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Comparative foliar anatomical traits of the endemic plant *Diospyros javanica* and its widely distributed relatives

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Abstract. *Rindyastuti R, Azrianingsih R, Junaedi DI, Damayani J, Arumingtyas EL. 2026. Comparative foliar anatomical traits of the endemic plant* Diospyros javanica *and its widely distributed relatives. Biodiversitas 27 (1): d270112. https://doi.org/10.13057/biodiv/d270112.* Anatomical characteristics may serve as diagnostic indicators that are useful for elucidating the identity of a plant species and its ecological adaptation to environmental changes. This study aimed to examine the foliar anatomical traits of *Diospyros javanica*, in comparison with its widely distributed relatives, *Diospyros cauliflora* and *Diospyros buxifolia*, which are crucial for understanding trait variation, taxonomic position, and environmental adaptation. Plant samples were collected from Purwodadi Botanic Garden, National Research and Innovation Agency (BRIN). Anatomical preparations were made using the paraffin-embedding method. Almost all qualitative variations could act as distinguishing traits among the species. Anatomical traits observed in this study include stomata, cuticle layer, epidermis, intracellular cavity, vascular bundles, sclerenchyma tissue, palisade and spongy mesophyll, supporting elements such as tracheids, mucilage cells, crystal oxalate, and metabolites such as phenolic deposits. Quantitative analysis of stomatal traits showed significant differences for all traits except stomatal density (p=0.18). All anatomical traits of leaf blades are significantly different across the three species, with p value of 0.0. The thinner photosynthetic structures of *D. javanica* indicate lower growth performance compared to that of its more widely distributed relatives. Research on ecophysiology related to growth rate and biochemical content that support plant adaptation could be conducted in the near future to inform species conservation.

Keywords: Adaptation, *Diospyros javanica*, endemic, leaf anatomy, plant traits

INTRODUCTION

Endemism is a biological phenomenon that threatens the existence and diversity of plant species due to its potential in restricting geographical occupancy of plants to a wider area (Fattorini 2017; Mir et al. 2020). Endemic plants are vulnerable both ecologically and anthropogenically, thus they are prioritized in conservation programs (Myers et al. 2000; Coelho et al. 2020). In general, endemic plants specifically adapt to high-pressure habitats, tolerate stress, show limited resource acquisition, and conserve higher nutrients (Lavergne et al. 2003; Lavergne et al. 2004).

van den Brink (1938) divided the genus *Diospyros* into 5 subgenera, i.e., *Cargillia, Eudiospyros, Hierniodendron, Maba,* and *Mabacea.* One of the endemic species of the genus *Diospyros* (Ebenaceae) recorded in Java and the Lesser Sunda Islands is *Diospyros javanica* Bakh. This species is distinguished morphologically by its leaves, which are either elongated or elongated-oval. The fruit is round to compressed round in shape, yellow to orange in color, and soft in the flesh. The fruit calyx is in the shape of a 4-lobed semi-square tube and wavy (van den Brink 1938; Wallnöfer 2001). *Diospyros cauliflora* Blume is a non-endemic plant species with a wide distribution from Indochina to Sumatra, Java, Lombok, Borneo, the Philippines, Celebes, and the Moluccas. *Diospyros buxifolia* (Blume) Hiern is widely distributed throughout Sumatra, Borneo, the Malay Peninsula, the Philippines, Moluccas, New Guinea, and Bali (POWO 2024). Three species in this study are included in the similar subgenus *Eudiospyros* (van den Brink 1938). The three species, especially *D. javanica*, demonstrated important ecological roles in Java (Rindyastuti et al. 2018; Febriana et al. 2020; Lestari and Darmayanti 2020).

Given the photosynthetic function of the leaves, which capture sunlight and absorb carbon dioxide for mass and energy production, leaf anatomical traits indicate the plant's adaptations to certain environments (Adams and Terashima 2018; Zhang et al. 2022). In the context of endemic and rare plants, some structural strategies used to cope with harsh environments are reflected by leaf anatomical traits that represent competitive ability and habitat specificity (Rossatto et al. 2015; de Paula et al. 2019). Sandratriniaina et al. (2023) reported high variation in anatomical features, such as the epidermal layer, mesophyll layer, and vascular strand, in *Diospyros* of Madagascar, which are useful for distinguishing species groups with taxonomic and conservation implications. Akinsulire et al. (2018) reported the diagnosis of epidermal and venation traits in *Diospyros*, suggesting that anatomical characters can serve as important diagnostic tools for species identification and subgenus classification. Kuster et al. (2016) also demonstrated that the adaxial epidermis and palisade layers were thicker at high altitude due to high light radiation, low humidity, and low temperature.

As part of ongoing investigations into plant traits associated with endemism, Rindyastuti et al. (2024) demonstrated that *D. javanica* exhibits specialized adaptations to the coastal habitats of Java and the Lesser Sunda Islands, particularly through its leaf morphology, including sub-cordate leaf apices and densely sericeous fruits and calyx trichomes. Building on these findings, we conducted comparative analyses of anatomical traits to examine differences between endemic and more widespread species. We predict that the endemic species *D. javanica* will also exhibit anatomical traits consistent with the environmental conditions to which it adapts. The present study aimed to identify anatomical trait variations among the three *Diospyros* species, to evaluate the taxonomic implications of these anatomical differences within the genus *Diospyros* and to assess how foliar anatomical traits reflect environmental adaptation in both endemic and widely distributed species. This study is important for species conservation, as insights into leaf internal structure enhance our understanding of adaptive strategies in Indonesian persimmon and ebony species.

MATERIALS AND METHODS

Plant materials

Anatomical study was conducted on the leaves of three species of *Diospyros*, the most common species growing in the tropical habitats of Java, namely *D. javanica*, *D. cauliflora*, and *D. buxifolia* (Figure 1). The study was conducted from May 2022 to December 2023. Leaves of three *Diospyros* species were sampled from the plant collection of the Purwodadi Botanic Garden of the National Research and Innovation Agency (BRIN), Pasuruan, East Java, Indonesia. The species identification was established formerly by the plant experts of botanic garden, then the morphological observation was established to reassure using species description by van den Brink (1938). Plant species, the origins, habitats, and distribution of the plant collection of Purwodadi Botanic Garden are presented in Table 1. Three plant trees were selected for each species. Therefore, three leaves per plant were sampled as replications for anatomical preparation. The leaves sampled for this study were collected from the outer canopy layers, ranging from the middle to the downward vertical canopy layers.

Table 1. Plant species, origin, habitats, and distribution

Species	Collection number	Sample origin	Habitat	Distribution
Diospyros javanica Bakh.	XX.H.3-3a-3b-3c	Trenggalek, East Java	Tropical lowland forest	Java and the Lesser Sunda Islands
Diospyros cauliflora Blume	XX.G.18	Malang, East Java	Tropical lowland forest	Indochina, Sumatra, Java, Lombok, Borneo, Philippines, Celebes, Moluccas
Diospyros buxifolia (Blume) Hiern	XVIII.C.063-063a	Pasuruan, East Java	Tropical lowland forest	Sumatra, Borneo, Malay Peninsula, Philippines, Moluccas, New Guinea, Bali

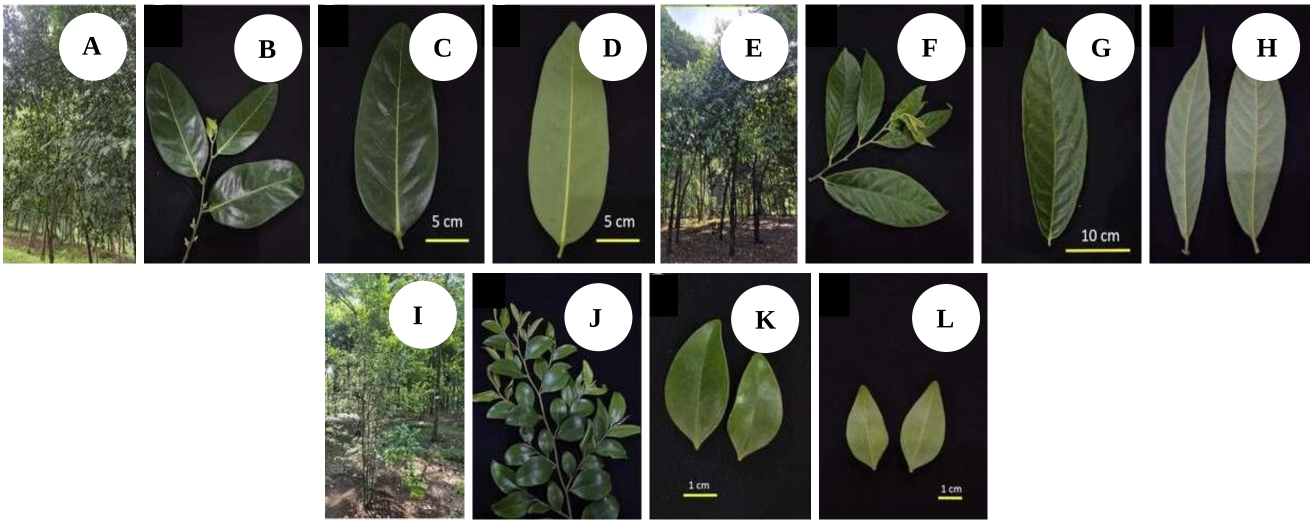

Figure 1. Habitus and leaf morphology of the three *Diospyros* species studied. *Diospyros javanica*: A. Habit, B. Leave and twigs, C. Lamina with upper surface, D. Lamina with downside surface. *Diospyros cauliflora*: E. Habitus, F. Leave and twigs, G. Lamina with upper surface, H. Lamina with downside surface. *Diospyros buxifolia*: I. Habitus, J. Leave and twigs, K. Lamina with upper surface, L. Lamina with downside surface

Procedures

Leaf anatomy was prepared using the paraffin embedding method (Ruzin 1999). The leaf lamina, together with the midvein, was sectioned approximately into 0.5 cm long and width sections. The samples were cut and then immersed in a fixative solution for 24 hours. The fixative solution consisted of 95% alcohol, glacial acetic acid, formalin, and distilled water in a ratio of 50:5:10:35. The samples from the fixative solution were sequentially transferred into a graded alcohol series, comprising 50% alcohol (2×30 minutes), 70% alcohol (2×30 minutes), 80% alcohol (2 × 30 minutes), 90% alcohol (2×30 minutes), and 96% alcohol (3×30 minutes). The leaves from the graded alcohol series were immersed in alcohol-xylene solution 1 (3:1) for 15 minutes, alcohol-xylene solution 2 (1:1) for 15 minutes, alcohol-xylene solution 3 (1:3) for 15 minutes, and then in pure xylene three times until the samples appeared transparent. The samples from pure xylene were transferred into a xylene-paraffin mixture for 15 minutes, followed by immersion in paraffin 1, paraffin 2, and paraffin 3, each for 15 minutes. They were then placed into molds and left at room temperature for 24 hours. The paraffin-embedded samples were mounted onto wooden blocks and left for an additional 24 hours. The paraffin blocks containing the leaf samples were sectioned using a rotary microtome to a thickness of approximately 10-12 µm, then placed on glass slides coated with glycerin albumin and moistened with distilled water. The sections on the slides were placed on a hot plate and left until dry and flattened. The samples on the glass slides were immersed in pure xylene for 20 minutes, then dipped in an alcohol-xylene solution (1:1) and subsequently in a graded alcohol series, each for 1 minute. The samples were then transferred into 1% aqueous safranin solution for 1 hour, rinsed with distilled water, dehydrated through a graded alcohol series (45% and 96%), stained with 0.1% fast green solution for 30 seconds, quickly immersed in absolute alcohol, de-alcoholized with an alcohol-xylene solution (1:1) for 30 seconds, cleared in pure xylene, and finally mounted using Entellan.

Anatomical samples were observed under a light microscope at 100× and 400× magnifications. Quantitative anatomical traits were observed and described for each sample and plant species. Important qualitative traits observed in this study are the stomatal types, the presence of cuticle, the layer number of palisade cells, the type of vascular bundles, the presence and layer number of sclerenchyma cells surround vascular bundles, the presence of phenolic deposits, mucilage cells, and crystal oxalate. Quantitative traits observed included Stomatal Density (SD), axis length, perimeter and area of stomata, aperture length, and Stomatal Index (SI). The traits of leaf blade were observed, including cuticle thickness, leaf thickness, sclerenchymal layers, phenolic deposits, and photosynthetic characteristics, which were measured using the P/S (palisade to spongy mesophyll) and P/T (palisade to total leaf thickness) ratios. All quantitative anatomical traits were measured using the Image Raster 3 software for Optilab 3.

Data analysis

An assessment of trait variation and adaptive abilities of *Diospyros* species to open habitats was conducted by examining the presence or absence and the measurement of important anatomical traits and specific adaptive anatomical features. For qualitative traits, anatomical traits were observed under the microscope, described and compared across three species. Several important qualitative traits to compare are the stomatal types, the presence of cuticle, the layer number of palisade cells, the type of vascular bundles, the presence and layer number of sclerenchyma cells surrounding vascular bundles, the presence of phenolic deposits, mucilage cells, and crystal oxalate.

Quantitative anatomical data, including data on leaf lamina, cuticle, epidermal cell, palisade, and spongy mesophyll layers, were measured and analyzed for the three *Diospyros* species under study using Analysis of Variance (ANOVA) and Tukey's Post-Hoc Test, with a 95% confidence level in SPSS 16.0. The comparison of the adaptive abilities of *Diospyros* species was based on statistical tests of various adaptive anatomical traits, including stomatal density, long axis length, short axis length, aperture length, and stomatal index. The leaf blade traits, including cuticle thickness, leaf thickness, sclerenchyma layers, phenolic deposits, and photosynthetic characteristics, were measured using the P/S (palisade to spongy mesophyll) and P/T (palisade to total leaf thickness) ratios (Hu et al. 2022; Li et al. 2022).

RESULTS AND DISCUSSION

Anatomical traits

Stomata

Stomata traits of the three *Diospyros* species and their trait comparison are represented in Figure 2, whereas the traits description of the stomata were displayed in Table 2. The stomata of three species studied are only found at the abaxial side; however, they have different stomata types (Table 2).

Based on the statistical test of ANOVA, all stomata traits, i.e., long axis length, short axis length, perimeter of stomata, area of stomata, aperture area, and Stomatal Index (SI), are significantly different (p=0), except Stomatal Density (SD) with a p=0.18 (Table 3). The values and species grouping showed that the endemic species *D. javanica* has the largest stomata among the three species, as confirmed by significant differences in stomatal size traits, i.e., long axis length, short axis length, perimeter, and area (Table 4). The big size of stomata is not compensated by the number of stomata, as shown by the non-significant difference, and the lowest of stomatal density among the three species (p<0.05). This comparison indicates that *D. javanica* has relatively large stomata and a high stomatal density, given the observed area and epidermal cell density (Figure 2). This result showed that stomatal traits have potential taxonomic value, serving as diagnostic traits for delimiting plant species and predicting plant adaptation to environmental conditions.

The ability to adapt to open environments is associated with leaf types that tolerate both sunny and shady habitats. Shade leaves lack the necessary adaptations to thrive in these environments (Dörken and Lepetit 2018). In response to increased light and drought stress in open habitats, plants develop thicker cuticles, multiple palisade layers, and spongy mesophyll (Wimalasekera 2019). In addition to leaf blade thickness, sun-adapted plants are characterized by a high density of stomata and trichomes (Ichie et al. 2016). Hughes et al. (2017) revealed that reduced stomatal density increases drought tolerance. Several crops exhibit relatively low stomatal densities; for example, barley ranges from 10 to 30 stomata/mm², wheat from 52 to 88 stomata/mm², and maize from 65 to 80 stomata/mm². In contrast, many woody tree species display significantly higher stomatal densities. According to Rindyastuti et al. (2024), *Artocarpus heterophyllus* reaches approximately 800 stomata/mm², *Canarium vulgare* about 500 stomata/mm², *Syzygium polyanthum* also up to 500 stomata/mm², *Garcinia dulcis* around 400 stomata/mm², *Durio zibethinus* approximately 550 stomata/mm², and *Cinnamomum sintoc* exceeds 600 stomata/mm². Compared to these species, *D. javanica* exhibits a moderate stomatal density, suggesting a correspondingly moderate tolerance to drought, though still less resilient than other woody trees. Conversely, *D. cauliflora* has the highest stomatal density among those studied, indicating greater adaptation to high-light environments; consequently, lower tolerance to drought stress relative to other species.

Stomatal density is another parameter associated with sun or shade-adapted characteristics. As stomata play a role in connecting the internal leaf space to the external atmosphere, stomatal density is a key feature in the direct regulation of CO_2 and H_2O exchange (Agurla et al. 2018; Driesen et al. 2020). The three species exhibit no significant differences in stomatal density, indicating that it is not possible to determine which species is more sun-adapted based on this parameter. Nevertheless, according to Agurla et al. (2018), smaller stomata can respond more rapidly to environmental changes by opening and closing more quickly, underscoring the role of stomatal size in this mechanism. *D. javanica* has the biggest stomatal size confirmed by all stomatal size traits that may influence the rate of plant transpiration and the slow opening-closing of the stomata. *D. buxifolia* has the smallest stomata, as measured by stomatal length and width, among the three species, suggesting a more efficient process of stomatal opening and closing. Coupled with a high stomatal density, it can increase stomatal conductance rapidly, facilitating enhanced CO_2 diffusion when conditions are favorable for photosynthesis. This comparison showed that *D. javanica* is more susceptible to drought stress and climate change, as indicated by rising temperatures.

Table 2. Stomata traits description and comparison among the three *Diospyros* species

Anatomical traits	***Diospyros javanica***	***Diospyros cauliflora***	***Diospyros buxifolia***
Stomata distribution	Hypostomatic	Hypostomatic	Hypostomatic
Stomata types	Actinosytic	Anisocytic, parasytic	Parasytic, actinosytic

Table 3. The output of ANOVA for the stomatal traits across the three *Diospyros* species

Anatomical traits	**Sum of squares**	**df**	**Mean square**	**F**	***p* values**
Stomatal Density (SD)	2662.4	2	1331.200	1.828	.180
Long axis length	105.41	2	52.705	25.243	.000
Short axis length	239.351	2	119.676	66.823	.000
Perimeter of stomata	3529.994	2	1764.997	237.462	.000
Area of stomata	408751.3	2	204375.64	271.485	.000
Aperture length	29.984	2	14.992	12.490	.000
Stomatal Index (SI)	86.083	2	43.041	35.007	.000

Table 4. Comparison of average stomatal parameters and species grouping of the three *Diospyros* species

Stomatal traits	***Diospyros javanica***	***Diospyros cauliflora***	***Diospyros buxifolia***
Stomatal Density (SD)	195.2±26.98^{a}	211.2±28.02^{a}	188.8±25.91^{a}
Long axis length (µm)	26.16±1.03^{a}	23.73±1.72^{b}	21.57±1.50^{c}
Short axis length (µm)	23.77±1.62^{a}	19.12±0.67^{b}	17.01±1.52^{c}
Perimeter of stomata (µm)	83.679± 2.51^{a}	69.966±2.33^{b}	57.113±3.24^{c}
Area of stomata (µm)	529.28±29.5^{a}	364.64±24.06^{b}	244.51± 28.43^{c}
Aperture length (µm)	11.85±0.99^{a}	11.51±1.27^{a}	9.58±1.04^{b}
Stomatal Index (SI)	6.80±0.56^{b}	12.50±1.36^{a}	8.43±1.23^{b}

Note: Data represents means±Standard Deviations (SD, n=9). Lowercase letters in each row indicate significant differences (ANOVA and Tukey's posttest, $p<0.05$)

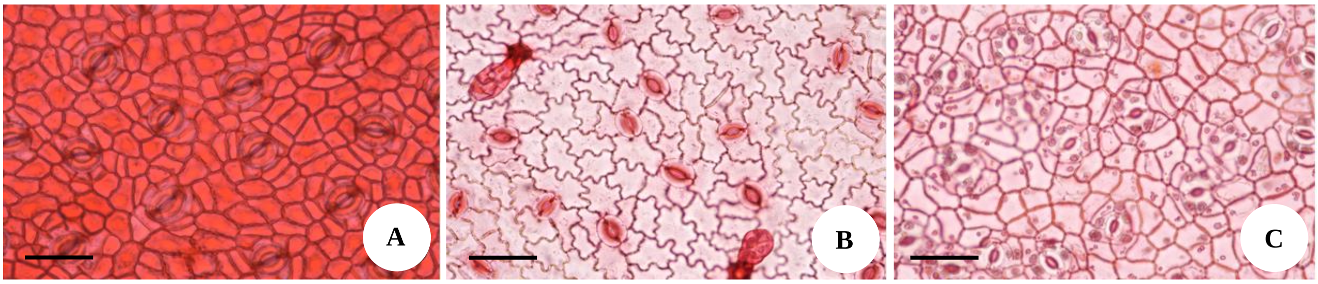

Figure 2. Leaf stomatal structures of the three *Diospyros* species. A. *Diospyros javanica*, B. *Diospyros cauliflora*, C. *Diospyros buxifolia* (magnification 40×). Scale bar: 50 µm

Leaf blades

Anatomical traits of leaf blades are explored through the transverse or cross-section of leaf blades. Through these traits, we could reveal the species variation and adaptation. All anatomical characteristics of the three *Diospyros* species are represented in Figure 3. The study revealed that qualitative traits, i.e., the cuticle layer thickness, palisade layers, spongy mesophyll layers, intracellular cavity, sclerenchyma tissue, the presence of mucilage cells, the position of crystal oxalate, and phenolic deposits, provide evidence that the anatomical traits of *Diospyros* vary at the species level.

Outermost structures

The outermost structures consist of mechanical components, including leaf blades, cuticle, and epidermal layers. The comparison of these structures was presented in Figure 4. The thickness of these two layers was measured and analyzed statistically. The ANOVA results and post-hoc grouping were presented in Tables 5 and 6.

The cuticle is the outermost layer of the leaf and plays a crucial role in protecting the plant by reducing absorbed solar radiation and temperature through the reflection of sunlight, as well as minimizing transpiration (Domínguez et al. 2017). Additionally, the cuticle prevents uncontrolled diffusion of water into the atmosphere (Ingram and Nawrath 2017; Arya et al. 2021). Therefore, the cuticles of leaves exposed to full sunlight are significantly thicker than those of shade leaves (Dörken and Lepetit 2018). Although cuticle layers play important roles in inner tissue protection, not all species of *Diospyros* possess this feature. Based on the statistical test results of ANOVA ($p<0.05$), cuticle thickness is significantly different across the three species, with a *p* value of 0.0 (Tables 5 and 6). Among the species examined, *D. javanica* possesses the thickest cuticle layer, whereas the other two species exhibit comparatively thinner layers. The variation in cuticle thickness among the three species suggests that this trait may serve as a useful diagnostic character for classification, identification, species grouping, and evolutionary studies. The taxonomic significance of this feature is further supported by previous studies that reported considerable variation in both the presence and thickness of cuticle layers across the genus *Diospyros*. For instance, in a study of 15 *Diospyros* species conducted by Sandratriniaina et al. (2023), *Diospyros rubripetiolata*, *Diospyros squamosa*, and *Diospyros toxicaria* were found to lack cuticle layers on their leaf surfaces.

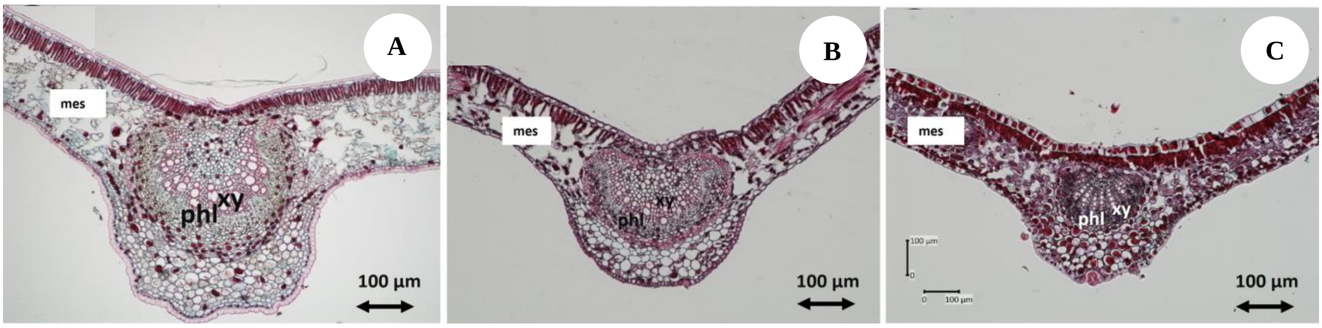

Figure 3. Leaf anatomical traits of the three *Diospyros* species. A. *Diospyros javanica*, B. *Diospyros cauliflora*, C. *Diospyros buxifolia* (magnification 40×). phl: Phloem, xy: Xylem

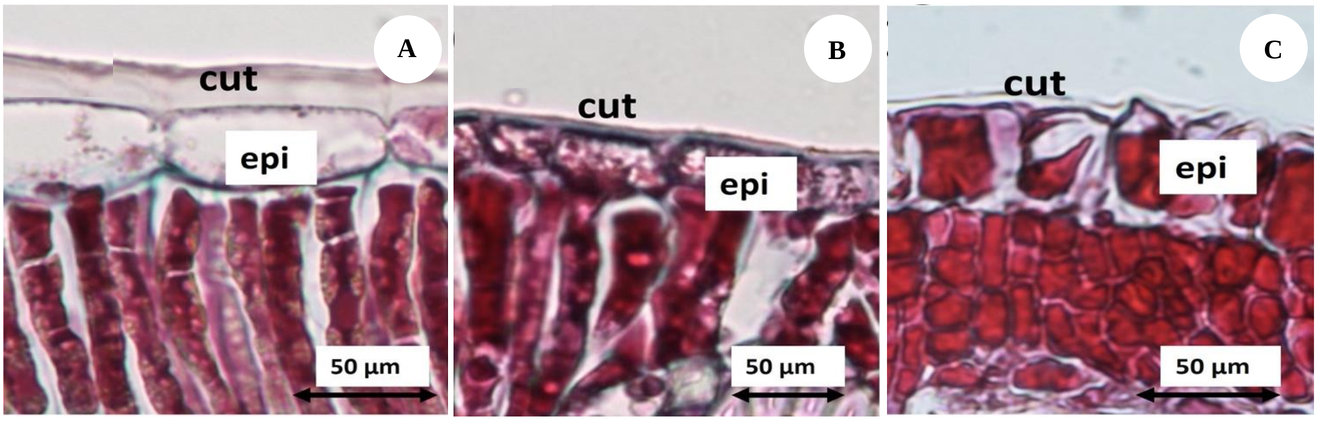

Figure 4. Outermost anatomical structures of the three *Diospyros* species: A. *Diospyros javanica*, B. *Diospyros cauliflora*, C. *Diospyros buxifolia* (magnification 400×). cut: Cuticle, epi: Epidermis

Table 5. The thickness of outermost structures of the three *Diospyros* species

Anatomical traits	***Diospyros javanica***	***Diospyros cauliflora***	***Diospyros buxifolia***
Leaf thickness (µm)	259.27±27.75^{a}	140.03±8.62^{b}	190.98±20.61^{c}
Cuticle thickness (µm)	8.31±0.63^{a}	2.98±0.52^{b}	3.47±0.49^{ab}
Upper epidermal cell thickness (µm)	13.67±2.36^{ab}	10.56±1.01^{b}	28.43±6.03^{a}

Note: Data represents means±Standard Deviations (SD, n = 9). Lowercase letters in each row indicate significant differences (ANOVA and Tukey's posttest, $p<0.05$)

Table 6. The output of ANOVA for the outermost anatomical traits across three *Diospyros* species

Anatomical traits	**Sum of squares**	**df**	**Mean square**	**F**	***p* values**
Leaf thickness (µm)	64430.78	2	32215.39	76.148	0.0
Cuticle thickness (µm)	140.36	2	70.18	234.428	0.0
Upper epidermis cell thickness (µm)	1641.019	2	820.51	57.308	0.0

Table 7. Outermost anatomical traits of the three *Diospyros* species

Anatomical traits	***Diospyros javanica***	***Diospyros cauliflora***	***Diospyros buxifolia***
Cuticle (*)	Thick	Thin	Thin
Trichome	Present	Present	Present
Trichome cells	Present, indistinct	Present, unicellular	Present, unicellular
Upper epidermal cells	Single layer, elongated	Single layer, square	Single layer, square

The epidermis, which is the outer cell layer of the leaf, functions as a barrier separating the leaf from the environment. This layer is essential for protecting the mesophyll cells from excessive solar radiation and for reducing the heat load on the leaf (Riglet et al. 2021; Zuch et al. 2022). All three species studied have single layers of epidermis. The epidermis of *D. javanica* is characterized by elongated epidermal cells on both the upper and lower surfaces, while the two other species feature square-shaped epidermal cells (Table 7). These anatomical features may serve as diagnostic characteristics for distinguishing between *Diospyros* species. However, Sandratriniaina et al. (2023) reported that most of the species in this genus studied in Madagascar have single-celled epidermis, while the other species could have 2-3 epidermal cells, such as *D. cupulifera*, *D. littoralis*, and *D. randrianasoloi*. In Nigeria, the morphology of the leaf epidermis of *Diospyros* has been recognized as a significant trait for differentiating different species, with variations observed in the cell shape, cell wall structure, thickness, and ornamentation (Ugbogu et al. 2016). Among the species studied, *D. buxifolia* has the thickest epidermal cells, while *D. cauliflora* has the thinnest. In addition, based on the statistical test results of ANOVA ($p<0.05$), all anatomical features of upper epidermis cell thickness are significantly different across three species with a *p* value of 0.0 (Table 5). In the environment with a greater level of exposure to light, shade-tolerant species tend to have a reduced epidermal cell thickness. Moreover, Ichie et al. (2016) presented that plants growing in open, hot, and drought-prone habitats tend to have hairier leaves compared to those of the same or similar species in more mesic or shaded environments.

Inner structure of leaf blades

The inner structure of the leaves, the mesophyll layer, was located between the upper and lower epidermis and plays a central role in photosynthesis. It is differentiated into two distinct layers, i.e., palisade and spongy mesophylls. The palisade mesophyll, positioned beneath the upper epidermis, consists of elongated cells rich in chloroplasts and functions as the primary site of photosynthesis. In contrast, the spongy mesophyll, located beneath the palisade layer, is composed of loosely arranged cells with abundant intercellular spaces that facilitate gas exchange. Together, these layers contribute to both photosynthetic efficiency and internal gas circulation within the leaf (Tian et al. 2016; Liu et al. 2021; Ohtake et al. 2021; Leite et al. 2023). Considering the functional importance of the palisade and spongy mesophyll, comparative analysis of these anatomical traits is particularly significant.

Diospyros javanica exhibits the thinnest palisade mesophyll, with only 1-2 cell layers, which contrasts with the more widely distributed *D. cauliflora* (2-3 layers) and *D. buxifolia* (3-4 layers) (Table 8). *D. javanica* exhibits more layers of spongy mesophyll (9-11 layers) (Figure 5), which contrasts with the more widely distributed *D. cauliflora* and *D. buxifolia* (7-9 layers) (Figures 6 and 7). The development of mesophyll cells is influenced by the plant's exposure to light (Johnson et al. 2005; Ohtake et al. 2021). Plant species with thicker mesophyll layers may have higher photosynthetic capacity, because of the increased number of chloroplasts (Castro-Díez et al. 2000; Liu et al. 2021). In addition, thicker layers of palisade and spongy mesophyll play a significant role in reducing tissue damage caused by intense solar irradiation and maintaining high photosynthetic rates (Liu et al. 2021). These trait differences may reflect variation on the species level, suggesting that they may be useful in aiding species delimitation and adaptive strategies associated with species distribution and ecological specialization.

Based on the statistical test results of ANOVA ($p<0.05$), all anatomical features of photosynthetic structures, including palisade layer thickness, spongy mesophyll thickness, and the ratios of P/T and P/S, are significantly different across three species with a *p* value of 0.0 (Tables 9 and 10). Palisade thickness, as described by the P/S ratio, is related to the light tolerance of plants and can vary depending on various environmental factors (Li et al. 2022). Plants grown in full sunlight typically develop greater palisade cell elongation in their leaves compared to those grown in the shade (Firmino et al. 2021; Leite et al. 2023; Nascimento et al. 2024). According to Tian et al. (2016), a thicker palisade mesophyll, which contains more chloroplasts, enhances light absorption, while a thicker spongy mesophyll facilitates gas exchange within the leaf. *D. javanica*, with its thinner palisade layers, has the thickest mesophyll layers, suggesting that in this species, the increased thickness of sun leaves is primarily due to the expansion of the mesophyll layers. Based on these findings, it can be inferred that this *D. javanica* is likely to have the lowest photosynthetic performance, while *D. cauliflora* is likely to have the highest.

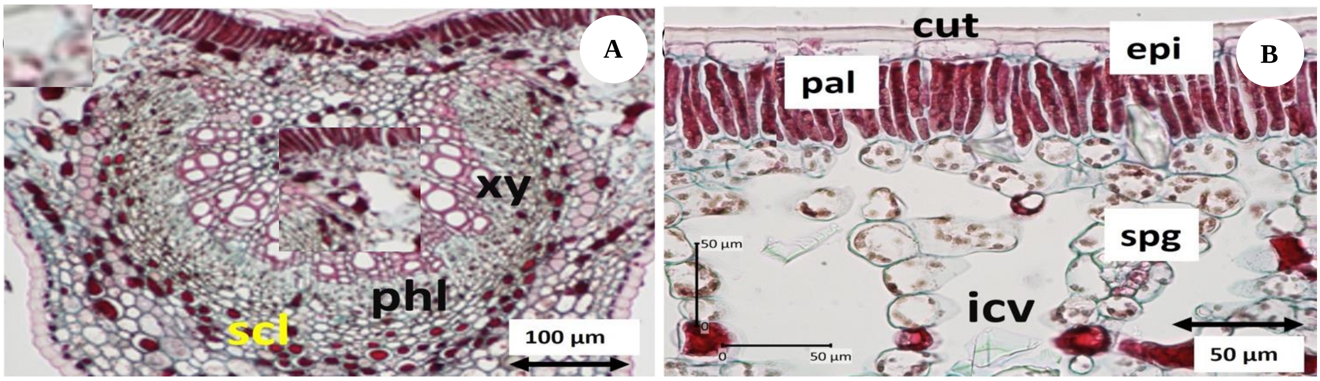

Figure 5. Inner leaf structures of the endemic species *Diospyros javanica*: A. Vascular bundle, containing xy: Xylem, phl: Phloem strands, and scl: Sclerenchyma layer (magnification 40×), B. The transverse section of the lamina, cut: Cuticle, epi: Epidermis, pal: Palisade layer, spg: Spongy mesophyll with very large intercellular cavity, icv: Intercellular cavity (magnification 40×)

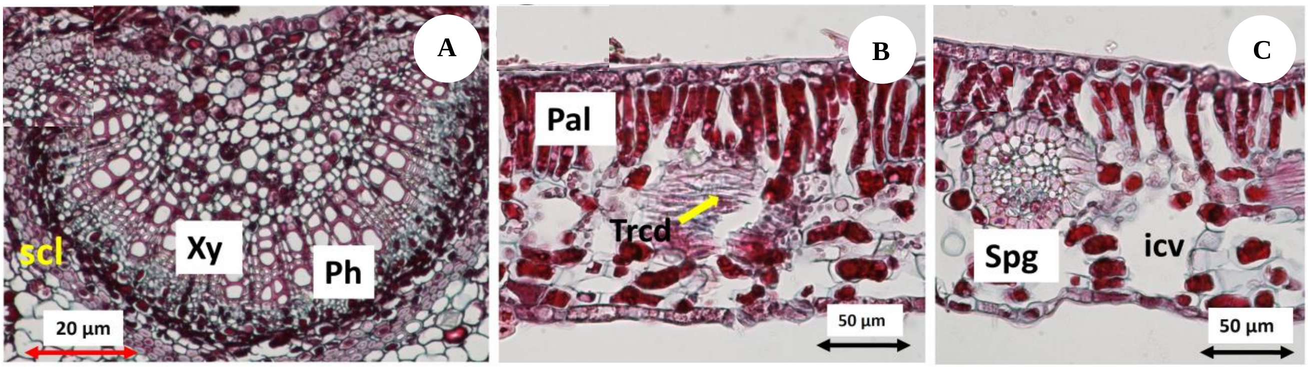

Figure 6. Inner leaf structures of the widely-distributed species *Diospyros cauliflora*: A. Vascular bundle, containing xy: Xylem, and ph: Phloem strands, scl: Sclerenchyma layer, B. The transverse section of the lamina, pal: Palisade layer (magnification 40×), C. Cut: cuticle, spg: Spongy mesophyll with large intercellular spaces, icv: Intercellular cavity (magnification 40×)

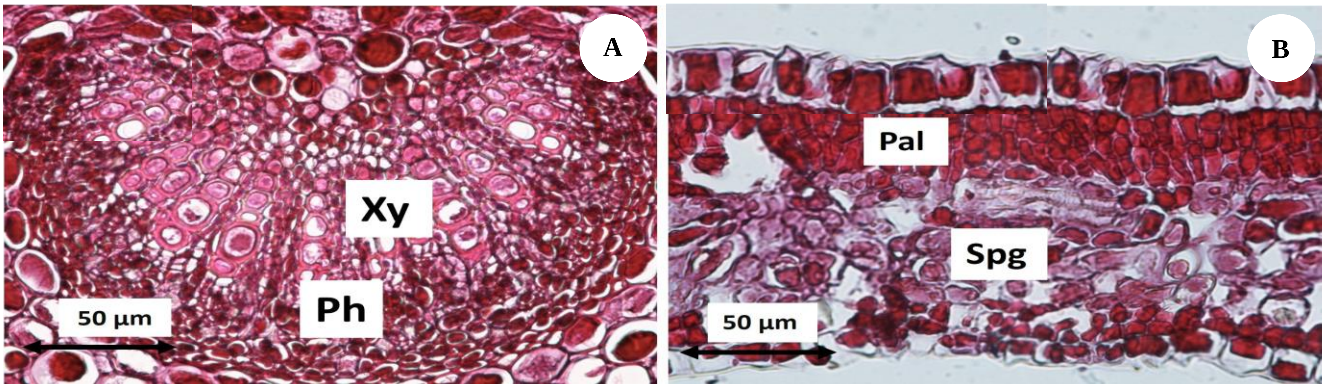

Figure 7. Inner leaf structures of the widely-distributed species *Diospyros buxifolia*: A. Vascular bundle, containing xy: Xylem, and ph: Phloem strands, B. The transverse section of the lamina, cut: Cuticle, pal: Palisade layer, spg: Spongy mesophyll with compact structure, icv: Intercellular cavity (magnification 40×)

Table 8. Inner leaf anatomical traits of the three *Diospyros* species

Anatomical traits	***Diospyros javanica***	***Diospyros cauliflora***	***Diospyros buxifolia***
Palisade	1-2 layers	2-3 layers	3-4 layers
Spongy mesophyll	9-11 layers	7-9 layers	7-9 layers
Development of vascular bundles	Highly developed	Highly developed	Well-developed
Vascular strands	U-type	U-type	U-type
Xylem and phloem configuration	Phloem almost surrounds xylem, xylem highly developed	Phloem almost surrounds xylem	Phloem almost surrounds xylem
Intracellular cavity	Very large	Large	Narrow
Sclerenchyma surround the vascular bundles (*)	Present, 3-4 layers	Present, 3-4 layers	Indistinct

Table 9. Anatomical structures of photosynthetic traits and the species grouping of the three *Diospyros* species

Anatomical traits	***Diospyros javanica***	***Diospyros cauliflora***	***Diospyros buxifolia***
Palisade thickness (µm)	39.07±3.38^{b}	51.58±4.32^{a}	43.09±6.35^{ab}
Spongy mesophyll thickness (µm)	149.4±4.68^{a}	73.07±9.09^{b}	103.58±20.93^{c}
P/S ratio (%)	49.18^{b}	59.08^{a}	35.98^{c}
P/T ratio (%)	13.6^{c}	43.9^{a}	26.7^{b}

Note: Data represents means±standard deviations (SD; n=9). Lowercase letters in each row indicate significant differences (ANOVA and Tukey's posttest; $p<0.05$)

Table 10. The output of ANOVA for the anatomical traits of leaf blades across three *Diospyros* species

Anatomical traits	**Sum of Squares**	**df**	**Mean Square**	**F**	***p* values**
Palisade layer thickness (µm)	734.154	2	367.077	15.62	0.0
Spongy layer thickness (µm)	26573.47	2	13286.74	73.478	0.0
P/T ratios	0.221	2	0.11	71.669	0.0
P/S ratios	0.97	2	0.485	52.67	0.0

The low ratio of photosynthetic structures in *D. javanica* may indicate limitations in growth performance and thicker palisade layers than in the widespread species *D. cauliflora*. In *Shorea disticha*, the increase in leaf thickness under full sunlight is mainly attributed to the expansion of palisade mesophyll, while the spongy mesophyll thickness actually decreases in full sunlight (Dörken and Lepetit 2018). Besides its association with photosynthesis and growth factors, spongy mesophyll thickness is related to the intercellular spaces or cavity that interconnects the parenchyma cells and other cell types. In plants, air spaces can form by cell separation or cell death (Whitewoods 2021). The intercellular spaces are filled with air, which is available for gas exchange, photosynthetic connections, water transport, and reducing incoming solar radiation to leaves (Jarvis et al. 2003). According to Whitewoods (2021), increased mesophyll cell density leads to an increase in photosynthetic capacity. *D. javanica*, with the largest intercellular cavity (Figure 5), may receive the least solar radiation; however, it possesses the lowest photosynthetic capacity. *D. javanica* tends to adapt to open habitats; however, at the same time has low effectiveness in growth and competition. *D. cauliflora* seems to have large intercellular cavity; however, it is not as large as those of *D. javanica*. *D. buxifolia* tends to have compact spongy mesophyll tissues because of narrow intercellular cavity. Variation in spongy mesophyll structures includes very large intercellular cavity, moderately large to almost absent intercellular cavity, and a supporting compact mesophyll structure (Sandratriniaina et al. 2023).

The shape of vascular strands is an important characteristic in Angiosperms. The U-type vascular bundle is a simple trace with a slightly curved shape, while the O-type bundle fuses to form a siphonostele (Song and Hong 2018). The three *Diospyros* species studied exhibit U-type vascular strands, indicating a close taxonomic relationship among them (Table 8). The conformation of the vascular bundles in the three species could not serve as distinctive traits for the subgenus. Meanwhile, Gomes and Lombardi (2010) used the vascular system of the petiole to classify Salacioideae (Celastraceae) into different genera. Moreover, different types of vascular strands are found among the 15 species of *Diospyros* in Madagascar (Sandratriniaina et al. 2023). The shape of vascular is ranging from flat-flat arc in *D. humbertiana*, slightly concave-slightly convex open arc in *D. cupulifera*, convex-flat, open arc in *D. rubripetiolata* to slightly biconvex, open arc in *D. lewisiae*. The shape of vascular strands can serve as a diagnostic feature at the inter-generic level, particularly if only different types are observed in other plant species.

The sclerenchyma cells surrounding the vascular bundles are a common traits found in the genus *Diospyros*. This tissue consists of a parenchymatous sheath of lignified cells surrounding the vascular bundle and is characterized by large lumens. It serves as a mechanical support in plant leaves and is closely related to leaf toughness and hardness, which decreases herbivory and increases life span (Terletskaya and Kurmanbayeva 2017; Dörken et al. 2020). According to Sandratriniaina et al. (2023), this trait is found in all 15 *Diospyros* species examined from Madagascar. However, this tissue is not found in the most widely-cultivated species in Asia, *D. kaki* (Paudel and Heo 2018). The sclerenchyma layers surrounding the vascular bundles in *D. buxifolia* are indistinct, but they are distinct in both *D. javanica* and *D. cauliflora*, with a total of 2-4 cell layers. This makes the leaves of the widely distributed species *D. buxifolia* have a tender texture, while those of the other two species are more rigid. The distinct features of sclerenchyma layers across plant species may have taxonomical implications. The presence or absence of sclerenchyma tissue in the three species suggests that this trait could serve as a diagnostic characteristic both within and across species, potentially clarifying classification at the sectional level in taxonomic hierarchies. Although the three species studied are all included in the same subgenus, *Eudiospyros*, they are classified into different sections. *D. buxifolia* is placed in the section *Brachycylix*, *D. cauliflora* is included in the section *Stelechantha*, and *D. javanica* is classified under the section *Nesindica* (van den Brink 1938).

Supporting elements and metabolites

Another important anatomical feature in *Diospyros* and other woody plants is the presence of tracheids. Tracheids are spiral and transparent elements embedded in palisade tissue. They are cylindrical with oblique ends (Keshavarzi and Zare 2006) and function primarily for mechanical support. All three *Diospyros* species studied contain tracheids scattered throughout the palisade layers (Figures 8, 9, and 10). The presence of such specialized structures may have significant implications for the taxonomic classification of plant groups, particularly when these features differ among species. Since the three species studied exhibit similar tracheid characteristics, these features have minimal implications for the taxonomic classification at the generic, subgeneric, or lower levels.

In the parenchymatous tissue, we also found the crystal configuration in all three species that has been commonly recognized as crystal oxalate (CaO). Thus, this similarity means this anatomical trait cannot serve as a diagnostic trait for the studied species. However, Gomes and Lombardi (2010) found prismatic monocrystals in the mesophyll layers, which may be used to define taxonomic classification among species in Salacioideae (Celastraceae). Prismatic crystals are not consistently present in all 15 *Diospyros* studied by Sandratriniaina et al. (2023). The results showed that a broader study of plant species will provide evidence that the presence of prismatic crystals could serve as a diagnostic trait within the genus *Diospyros*.

Phenolic deposits are a common feature in Angiosperms, often filling the lumen of epidermal or parenchymatous cells. These deposits can be found within the cell wall, in the cytoplasmic vacuole, or scattered throughout the cytoplasm (Stešević et al. 2016). In this study, the deposits were observed as cytoplasmic vacuoles. The deposits may consist of tannins or other phenolic compounds. Chemical analysis can be used to determine the composition of the phenolic deposits (Mitsopolous et al. 2016; Aoussar et al. 2020; Barbouchi et al. 2020). *D. ramulosa* has been reported to have phenolic deposits containing tannin compounds (Jordaan and Theunissen 1992). All three species examined in this study were found to contain phenolic deposits, although their abundance and tissue distribution vary. Moreover, according to Sandratriniaina et al. (2023), all *Diospyros* species in Madagascar, including *Diospyros* of Madagascar, such as *D. analamerensis*, *D. bardotiae*, *D. baronii*, *D. chitoniophora*, *D. cupulifera*, *D. gracilipes*, *D. humbertiana*, *D. lewisiae*, *D. littoralis*, etc. do not exhibit phenolic deposits. Paudel and Heo (2018) also reported the absence of phenolic deposits in the subtropical species D. kaki, as evidenced by the clear cytoplasm of mesophyll cells. Therefore, these characteristics may serve as diagnostic features for distinguishing between the three species, given the notable differences observed among them.

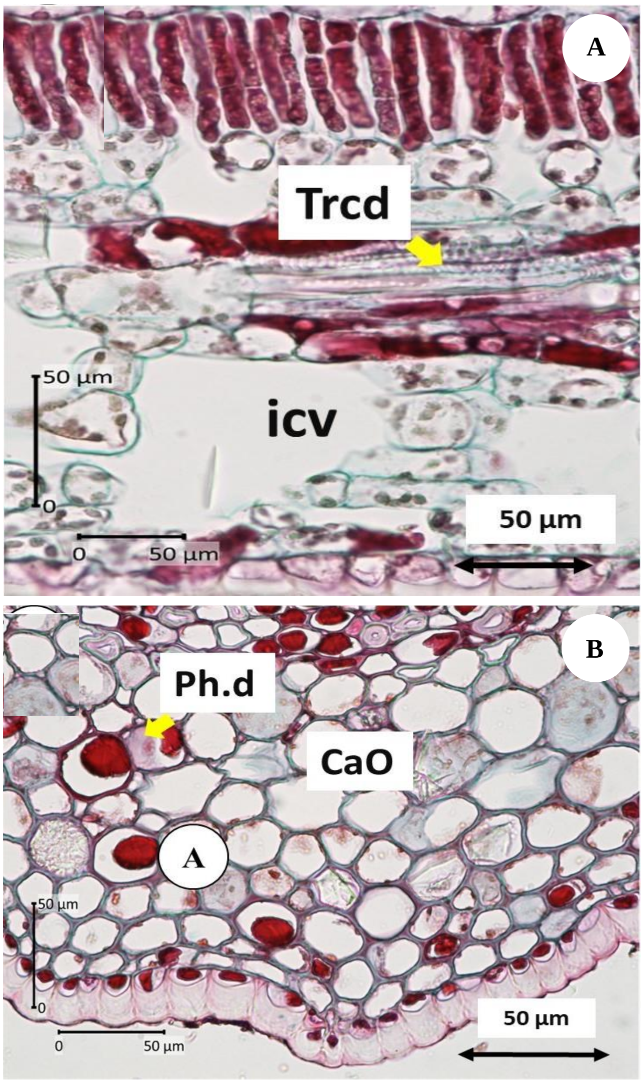

Figure 8. Supporting elements and metabolites of the endemic species *Diospyros javanica*: A. Trcd: Tracheides (magnification 40×), B. Ph.D: Phenolic deposits and CaO: Crystal oxalate (magnification 40×). Anatomical features indicated by yellow arrows

Table 11. Supporting elements and metabolites of the three *Diospyros* species

Anatomical traits	***Diospyros javanica***	***Diospyros cauliflora***	***Diospyros buxifolia***
Crystal oxalate (*)	Present, abundant, scattered in the mesophyll	Present, quite abundant, scattered in the mesophyll	Present, few, big, embedded in the palisade
Mucilage cells	Indistinct	Distinct	Indistinct
Scattered tracheides	Distinct	Distinct	Distinct
Phenolic deposits (*)	Present, few, in the mesophyll	Present, abundant, scattered in the mesophyll	Present abundant, scattered in the mesophyll
Secretory cells	Indistinct	Indistinct	Indistinct

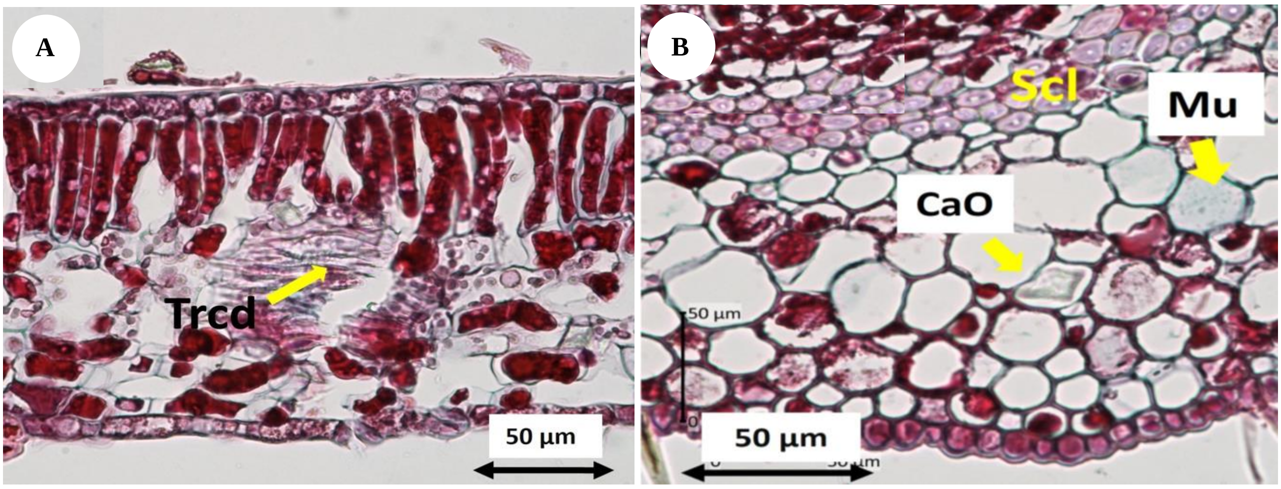

Figure 9. Supporting elements and metabolites of the widely-distributed species *Diospyros cauliflora*: A. trcd: Tracheides (magnification 40×), B. Ph.D: Phenolic deposits and CaO: Crystal oxalate, Mu: Mucilage cells (magnification 40×). Anatomical features indicated by yellow arrows

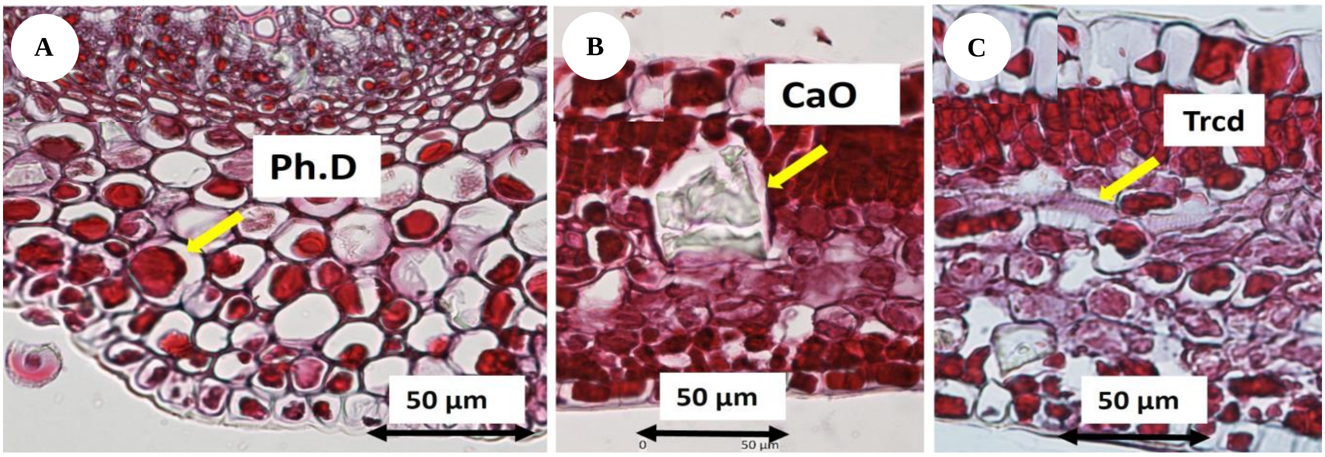

Figure 10. Supporting elements and metabolites of the widely-distributed species *Diospyros buxifolia*: A. Ph.D: Phenolic deposit, B. CaO: Crystal oxalate embedded in the palisade layer, C. Trcd: Tracheids (magnification 40×). Anatomical features indicated by yellow arrows

Table 12. Assessment of the adaptive anatomical traits of the three *Diospyros* species

Species	**Leaf thickness**	**Cuticle thickness**	**Upper epidermal cell thickness**	**Palisade thickness**	**Spongy mesophyll thickness**	**Phenolic deposits**
Diospyros javanica	+++	++++	++	++	++++	++
Diospyros cauliflora	+	+	++	+++	++	+++
Diospyros buxifolia	++	+	++++	++	+++	+++

Jordaan and Theunissen (1992) reported the relationship between anatomical structures and plant adaptation in D. *ramulosa* and other xerophytic plants in southern Africa. Moreover, according to Mohamed et al. (2018), phenolic deposits and the high tannin content of xerophytic plants increase under abiotic stress. However, many other species of *Diospyros* in Madagascar, such as *D. analamerensis, D. bardotiae, D. baronii, D. chitoniophora, D. cupulifera, D. gracilipes, D. humbertiana, D. lewisiae, D. littoralis* etc. did not show the presence of phenolic deposits that were indicated by the clear cell cytoplasm of the parenchym tissue (Sandratriniaina et al. 2023).

Paudel and Heo (2018) also revealed the absence of phenolic deposit in the subtropical species *D. kaki*. The presence of phenolic deposits and high tannin content is associated with adaptation to dry, high-light-exposure habitats. According to Adu et al. (2022), phenolic compounds function as antioxidants against pests and pathogens. It seems that climatic conditions influence the presence and abundance of phenolic deposits in *Diospyros* species worldwide. All three species studied were found to have phenolic deposits, but they differ in terms of abundance and tissue distribution. *D. buxifolia* and *D. cauliflora* exhibit abundant phenolic deposits in the epidermal and mesophyll layers, whereas *D. javanica* has the fewest phenolic deposits, particularly in the mesophyll layers. Based on these traits, *D. javanica* tends to adapt better to a dry, high-light-exposure climate than other species. Additional biochemical analyses on phenolic content are required to understand the adaptation and susceptibilities of tropical plants to light exposure, high temperatures, and solar radiation. Plant adaptation to the environment is often reflected in modifications to internal structures, particularly those of leaf organs, whose presence or size can be identified.

Implications for plant's ecology and conservation

Diospyros javanica is mostly found in coastal habitats of tropical forests in Java; however, it can also thrive in the ecotone between coastal and lowland forests. This endemic species is susceptible to high solar radiation and high temperatures due to its large size and relatively dense stomata. Moreover, the three *Diospyros* species studied here grow in medium-humidity habitats within an intermediate stratum of the forest (van den Brink 1938). Thinner palisade layers indicate slow-growing plants and fewer phenolic deposits, especially in the mesophyll, suggesting hygromorphic adaptation (Chalker-Scott and Fuchigami 1989). For plant species with sun-adapted leaves, the greater the exposure to light, the thicker the leaf blade tends to be (Mishio and Kawakubo 2015; Dörken and Lepetit 2018). From this anatomical observation and the habitat information, *D. javanica*, which has the thickest leaf blade, appears to be the most sun-adapted species, while *D. buxifolia* and *D. cauliflora*, which have a thinner leaf blade, seem to possess shade leaves (Table 12) (Adams and Terashima 2018; Zhang et al. 2019). This result showed that these widely distributed species were predicted to perform better in growth and did not adapt to specific habitats.

The anatomical and ecological traits of *D. javanica* highlight its vulnerability to environmental changes. Although it can grow across the ecotone between coastal and lowland forests, its large stomatal size and relatively dense stomatal distribution suggest susceptibility to high solar radiation and elevated temperatures (Mishio and Kawakubo 2015; Dörken and Lepetit 2018). This sensitivity is particularly concerning given the ongoing intensification of heat stress and coastal habitat degradation in Java. Conservation strategies for *D. javanica* should prioritize the protection and restoration of coastal and transitional forest zones, while also considering microhabitat conditions that buffer extreme sunlight and temperature. Ex-situ conservation, assisted plant transfer to suitable shaded microhabitats, and long-term monitoring of physiological responses to climate stress may further support its persistence.

In contrast, the more widely distributed *D. buxifolia* and *D. cauliflora* exhibit traits associated with shade-adapted species, including thinner leaf blades and reduced palisade layers, which indicate hygromorphic adaptations (Chalker-Scott and Fuchigami 1989; Adams and Terashima 2018). Their broader ecological tolerance and lack of specialization to coastal or drier habitats suggest higher resilience to environmental variability, a pattern commonly observed in species with shade-type leaf anatomy (Zhang et al. 2019). However, their dependence on medium-humidity habitats within intermediate forest strata means that widespread forest fragmentation still poses threats, as such disruptions significantly alter understory microclimates and reduce habitat continuity. Conservation efforts for these species should therefore prioritize maintaining continuous forest canopies, preserving understory humidity, and ensuring landscape connectivity. By integrating anatomical evidence with habitat information, this research underscores the importance of species-specific strategies that reflect physiological limitations and adaptive capacities across the *Diospyros* lineage.

In conclusion, the foliar anatomical traits, including cuticle layer thickness, stomatal types, intracellular cavity, sclerenchyma tissue, the presence of mucilage cells, the position of crystal oxalate, and phenolic deposits, varied across the three *Diospyros* species studied. Through foliar anatomical traits, we could justify that all three species belong to the same subgenus, *Eudiospyros,* however are classified into different sections. The foliar characteristics of the endemic *D. javanica* differ markedly from those of the more widespread *D. cauliflora* and *D. buxifolia*. Its thicker leaves, greater phenolic deposition, and larger intercellular cavity suggest adaptation to more open or stressful environments, yet its thinner photosynthetic-related traits indicate lower growth potential than those of its relatives. Future research should integrate ecological and physiological approaches, such as growth-rate assessment and biochemical profiling, to better understand adaptive strategies in *D. javanica*.

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