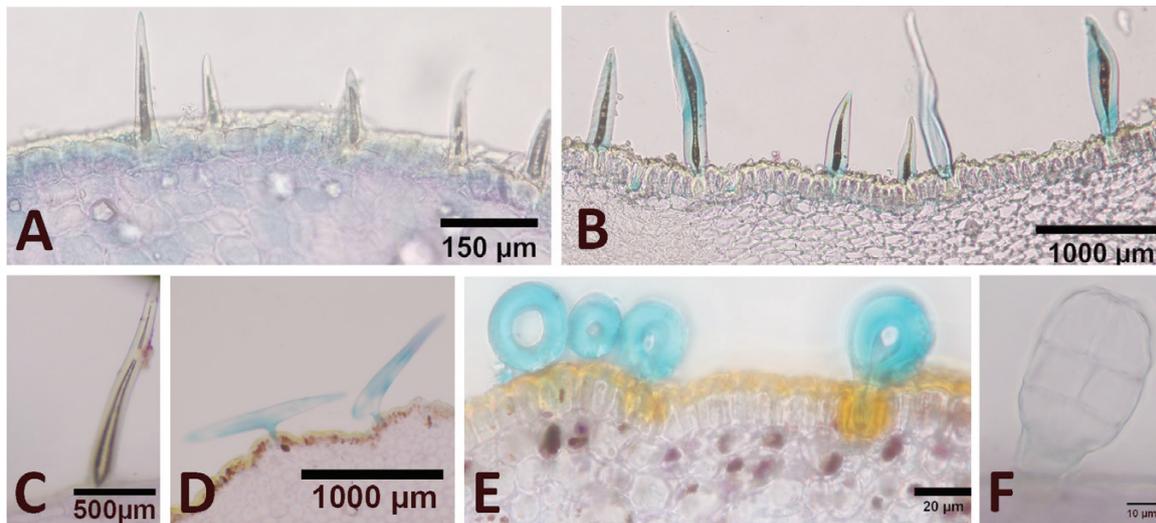


Figure 3. Indumentums. A, *D. analamerensis*, simple non-glandular hairs. B, *D. baronii*, simple non-glandular hairs. C, *D. randrianasoloi*, bicellular hair. D, *D. malandy*, unicellular with two unequal arms non-glandular trichomes. E, *D. lewisiae*, unicellular doughnut-shaped trichomes. F, *D. randrianasoloi*, pluricellular glandular trichomes.

Regarding glandular indumentum, two types of trichomes were observed: unicellular annular trichomes in the petiole of *D. lewisiae* and *D. malandy*, and pluricellular trichomes consisting of a short stalk and a multicellular head on the lamina of *D. randrianasoloi* (Fig. 3E, F).

Petiole

Each vascular bundle in the proximal part of the petiole is formed by an arc in all the studied species. The shape of the arc, which differs among species (Table 3), may be an open arc (Figs 4A, 5A), an open arc with invaginated ends (Figs 4B, 5B), a closed sinuous arc (Figs 4C, 5C), and a simple closed arc (Figs 4D, 5D).

Four patterns of vascularization were observed in the distal part of each petiole, an open arc (Figs 5E, 6A), an open arc with lateral accessory vascular bundles (Figs 5F, G, 6B, C), an open arc with one or two flat adaxial vascular bundles (Figs 5H, 6D), and a closed arc vascular cylinder that is slightly flattened on the adaxial side with lateral vascular bundles (Figs 5I, 6E).

The presence of stone cells in the petiole (Figs 5A, C, 6B, C) is a particular character of *D. chitoniophora*, *D. littoralis*, and *D. toxicaria*. Stone cells are absent in all other species.

At the distal end of the petiole, *D. baronii* has extrafloral nectaries composed of a mass of many small cells, subtended by a sheath of one to two layers of thick-walled cells on the abaxial side (Fig. 6F, G). They are absent in all other studied species.

Table 3. Vascularization at the petiole.

Species	Proximal	Distal
<i>Diospyros analamerensis</i>	Open arc	Open arc with lateral accessories vascular bundles
<i>Diospyros bardotiae</i>	Closed arc	Closed vascular cylinder slightly flattened on the adaxial size with lateral accessories vascular bundles
<i>Diospyros baronii</i>	Closed arc	Open arc with one or two adaxial plate vascular bundles
<i>Diospyros chitiniophora</i>	Closed sinuous arc	Open arc with lateral accessories vascular bundles
<i>Diospyros cupulifera</i>	Open arc with invaginated ends	Open arc with lateral accessories vascular bundles
<i>Diospyros gracilipes</i>	Open arc with invaginated ends	Open arc with lateral accessories vascular bundles
<i>Diospyros humbertiana</i>	Open arc	Open arc
<i>Diospyros lewisiae</i>	Open arc with invaginated ends	Open arc with lateral accessories vascular bundles
<i>Diospyros littoralis</i>	Open arc with invaginated ends	Open arc with lateral accessories vascular bundles
<i>Diospyros malandy</i>	Closed sinuous arc	Closed vascular cylinder slightly flattened on the adaxial size with lateral accessories vascular bundles
<i>Diospyros randrianasoloi</i>	Closed arc	Closed vascular cylinder slightly flattened on the adaxial size with lateral accessories vascular bundles
<i>Diospyros rubripetiolata</i>	Open arc with invaginated ends	Open arc with lateral accessories vascular bundles
<i>Diospyros squamosa</i>	Open arc with invaginated ends	Open arc with lateral accessories vascular bundles
<i>Diospyros toxicaria</i>	Open arc	Open arc
<i>Diospyros tropophylla</i>	Open arc	Open arc

Lamina

The shape of the leaf midrib varies among the studied species of *Diospyros* (Fig. 7). The adaxial surface is slightly concave and the abaxial surface slightly convex in *D. analamerensis* and *D. cupulifera*, and hence ‘slightly concave–slightly convex’ (Fig. 7A). In *D. humbertiana*, both the adaxial and the abaxial surfaces are flat, and hence ‘flat–flat’ (Fig. 7B). However, in *D. rubripetiolata*, the adaxial surface is convex but the abaxial surface is flat and thus ‘convex–flat’ (Fig. 7C). In *D. chitoniophora*, the adaxial surface is slightly convex and the abaxial surface is convex (‘slightly convex–convex’) (Fig. 7D). Both surfaces are slightly convex in *D. lewisiae*, and hence ‘slightly biconvex’ (Fig. 7E). In *D. baronii* and *D. toxicaria*, the adaxial surface is concave and the abaxial surface is convex, and thus ‘concave–convex’ (Fig. 7F). Both surfaces are convex in *D. gracilipes*, *D. littoralis* and *D. randrianasoloi*, and hence ‘biconvex’ (Fig. 7G).

In the leaf midrib, three vascularization patterns are observed: an open arc (Fig. 7A–E) in most species, an open arc with strongly incurved ends (Fig. 7F) in *Diospyros baronii*, and an open arc with two adaxial vascular bundles (Fig. 7G–I) in *D. bardotiae*, *D. malandy*, *D. randrianasoloi*, and *D. gracilipes*. The vascular bundles are surrounded by layers of sclerenchyma in all species examined.

All of the studied species present some layers of angular collenchyma on the abaxial side of the midrib except in *D. cupulifera*, *D. humbertiana*, *D. rubripetiolata*, and *D. squamosa*. In *D. malandy*, layers of sclerenchyma are observed between the epidermis and the vascular bundle on the adaxial side (Fig. 7I).

The palisade parenchyma is generally uniseriate (Fig. 8A–C) except in *D. baronii*, *D. cupulifera*, *D. malandy*, *D. randrianasoloi*, and *D. tropophylla*, where it is bi- or pluriseriate (Fig. 8D–F). Spongy parenchyma is compact (Fig. 8A, D) in most of the studied species but with some intercellular spaces (Fig. 8B, C) in *D. randrianasoloi*, *D. rubripetiolata*, *D. squamosa*, and *D. toxicaria*,

and with abundant intercellular spaces (Fig. 8E, F) in *D. baronii* and *D. humbertiana*.

In *D. chitiniophora* and *D. cupulifera*, solitary or clustered sclereids (Fig. 8A) are present beneath the adaxial epidermis, whereas they are absent in other studied species. A large number of filiform sclereids (Fig. 8B) are observed in *D. lewisiae*, and extensions of the sclerenchymatic sheaths to the adaxial epidermis (Fig. 8A, E) are found in *D. analamerensis*, *D. baronii*, *D. chitiniophora*, *D. malandy*, and *D. toxicaria*. They were not observed in the other species studied.

A cuticle was present in all studied species except in *D. rubripetiolata*, *D. squamosa*, and *D. toxicaria*. The epidermal cells are larger on the adaxial surface than on the abaxial surface in *D. analamerensis*, *D. chitoniophora*, *D. cupulifera*, *D. humbertiana*, *D. rubripetiolata*, *D. squamosa*, and *D. toxicaria*, but they are similar size in *D. bardotiae*, *D. baronii*, *D. gracilipes*, *D. lewisiae*, *D. littoralis*, *D. malandy*, *D. randrianasoloi*, and *D. tropophylla*. The epidermis is generally pluristratified (Fig. 8F) except in *D. analamerensis*, *D. chitoniophora*, *D. humbertiana*, and *D. rubripetiolata*, where it is unistratified. A hypodermis is usually absent in all species.

Crystals

Prismatic crystals are commonly present in young stems and leaves in all studied species.

Data analysis

MCA showed that the three first dimensions explained 62% of the total variance, respectively accounting for 29, 20, and 13% of the variability. A summary of the MCA results is provided in [Supporting Information 1](#).

Characters most strongly correlated with the first dimension of the MCA were midrib vascularization pattern, proximal and distal vascularization patterns, epidermis size and stratification, presence of extrafloral nectaries, stem indumentum, and

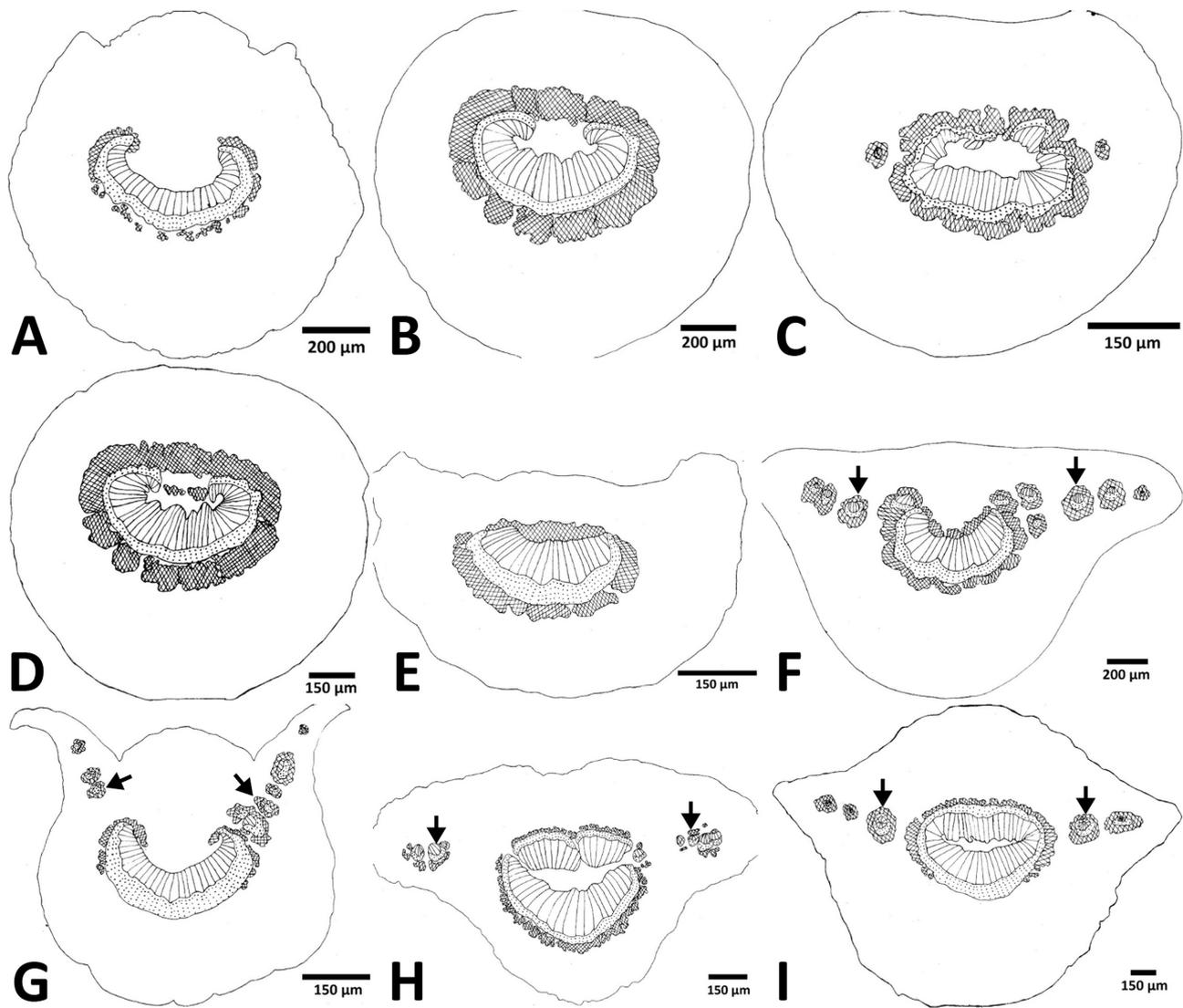


Figure 4. Vascularization pattern of the petiole at the proximal region (A–D): A, open arc; B, open arc with invaginated ends; C, closed sinuous arc; D, simple closed arc. Vascularization pattern of the petiole at the distal region (E–I): E, open arc, F–G, open arc with lateral accessory vascular bundles; H, open arc with one or two plate adaxial vascular bundles; I, closed arc vascular cylinder slightly flattened on the adaxial side with lateral vascular bundles. Arrow = accessory bundles, dot-filled areas = phloem, striped areas = xylem, tile areas = sclerenchyma.

presence/absence of a cuticle. The second factor was highly influenced by distal and midrib vascularization patterns, spongy parenchyma structure, and presence/absence of extrafloral nectaries, while characters most strongly correlated with the third factor were presence/absence of papillae and spongy parenchyma structure (Fig. 9A). Extrafloral nectaries, midrib and distal vascularization patterns, and epidermis stratification were related to both the first and the second dimensions.

The first dimension segregates the studied species into two major groups, distinguished primarily by epidermal characteristics. Members of the first group are characterized by adaxial epidermis cells larger than abaxial epidermis cells while those of the second group are characterized by adaxial and abaxial epidermis cells of the same size (Fig. 9). The first dimension contrasts *D. rubripetiolata* and *D. toxicaria* with *D. bardotiae* and *D. baronii*. *Diospyros rubripetiolata* and *D. toxicaria* have the following characteristics: young glabrous stems, leaves with non-stratified epidermis, cells of the adaxial epidermis larger than the cells of

the abaxial epidermis, cuticle absent, midrib vascularization pattern in an open arc, and distal vascularization pattern in an open arc with lateral accessory vascular bundles. *Diospyros bardotiae* and *D. baronii*, by contrast, show young stems covered by indumentum, leaves with pluristratified epidermis, cells of the adaxial and abaxial epidermis of the same size, with thick cuticles, and other patterns of vascularization at the level of the midrib and of the distal part of the petiole (Fig. 9).

The second dimension of the AMC contrasts *D. baronii*, which has lacunar spongy parenchyma, extrafloral nectaries, midrib vascularization pattern in an arc with strongly incurved ends, distal vascularization pattern in an open arc with one or two adaxial vascular bundles, and pluristratified epidermis, with *D. bardotiae* and *D. malandy*, which have compact spongy parenchyma, midrib vascularization pattern in one arc with two adaxial vascular bundles, and distal vascularization pattern in closed vascular cylinders with lateral vascular bundles that lack glandular tissues.

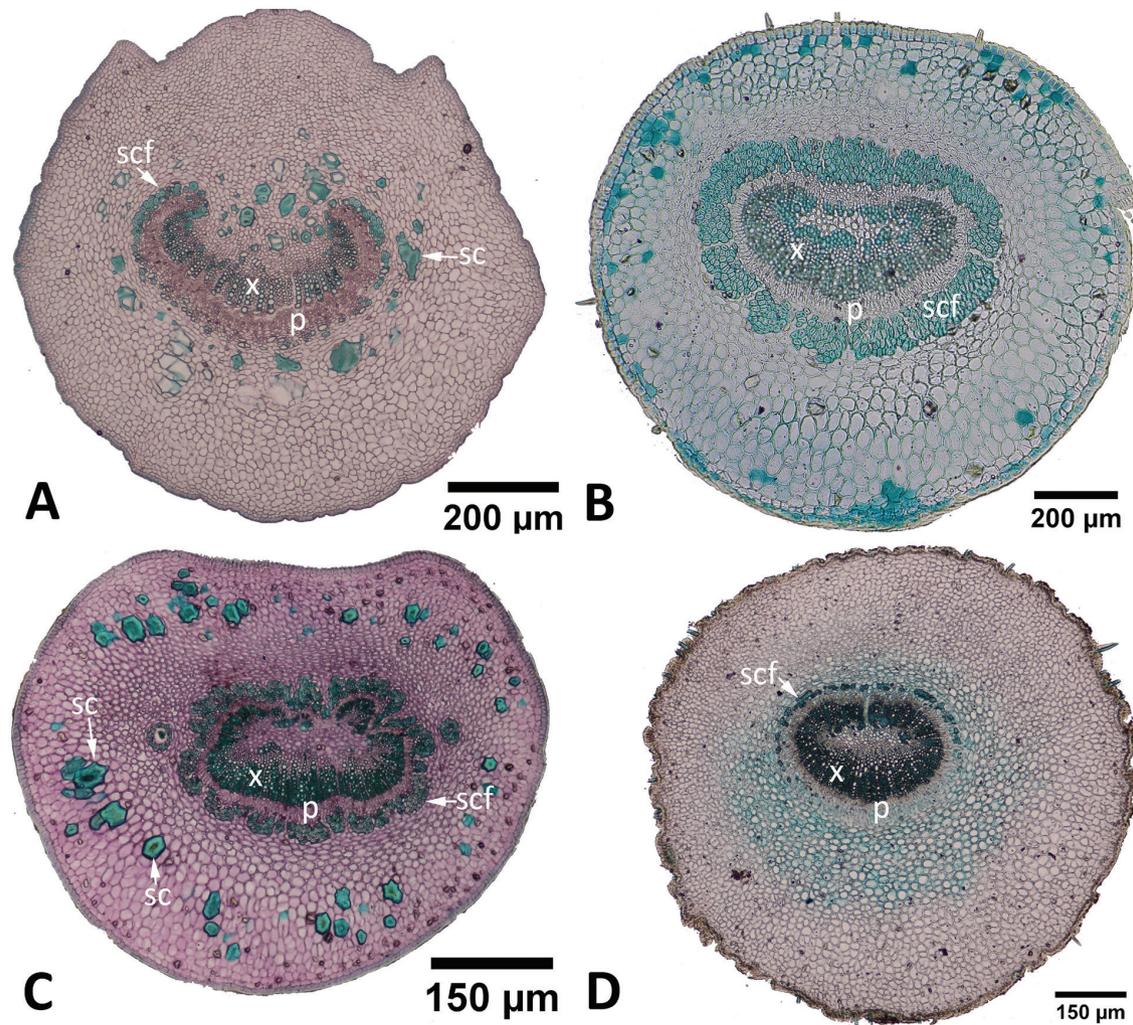


Figure 5. Cross-sections of the petiole at the proximal region. A, open arc in *D. toxicaria*. B, open arc with invaginated ends in *D. analamerensis*. C, closed sinuous arc in *D. chitoniophora*. D, simple closed arc in *D. randrianasoloi*. Abbreviations: p = phloem; scf = sclerenchymatous fibres; sc = stone cells; x = xylem; vb = vascular bundles.

The third dimension separates *D. analamerensis*, *D. chitoniophora*, and *D. cupulifera*, which have epidermis without papillae and compact spongy parenchyma, from *D. humbertiana*, *D. randrianasoloi*, and *D. toxicaria*, which have epidermal papillae and moderately compact or lacunar spongy parenchyma.

Cluster analysis carried out on the 15 studied species revealed six clusters (1–6 in Fig. 10A). The main characters that separate these clusters are distal vascularization pattern, spongy parenchyma structure, midrib vascularization pattern, size of epidermis, glandular tissue, papillae, cuticle, and epidermis stratification.

Diospyros baronii forms an isolated external group (Fig. 10B, first cluster) in relation to the others by the presence of extrafloral nectaries at the petiole, midrib vascularization pattern in an arc with strongly incurved ends, and distal vascularization pattern in an open arc with one or two adaxial vascular bundles.

Clusters 2 and 3 are then separated from clusters 4–6 by epidermal cell size. Species belonging to clusters 2 and 3 have cells of the adaxial epidermis larger than those of the cells of the

abaxial epidermis in species belonging to clusters 4 and 5, which have adaxial and abaxial epidermis cells of the same size. Cluster 2 comprises *D. malandy*, *D. randrianasoloi*, and *D. bardotiae*. These species share the midrib vascularization pattern in an arc with two adaxial vascular bundles and the distal vascularization pattern in closed vascular cylinder with lateral vascular bundles. This distal vascularization pattern differs from that in species belonging to cluster 3, which includes *D. lewisiae*, *D. gracilipes*, *D. littoralis*, and *D. tropophylla*, which have a distal vascularization pattern in an open arc with lateral accessory vascular bundles.

Diospyros humbertiana forms cluster 5. This species differs from those belonging to clusters 4 and 6 by the distal vascularization pattern, which is in an open arc with lateral accessory vascular bundles. Cluster 4 includes *D. analamerensis*, *D. chitoniophora*, and *D. cupulifera*, which have glabrous young stems with no papillae on leaves or stems. The last group, cluster 6, includes *D. toxicaria*, *D. squamosa*, and *D. rubripetiolata*. Shared characteristics of these species are the absence of cuticle, spongy lacunar parenchyma, and non-stratified leaf epidermis.

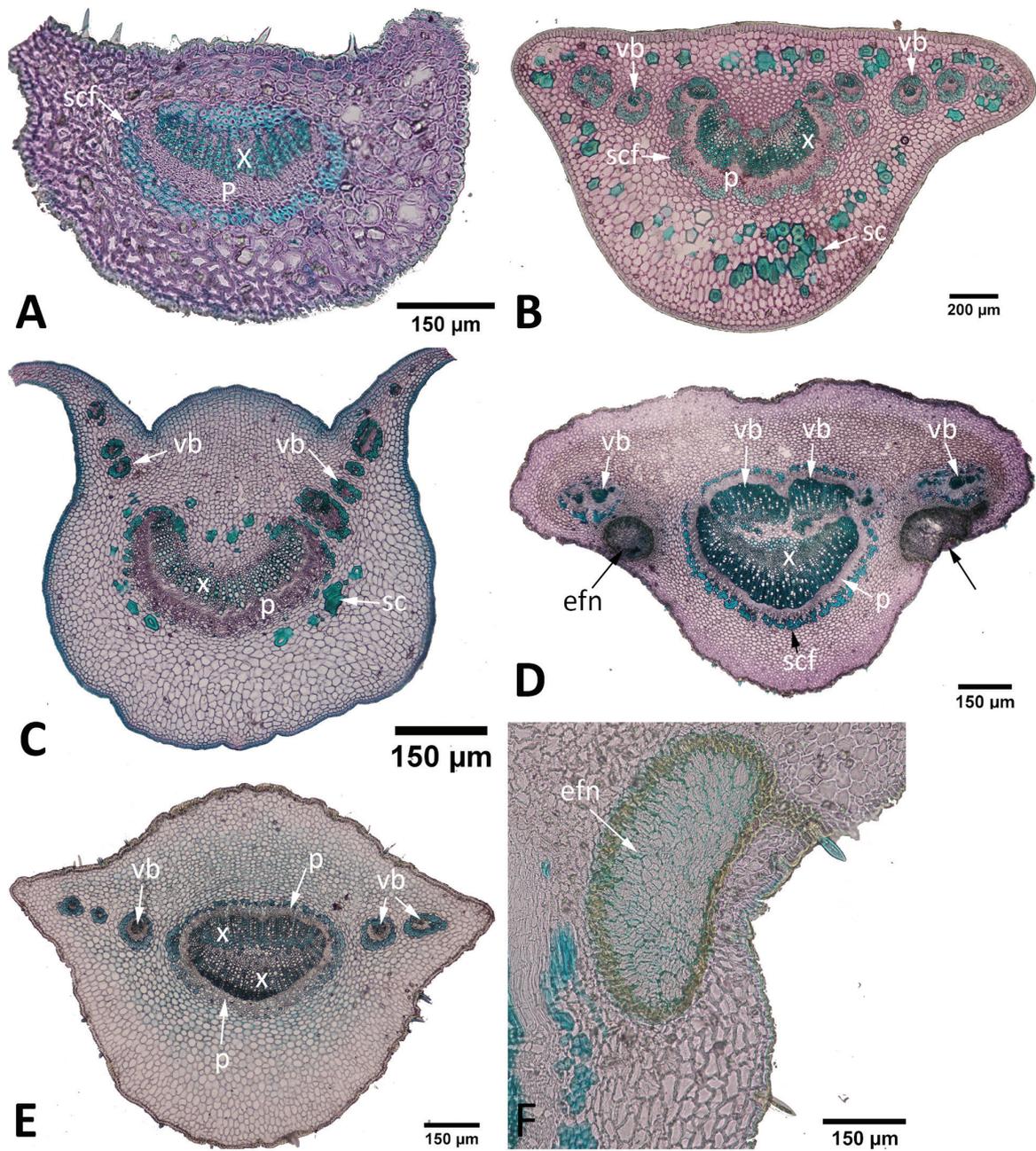


Figure 6. Cross-sections of the petiole at the distal region. A, open arc in *D. humbertiana*. B, open arc with lateral accessory vascular bundles in *D. chitoniphora*. C, open arc with lateral accessory vascular bundles in *D. toxicaria*. D, open arc with one or two plate adaxial vascular bundles in *D. baronii*. E, closed arc vascular cylinder slightly flattened on the adaxial side with lateral vascular bundles in *D. randrianasoloi*. F, extrafloral nectaries in *D. baronii*. Abbreviations: p = phloem; scf = sclerenchymatous fibres; sc = stone cells; x = xylem; vb = vascular bundles; efn = extrafloral nectaries.

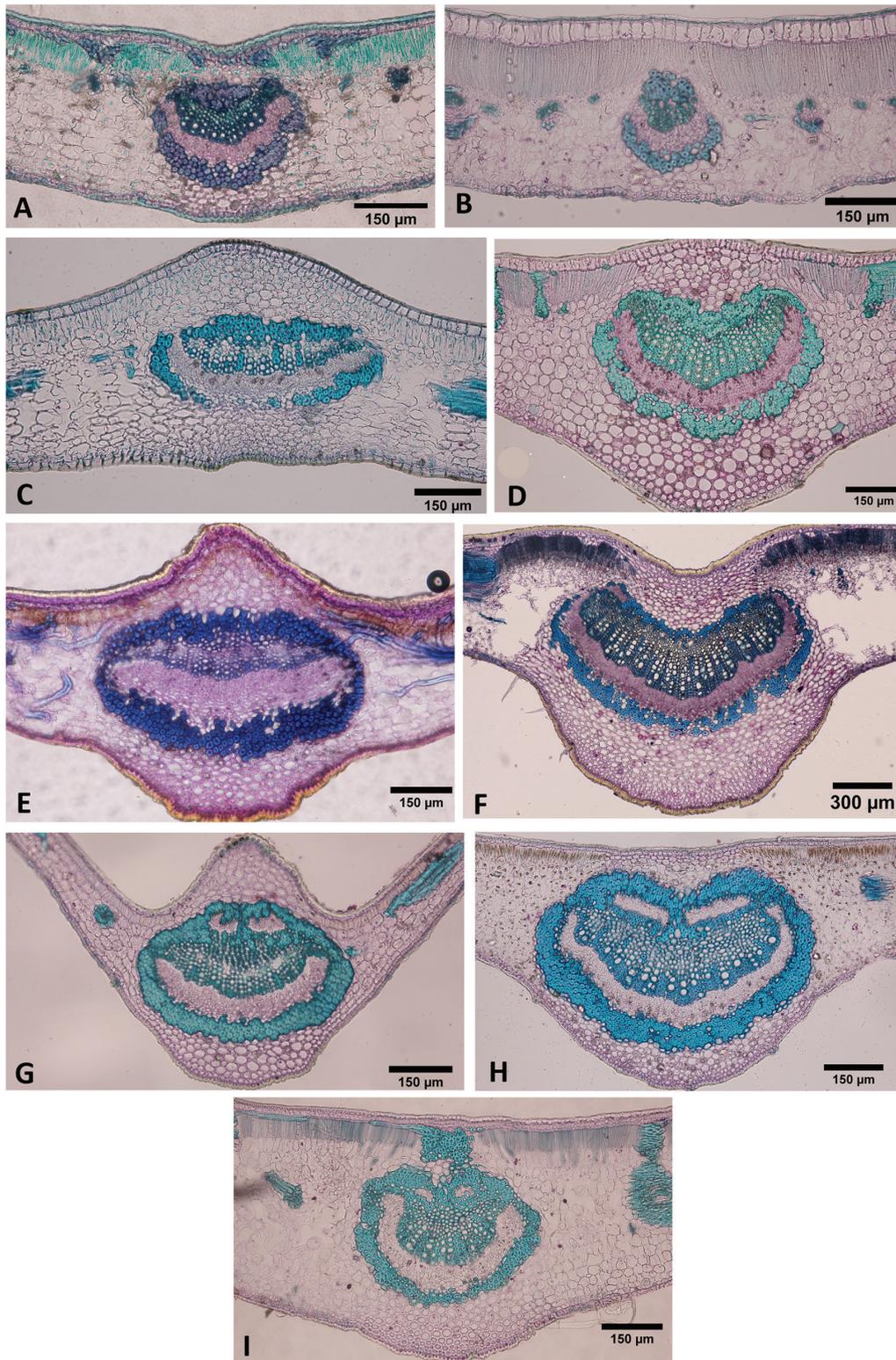


Figure 7. Cross-sections of the midrib, showing shape and vascularization. A, slightly concave–slightly convex, open arc in *D. cupulifera*. B, flat–flat, open arc in *D. humbertiana*. C, convex–flat, open arc in *D. rubripetiolata*. D, slightly convex–convex, open arc in *D. chitoniophora*. E, slightly biconvex, open arc in *D. lewisaie*. F, concave–convex, open arc with strongly incurved ends in *D. baronii*. G, biconvex, open arc with two adaxial vascular bundles in *D. gracilipes*. H, flat–convex, open arc with two adaxial vascular bundles in *D. bardotiae*. I, flat–slightly convex, open arc with two adaxial vascular bundles in *D. malandy*.

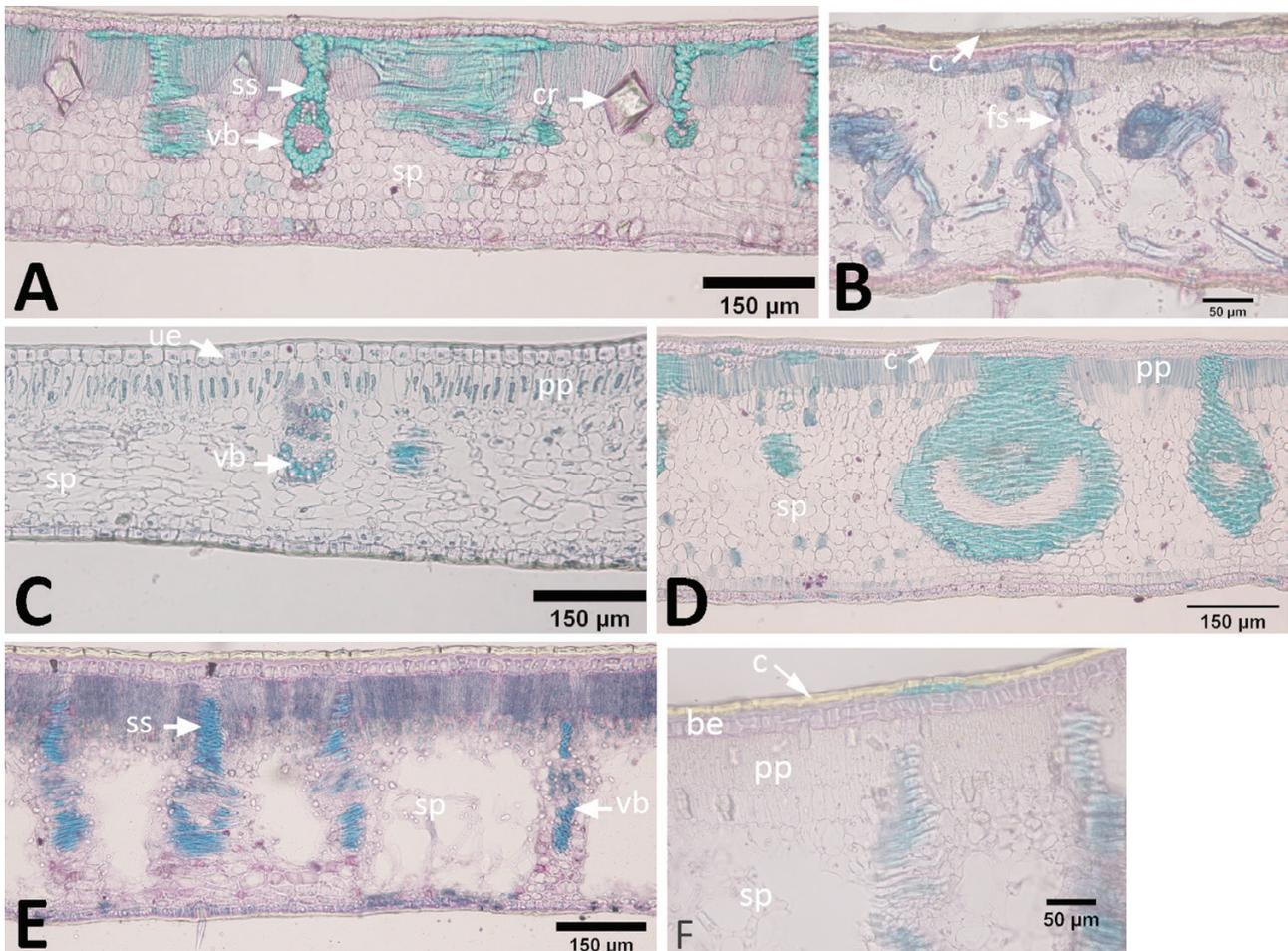
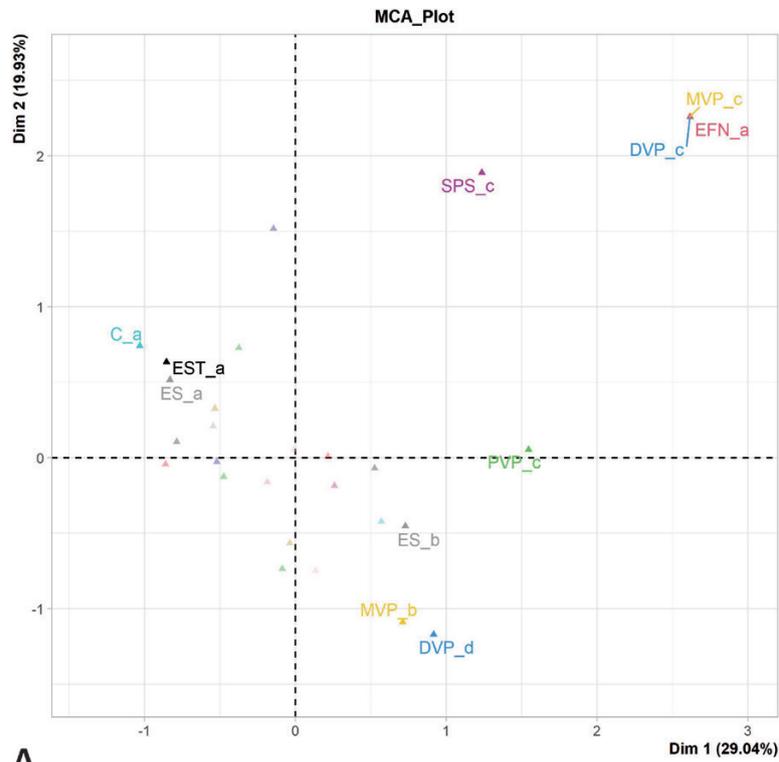
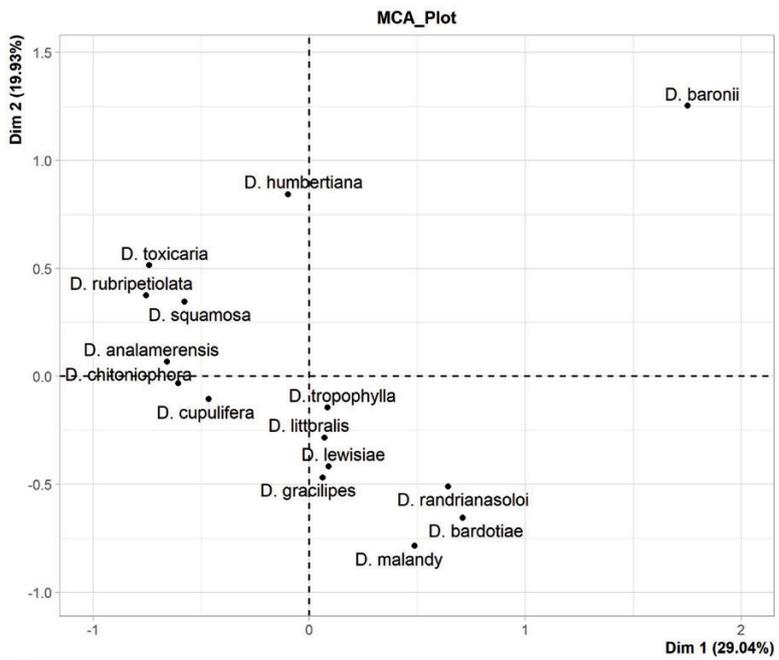


Figure 8. Cross-sections of the mesophyll. A, *D. chitoniophora*: palisade parenchyma uniseriate, compact spongy parenchyma, vascular bundles with sclerenchyma sheath extensions to the adaxial surface. B, thick cuticle, filiform sclereids in *D. lewisiae*. C, palisade parenchyma uniseriate and spongy parenchyma moderately compact in *D. rubripetiolata*. D, thick cuticle, palisade tissue biseriate and spongy parenchyma compact in *D. malandy*. E and F, thick cuticle, pluristratified epidermis, palisade biseriate tissue and spongy parenchyma with many intercellular spaces in *D. baronii*. Abbreviations: c = cuticle; be = biseriate epidermis; cr = crystal; fs = sclerenchyma filiformis; pp = palisade parenchyma; sp = spongy parenchyma; ss = sclerenchyma sheath extension; vb = vascular bundles.



A



B

Figure 9. MCA results. Projection of the first two dimensions. A, points represent character states; B, points represent species.

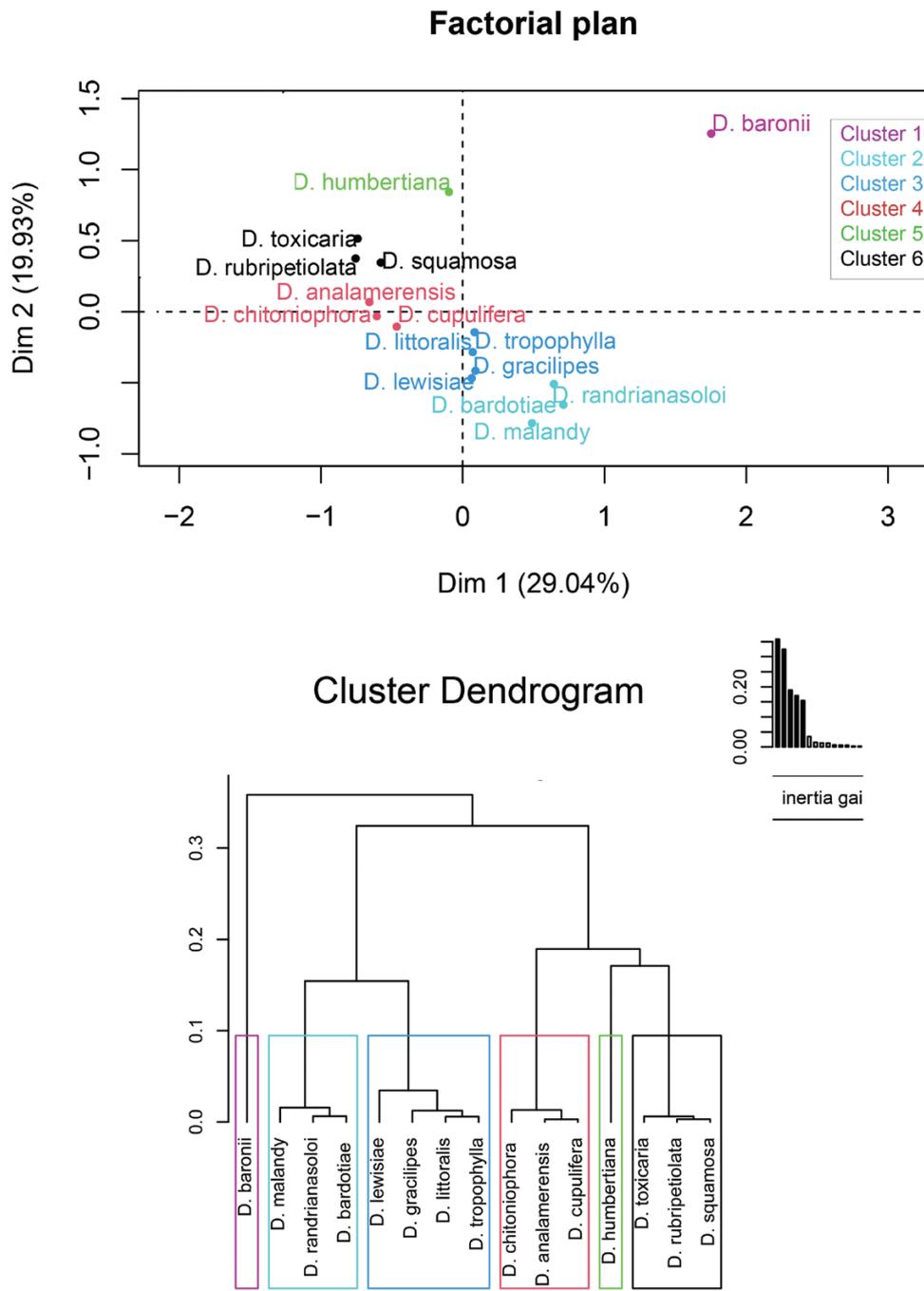


Figure 10. Cluster analysis of *Diospyros* species based on young stem and leaf anatomical characters: A, factorial plan of the first two dimensions; B, cluster dendrogram with cluster 1 at left and cluster 6 at right.

Identification key of the 15 *Diospyros* species examined based on young stem and leaf anatomy

1. Extrafloral nectaries present, distal vascular pattern in open arc with 1 or 2 adaxial vascular bundles, midrib vascular pattern in arc with strongly incurved ends ***D. baronii***
- Extrafloral nectaries absent, others distal vascular pattern, others midrib vascular pattern..... 2
2. Cells of adaxial epidermis larger than those of the abaxial epidermis 3
- Cells of adaxial and abaxial epidermis of the same size 9
3. Distal vascular pattern in a closed vascular cylinder, with lateral vascular bundles 4
- Distal vascular pattern in an open arc, with lateral accessory vascular bundles 6
4. Proximal vascular pattern in a closed sinuous arc, leaf midrib flat–slightly convex ***D. malandy***
- Proximal vascular pattern in a closed arc, leaf midrib shape flat–convex/biconvex..... 5
5. Spongy parenchyma compact, leaf midrib flat–convex ***D. bardotiae***
- Spongy parenchyma moderately compact, leaf midrib biconvex ***D. randrianasoloi***
6. Filiformis sclerenchyma present, leaf midrib slightly biconvex ***D. lewisiae***
- Filiformis sclerenchyma absent, leaf midrib biconvex or flat–convex 7
7. Stem indumentum absent, midrib vascular pattern in an arc, with 2 adaxial vascular bundles..... ***D. gracilipes***
- Stem indumentum present, midrib vascular pattern in open arc..... 8
8. Midrib biconvex..... ***D. littoralis***
- Midrib flat–convex ***D. tropophylla***
9. Papillae absent, spongy parenchyma compact 10
- Papillae present, spongy parenchyma moderately compact or lacunar 12
10. Proximal vascular pattern in a closed sinuous arc, midrib slightly convex–convex..... ***D. chitoniophora***
- Proximal vascular pattern in an open arc, with invaginated ends, midrib slightly concave–slightly convex..... 11
11. Epidermis unistratified ***D. analamerensis***
- Epidermis bi- or pluristratified..... ***D. cupulifera***
12. Spongy parenchyma lacunar ***D. humberiana***
- Spongy parenchyma moderately compact 13
13. Proximal vascular pattern in an open arc, midrib concave–convex..... ***D. toxicaria***
- Proximal vascular pattern in an open arc, with invaginated ends, midrib convex–flat or flat–convex 14
14. Stem indumentum absent, midrib convex–flat ***D. rubripetiolata***
- Stem indumentum present, midrib flat–convex..... ***D. squamosa***

DISCUSSION

The results of this study agree largely with the general descriptions provided by Metcalfe and Chalk (1957) and Wallnöfer (2001), as well as descriptions of some *Diospyros* species (Jordaan and Kruger 1992, Ugbogu *et al.* 2016, Paudel and Heo 2018), which mentioned characters such as trichomes mostly unicellular, prismatic crystals present, presence of epidermal papillae, hypodermis generally absent, and a pericycle usually containing a composite continuous ring of sclerenchyma. Some anatomical features seen in the studied species appear to be of potential taxonomic value and may therefore be useful as a tool for species identification.

The epidermis layer of the young stem can be used to distinguish *D. cupulifera*, *D. littoralis*, and *D. randrianasoloi* from the other species examined. These three species have two or more layers of epidermal cells while the others always have only a single layer.

Doughnut-shaped trichomes are found on the stems in *D. baronii* and *D. humberiana* and on both stems and leaves in *D. lewisiae* and *D. malandy*. This type of trichome was not previously mentioned in the general anatomical descriptions of Ebenaceae by Metcalfe and Chalk (1979) or Wallnöfer (2001), or in descriptions of 30 *Diospyros* species from Nigeria by Ugbogu *et al.* (2016). Pluricellular trichomes constituted by a short stalk and multicellular head in the leaf lamina occur in *D. randrianasoloi*; this type of trichome is called ‘club shaped’ by Metcalfe and

Chalk (1979) and Wallnöfer (2001). Unicellular, two-armed trichomes with short stalks occur only in *D. malandy*. According to Evert (2006), differences in trichome type are important for taxonomy and are of value for the systematics and identification of species or genera (Metcalfe and Chalk 1957). According to Stuessy (2009), trichomes have mostly been used taxonomically to compare species within a genus. For example, trichome types are considered among the most important characters used in the identification key of Malpighiaceae species (Araújo *et al.* 2010).

Most of the studied species have papillae except *D. analamerensis*, *D. chitoniophora*, and *D. cupulifera*. According to Metcalfe and Chalk (1957), Wallnöfer (2001), and Ugbogu *et al.* (2016), epidermal papillae on leaf surfaces occur in several species of *Diospyros*. The absence of papillae in the three species mentioned above can thus be used to distinguish them from the other species examined. The occurrence of papillae is a potential important systematic feature according to Dickison (2000).

Some anatomical characteristics of the petiole, such as vascularization pattern, the occurrence of stone cells, and the presence of extrafloral nectaries, can be used to distinguish the studied species. According to Metcalfe and Chalk (1957), the petiole is of considerable taxonomic importance since its structure appears to be less affected by the environment than other leaf features.

Among the 15 studied species, *D. chitoniophora*, *D. littoralis*, and *D. toxicaria* contain stone cells in the parenchymatous

portions of the veins of petioles. According to Wallnöfer (2001), the presence of stone cells in the petiole is a common character in *Diospyros* and *Euclea* (another genus of Ebenaceae). They can therefore be considered as diagnostic to separate these three species from the others. To our knowledge, studies on the anatomy of the young stem and leaves of Ebenaceae are rare or non-existent. We could not find available data on the two other genera, *Lissocarpa* and *Royena*, for comparison.

The presence of extrafloral nectaries in the distal part of the petiole distinguishes *D. baronii* from all other studied species. According to Metcalfe and Chalk (1957), Contreras and Lersten (1984), Wallnöfer (2001), and Weber and Keeler (2013), the presence of extrafloral nectaries on the abaxial side of leaves is frequent in many species of Ebenaceae. Of the 169 *Diospyros* species examined by Contreras and Lersten (1984), 127 exhibited this character on the abaxial side of the lamina, where it was associated with vascular tissue in the blade but not in the petiole. The other 14 species examined in this study may have extrafloral nectaries on other parts of their leaves. According to Wallnöfer (2001), in Ebenaceae they are often located near the base of the leaf blade or near the midrib and apex.

Vascularization at the distal part showed four types of patterns. According to our results, this character is useful to group and to distinguish *Diospyros* species. In general, the studied species show an open arc with lateral vascular bundles, but other forms are found in some species, which allow them to be differentiated. *Diospyros humbertiana* and *D. baronii* each present very particular forms of vascularization: an open arc without lateral vascular bundles and a closed vascular cylinder with lateral vascular bundles in *D. humbertiana*, whereas *D. baronii*, *D. bardotiae*, *D. malandy*, and *D. randrianasoloi* present closed vascular cylinders that are slightly flattened on the adaxial side with lateral vascular bundles. Some researchers have reported that the vascularization system patterns are also useful for identification of certain species in Dipterocarpaceae (Ruzi *et al.* 2009, Noraini *et al.* 2016, Talip *et al.* 2017) and in *Curcuma* (Zingiberaceae) (Anu and Dan 2020).

Variation in the shape of the midrib observed in the studied species is useful to distinguish among them. Ten shapes of the midrib were observed in the 15 species analysed. This character was also reported as useful to develop an identification key of species of *Banisteriopsis* C.B. Rob. (Malpighiaceae) (Araújo *et al.* 2020).

Most of studied species except *D. baronii* and *D. humbertiana* have a mesophyll that lacks intercellular spaces. The presence of lacunar mesophyll is useful to distinguish these species from the others studied here. According to Evert (2006), the presence or absence of intercellular spaces may be a systematically significant character.

Cluster analysis places *D. gracilipes* and *D. lewisiae* together in the same group (cluster 3). According to Linan *et al.* (2019), *D. lewisiae* is close to *D. gracilipes*. These two species along with *D. randrianasoloi* belong on the Gracilipes group, one of the dozen informal Malagasy groups recognized on the basis of vegetative and reproductive features. These three species share the following anatomical characteristics: presence of epidermal papillae, bi- or pluristratified epidermis, and adaxial epidermis cells larger than abaxial epidermis cells. However, *D. lewisiae*

has abundant filiform sclerenchyma in the mesophyll that distinguishes it from all other studied species. Other anatomical characteristics that can be used to distinguish them are the vascularization pattern and the shape of the midrib.

On the basis of anatomical characters, *D. analamerensis*, *D. chitoniophora*, and *D. cupulifera* form a distinct group according to the results of cluster analysis. They have many anatomical characters in common. What distinguishes these three species from all other studied species is the absence of papillae on the stems and leaves. According to Linan *et al.* (2019), *D. analamerensis*, *D. chitoniophora*, and *D. cupulifera* are closely related. Linan *et al.* (2021) included these species in *Diospyros* section *Forbesia*, whose members share similar morphological characteristics of their leaves, flowers, and fruits. The anatomical characters that can be used to distinguish them are: the vascularization pattern at the proximal end of the petiole, a sinuous arc in *D. chitoniophora* versus an open arc with invaginated ends in *D. analamerensis* and *D. cupulifera*, and epidermis stratification. *Diospyros chitoniophora* has stratified epidermis but *D. cupulifera* has non-stratified epidermis.

In conclusion, our results show that anatomical characters of young stems and leaves provide useful data for differentiating the 15 *Diospyros* species studied and indicate that many traits appear to have potential taxonomic value, suggesting that they may be useful in aiding species delimitation and supporting identification of the more than 250 species of *Diospyros* found on Madagascar. Based on these anatomical characteristics, an identification key for the selected members of the genus in Madagascar is given for the first time. Our research plans are to extend the anatomical studies to other large-tree *Diospyros* species and to continue analyses on the other currently recognized groups to assess whether anatomical data support any of them. It would also be interesting to extend this research to other Malagasy genera.

SUPPLEMENTARY DATA

Supplementary data are available online at the *Botanical Journal of the Linnean Society*.

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