Anatomy and foliar architecture of two Mexican species of *Ternstroemia* Mutis ex L.f. (Pentaphylacaceae)

Anatomía y arquitectura foliar de dos especies mexicanas de *Ternstroemia* Mutis ex L.f. (Pentaphylacaceae)

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Abstract

Background: Many Mexican *Ternstroemia* species mainly inhabit the tropical montane cloud forest. These species have been misidentified in herbaria due to their morphological resemblance.
Question: Are there foliar anatomical and micromorphological characters with diagnostic value that allow discriminating between the Mexican species of *Ternstroemia*?
Study species: *Ternstroemia sylvatica* Schltdl. & Cham. and *T. lineata* DC. subsp. *lineata* (Pentaphylacaceae Engl.), 2018-2019.
Study site and date: Tropical montane cloud forests at the Trans-Mexican Volcanic Belt and Sierra Madre Oriental.
Methods: Botanical specimens were collected and removed from herbarium sheets. The leaves were prepared through cleared, dehydration and paraffin inclusion techniques to observe and describe them.
Results: The *Ternstroemia* species showed specific features of plants in humid environments, such as thin cuticle, mesophyll with scarce parenchyma palisade, and abundant intercellular spaces in the spongy parenchyma, and specific pigments that help to capture light and serve as protection. The species share a festooned broquidodromous venation pattern, anomocytic stomata, and closed collateral vascular bundle surrounded by perivascular fibers. Both species are differentiated by leaf shape, abundance of collenchyma layers, lenght of palisade parenchyma, and number of stomata per square millimeter on the abaxial surface.
Conclusions: We support and validate some features recorded for *Ternstroemia* species and reject some previous generalizations made worldwide to the genus. This research is the first attempt to study anatomy, architecture, and foliar micromorphology of Mexican Pentaphylacaceae.
Key words: anatomy, foliar architecture, micromorphology, *Ternstroemia*, tropical montane cloud forests, ultrastructure.

Resumen

Antecedentes: Las especies mexicanas del género *Ternstroemia* habitan principalmente el bosque templado húmedo de montaña y han sido confundidas en los herbarios por su gran parecido morfológico.
Pregunta: ¿Existen caracteres foliares, anatómicos y micromorfológicos, con valor diagnóstico que permitan separar a dos de las especies mexicanas de *Ternstroemia*?
Especies de estudio: *Ternstroemia sylvatica* Schltdl. & Cham. y *T. lineata* DC. subsp. *lineata* (Pentaphylacaceae Engl.).
Sitio de estudio y fecha: Bosques templado húmedos de montaña de la Faja Volcánica Transmexicana y Sierra Madre Oriental, 2018-2019.
Métodos: Se recolectaron en campo y removieron de ejemplares de herbario hojas de las dos especies. Las hojas se prepararon con técnicas de diafanizacion, deshidratación e inclusión en parafina para su observación y análisis.
Resultados: Ambas especies de *Ternstroemia* mostraron características propias de las plantas de ambientes húmedos, como una cutícula delgada, escaso parénquima de empalizada, parénquima esponjoso laxo, esclereidas y pigmentos específicos que ayudan a captar la luz y sirven de protección. Las especies comparten un patrón de venación broquidódromo festoneado, estomas anomocíticos y haz vascular colateral cerrado rodeado por fibras perivasculares. Se diferencian por la forma de la hoja, abundancia en capas de colénquima, largo del parénquima en empalizada y número de estomas por milímetro cuadrado en la superficie abaxial.
Conclusiones: Apoyamos y validamos algunas características registradas en el pasado para las especies de *Ternstroemia* a nivel mundial y rechazamos algunas generalizaciones previas hechas al género. Esta investigación es el primer estudio de la anatomía, arquitectura y micromorfología foliar de Pentaphylacaceae mexicanas.
Palabras clave: Anatomía, arquitectura foliar, bosque mesófilo de montaña, micromorfología, *Ternstroemia*, ultraestructura

The family Pentaphylacaceae Engler *nom. cons.* (Ternstroemiaceae Mirb. ex DC. *sensu* Weitzman *et al.* 2004, Luna-Vega & Ochoterena 2004) belongs to the order Ericales (Judd *et al.* 2016) and is composed of 14 genera and ≈ 337 species distributed in tropical and subtropical areas of Hemispheres (Stevens 2021). This family was earlier included in the Theaceae Mirb. (Cronquist 1981, Takhtajan 1997), but by morphological (Luna-Vega 1997, Luna-Vega & Ochoterena 2004), molecular (Soltis *et al.* 2000, Prince & Parks 2001, Anderberg *et al.* 2002, Judd *et al.* 2016), and embryological evidence (Tsou 1995), it was concluded that Theaceae is not a monophyletic group. Pentaphylacaceae is represented in Mexico by four genera: *Cleyera* Thunb., *Freziera* Willd., *Symplococarpon* Airy Shaw and *Ternstroemia* Mutis ex L. f. (Luna-Vega & Alcántara-Ayala 2008). *Ternstroemia* belongs to the Ternstroemieae DC. subfamily with ≈ 100 species (Stevens 2021) widely distributed in temperate and humid forests in tropical and subtropical regions worldwide (Luna-Vega & Contreras-Medina 2000). The current IUCN Red List of Threatened Species (www.iucnredlist.org) includes six Mexican species of *Ternstroemia*, four considered as vulnerable (*Ternstroemia acajatensis* Cast.-Campos & Pal.-Wass., *T. dentisepala* B.M. Barthol., *T. huasteca* B.M. Barthol., *T. maltbyi* Rose) and two as least concern (*T. lineata* DC., *T. sylvatica* Schltdl. & Cham.). The species studied in this manuscript are listed in the last risk category because the authorities think their populations are stable. Despite this, the Mexican Official Norm NOM-059-ECOL-2010 (SEMARNAT 2010) does not include any species of the Pentaphylacaceae. Several Pentaphylacaceae species are characteristic or diagnostic of the Mexican tropical montane cloud forest (TMCF; 'bosque mesófilo de montaña' *sensu* Rzedowski 1996, Luna-Vega *et al.* 2006), considered in itself as a threatened vegetation type (Luna-Vega *et al.* 2006, Bruijnzeel *et al.* 2010).

The nine species of Mexican *Ternstroemia* are endemic or almost restricted to the country (Luna-Vega & Alcántara-Ayala 2008, Castillo-Campos & Palacios-Wassenaar 2019). They are locally known as "tilas" o "trompillos" (Luna-Vega *et al.* 2004). The fruits are sold in the traditional Mexican markets to make a decoction to alleviate anxiety, fears, and sleep disorders (Aguilar *et al.* 1994, Luna-Vega & Ochoterena 2004). Unfortunately, the active principles have not been identified yet, and some authors (Balderas-López *et al.* 2013) showed that the fruits of *T. sylvatica* are toxic rather than an antidepressant. The leaves of *T. sylvatica* are used effectively in alcoholic cataplasms to treat inflammatory and rheumatic processes (Moreno-Quirós *et al.* 2017).

The Mexican species of *Ternstroemia* have certain sameness in their characters, making it very difficult to be taxonomically delimited (Luna-Vega & Alcántara-Ayala 2002, Luna-Vega & Ochoterena 2004). Consequently, specimens of several species of Mexican *Ternstroemia* are misidentified and confused in the herbaria. In this context, the anatomy and foliar architecture can provide evidence to support or refute the classification of these species (Martínez-Cabrera *et al.* 2007).

Ternstroemia lineata subsp. *lineata* and *T. sylvatica* are represented in the tops or ridges of hills in mountainous areas, at elevations above 1,000 m asl, mainly in TMCF, humid pine-oak, and oak forests, primary or disturbed. Both species are trees or shrubs with white axillary, solitary flowers, fruit baccate, coriaceous, and indehiscent. These species are represented in the floristic provinces of the Sierra Madre Occidental, Sierra Madre Oriental, Serranías Meridionales, and Serranías Transístmicas, in the Mesoamerican montane region *sensu* Rzedowski (1978) (Alcántara-Ayala *et al.* 2002).

Weitzman *et al.* (2004) suggested that the most common venation of the Pentaphylacaceae is brochidodromous and sometimes reticulodromous. The last authors and Stevens *et al.* (2004) indicated that the thickness and size of the leaf, the venation, and the presence of glandular deciduous "theoid" teeth are characteristic of the Pentaphylacaceae and Theaceae. Some of the members of the subfamilies Ternstroemieae and Freziereae Willd. share the presence of sclerified idioblasts in the mesophyll, as well as in the cortex and pith. Both columnar and isodiametric sclereids may be densely clustered around the veins or spread evenly throughout the leaf (Stevens 2021, Weitzman *et al.* 2004). In addition, on the adaxial surface of the lamina, there may be one or two hypodermal layers of exclusively isodiametric cells (Weitzman *et al.* 2004).

The leaves of the Pentaphylacaceae have infrequent unicellular trichomes (Weitzman *et al.* 2004), tannins are present on the abaxial surface in some *Ternstroemia* species (Metcalfe & Chalk 1972), they are dorsiventral, and the mesophyll consists of a variable number of layers, one to three layers of palisade parenchyma, together with a region

of lacunar spongy parenchyma occupying two-thirds of the thickness of the lamina (Weitzman *et al.* 2004). The vascular bundle of veins shows a sclerenchyma sheath and the midrib usually contains a V- or U-shaped vascular bundle (Metcalfe & Chalk 1972). Anomocytic stomata occur in *Ternstroemia* (Zhang & Zhuang 2004) and are surrounded by 2 to 5 subsidiary cells. Weitzman *et al*. (2004) suggested that stomata are confined to the abaxial epidermis. The petiole of some *Ternstroemia* species exhibits an arc, U, or V-shaped vascular bundle in cross-section at the distal end (Metcalfe & Chalk 1972). The nodes are unilacunar and with a foliar trace (Beauvisage 1920, Keng 1962, Schofield 1968, Weitzman *et al*. 2004); there are one to five bundles in the petiole (Weitzman *et al*. 2004).

Given the morphological similarity between *T. lineata* ssp. *lineata* and *T. sylvatica*, they are misidentified in the herbaria collections (Luna-Vega & Ochoterena 2004, Alcántara-Ayala *et al.* 2020). In this study, we analyzed other sources of evidence to find characters that serve as taxonomic markers. Our main aim was to describe the anatomy and foliar architecture of two endemic *Ternstroemia* species inhabiting the Mexican tropical montane cloud forest.

Material and methods

Recollection. We obtained four leaf samples (including petiole and lamina) per individual of *Ternstroemia lineata* ssp. *lineata* and *T. sylvatica* in six localities of Mexican TMCF in 2013 and 2014 (three individuals per locality = 18 individuals = 72 leaves). We selected randomly well-developed trees with healthy foliage. We got these leaf samples from the middle part of the trees. The voucher specimens were deposited in the FCME Herbarium of the Facultad de Ciencias, UNAM (Table 1). We also removed four fully developed and healthy leaves (petiole and lamina) of herbarium specimens from three individuals from each of the populations of the chosen species (Table 1).

Table 1. Location, features, and specimen data of *Ternstroemia sylvatica* and *T. lineata* ssp. *lineata*.

Locality	Species	Source	Coordinates, longitude/altitude	Collection number
Gómez Farías **TAMAULIPAS**	*T. sylvatica*	Herbaria specimen*	-99° 13' 49.0"/23° 2' 50.0"	6305
Zacualtipán **HIDALGO**	*T. sylvatica*	Herbaria specimen*	-98° 39' 31.0"/20° 38' 47.0"	6757
		Field collection #	-98° 40' 35.0"/20° 39' 40.0"	4345
Capulálpam **OAXACA**	*T. sylvatica*	Herbaria specimen*	-96° 33' 12.0"/17° 32' 0.0"	6789
La Concordia **SINALOA**	*T. lineata* ssp. *lineata*	Herbaria specimen*	-105° 50' 11.0"/23° 33' 44.0"	7865
Ocuilan **ESTADO DE MÉXICO**	*T. lineata* ssp. *lineata*	Herbaria specimen*	-100° 16' 45.0"/19° 22' 1.0"	7678
		Field collection #	-100° 17' 55.0"/19° 23' 2.0"	5434
Tuxtla Gutiérrez **CHIAPAS**	*T. lineata* ssp. *lineata*	Herbaria specimen*	-92° 41' 18.0"/16° 47' 6.0"	7645

*Collector and determiner: O. Alcántara-Ayala. # Collector and determiner: E.I. García-Gómez. Specimens deposited in FCME, Facultad de Ciencias, UNAM.

Foliar architecture. The leaves from herbaria and collected in the field were treated with 20 % sodium hydroxide (NaOH) at 60 ºC for 24 h, then washed with running water, followed by adding 50 % sodium hypochlorite (commercial chlorine, Clorox®) for 3 h until a whitish color was obtained. The leaves were then washed and dehydrated with a series of ethanol (50, 70, and 96 %) every 24 h, and placed in a modified BB1/4 solution (Martínez-Cabrera *et al*. 2007) for 4 to 8 days until the leaves got translucent. Immediately, they were placed in 96 % ethanol for 24 h to eliminate the remains of the clarifying solution and stained with safranin for 1 h, rinsed in ethanol (96 and 100 %) until the desired contrast obtained, and mounted with synthetic resin (Martínez-Cabrera *et al*. 2007).

The width and length of the leaf, venation pattern, vein branching, and areole development were characterized for each individual. We quantified the number of sclereids per mm^2, the total number of epidermal cells, and stomata per mm^2 in 25 different fields. We calculated the stomatal index using the last two parameters (Salisbury 1928). The foliar architecture was based on Hickey (1979) and Ellis *et al.* (2009).

Foliar anatomy (petiole and lamina). For the anatomical study, we fixed the laminas and petioles from the field and herbaria in formaldehyde-glacial acetic acid-ethyl alcohol (FAA; Ruzin 1999) for 24 h. Next, we added 50 % ethyl alcohol. Then the samples were dehydrated in ascending concentrations of ter-butanol (TBA, 50 - 100 %) in an automatic tissue processor (Leica, TP1020) for 24 h and embedded in paraplast (melting point 60°C). Transverse and paradermal sections 12 to 16 μm thick were obtained with a rotary microtome (Leica, RM2125), stained with safranin-fast green, and mounted with synthetic resin (Johansen 1940).

For each individual, we described the cuticle, epidermis, mesophyll, vascular tissue of the lamina and the epidermis, collenchyma, and vascular tissue of the midrib. In the case of the petiole, we described the morphology, epidermis, cortex, and vascular tissue. In addition, we made in each individual 25 measurements in the transverse sections of the leaves of wide of lamina, thickness of cuticle, length and wide of epidermal cells of the adaxial and abaxial surfaces, and length of palisade parenchyma. The anatomical descriptions of the foliar lamina followed the terminology of Metcalfe & Chalk (1979), for the leaf surface, we followed Wilkinson (1979) and Koch *et al.* (2009), and for petiole Howard (1979).

All observations, microphotographs, and measurements were carried out with an Olympus COVER-018 stereomicroscope and an Olympus BX-51 light microscope adapted to an Image-Pro Plus version 7.1 image analyzer (Media Cybernetics 2006). In addition, we carried out statistical procedures and analyses with the R statistical software (R Core Team 2020).

Foliar micromorphology SEM. Only the material collected in the field was used to study the leaf surface in the abaxial and adaxial surfaces through SEM. The were fixed in the field with absolute ethyl alcohol-glacial acetic acid solution (Farmer technique, Ruzin 1999). We kept some others in cardboard boxes to avoid epicuticular wax modifications. The fixed material was washed with distilled water and cut into sections of the 4.0-5.0 mm foliar leaf. They were gradually dehydrated with ethyl alcohol (30 - 100 %). All the samples were placed in a critical point drier (Hitachi-S-2460N), and mounted in an aluminum sample holder using carbon-based glue and metalized with gold (Hitachi-S-2460N). Each surface was observed and photographed with the SEM (Hitachi-S-2460N SEM) of the Instituto de Biología, UNAM. We described from each photograph the shape of epidermal cells, the limits of anticlinal walls (relief and curvature), and the sculpture of outer periclinal walls (relief, convexity, and microrelief). We mounted 4.0-5.0 mm of the lamina from the material stored in the cardboard box in an aluminum sample holder to study the epicuticular wax, following the method described above. The description of micromorphology followed Barthlott *et al.* (1998) and Koch *et al.* (2009).

Results

Quantitative characters are shown with their standard deviations in Table 2 and Table 3 and summarize the most distinctive characteristics of the studied species.

Foliar architecture. Leaves simple, alternate, petiolate, symmetrical, coriaceous, 75.02 mm long, 19.63 mm wide in *Ternstroemia lineata* ssp. *lineata*; and slightly coriaceous in *T. sylvatica* with 58.13 mm long and 19.45 mm wide. Oblanceolate to obovate in *T. lineata* ssp. *lineata* (Figure 1A) and narrow-elliptical to obovate shape in *T. sylvatica* (Figure 1B). Microphyllous in both species. Apex straight to round shape, with an acute angle (less than 90°) and a retuse ending in *T. lineata* ssp. *lineata*, while in *T. sylvatica* apex slightly acuminate to straight with an acute angle, and the termination non-retuse. Base symmetrical and decurrent with an acute angle in both species. The leaf with

diminutively serrated margin in *T. lineata* ssp. *lineata* and entire margin in *T. sylvatica*. The petiole slightly winged, more evident near the leaf base due to the extension of the lamina and attached to the leaf margin in both species.

The primary venation pinnate, with a basal and primary vein following a straight course, moderately thick, 502.78 μm wide in both *Ternstroemia* species (Figure 1A-C). The secondary venation brochidodromous festooned, more developed in *T. lineata* ssp. *lineata* (Figure 1A, B). The secondary veins moderately thick; space between them irregular and decurrent, with an acute divergence angle. Intersecondary veins weak to robust, with approximately one per intercostal area (Figure 1A, B). Random reticulate pattern in tertiary veins, with a slightly sinuous course and obtuse angle to the primary vein (Figure 1D). The fourth-order venation irregular, reticulate, with freely branching fifth-order venation and areolae moderately developed, *e.g.,* irregular in shape and more or less variable in size, predominating four to seven-sided areolae (Figure 1D, E). Most veinlets have two or more unequal branches (Figure 1H). Astrosclereids highly branched, usually large, evenly distributed in the mesophyll (Figure 1E, F), but well organized and continuous throughout the margin (Figure 1G, H). Sclereids/mm^2 27.77 in *T. lineata* ssp. *lineata* and 20.68 sclereids/mm^2 in *T. sylvatica*, with significant differences between both species (Table 2). The ultimate marginal venation incomplete in both species (Figure 1G, H), and marginal, glandular, deciduous serrate glands with frequent scars in *T. lineata* ssp. *lineata* and infrequent in *T. sylvatica* (Figure 1H-J). The mucilage all over the leaf lamina of both species visible as brown spots.

Table 2. Mean characters values of the leaf of *Ternstroemia sylvatica* and *T. lineata* spp. *lineata*, and differences by *Student's t test.**

Character	T. sylvatica	T. lineata spp. lineata
Cuticle thickness (μm)	1.75 ± 0.33 [a]	1.89 ± 0.41 [a]
Adaxial epidermal cell height (μm)	18.33 ± 3.02 [a]	18.20 ± 1.43 [a]
Abaxial epidermal cell height (μm)	12.49 ± 1.79 [a]	10.04 ± 1.89 [a]
Lamina thickness (μm)	233.86 ± 40.73 [a]	281.80 ± 73.58 [b]
Palisade parenchyma thickness (μm)	40.04 ± 2.92 [a]	57.81 ±7.25 [b]
Spongy parenchyma thickness (μm)	193.82 ± 37.77 [a]	223.99 ± 66.33 [b]
Guard cells height (μm)	18.58 ± 3.11 [a]	20.71 ± 2.41 [b]
Abaxial epidermal cell height of the midrib (μm)	16.21 ± 2.48[a]	20.37 ± 3.98[b]
Number of stomata/mm^2 (abaxial)	5.68 ±1.02 [a]	10.76 ± 0.34 [b]
Number of sclereids/mm^2	20.68 ± 10.90 [a]	27.77 ± 12.41 [b]
Petiole area (mm^2)	7.35 ± 0.88 [a]	8.21 ± 0.13 [b]

*Different letters indicate significant differences per file (Student's t test $p < 0.05$).

Foliar lamina. In the surface view, the typical epidermal cells of the adaxial and abaxial epidermis tetragonal and polygonal in shape with irregular sides in *T. lineata* ssp. *lineata* and tetragonal in *T. sylvatica* (Figure 2A). These cells have straight or slightly curved anticlinal walls, rounded ends in both species. Anomocytic stomata surrounded by four to five ordinary epidermal cells (Figure 2A), randomly distributed on both surfaces (amphistomatic leaf). Both, length of guard cells (20.71 μm in *T. lineata* ssp. *lineata*; 18.58 μm in *T. sylvatica*) and stomata/mm^2 (10.76 in *T. lineata* ssp. *lineata*; 5.68 in *T. sylvatica*) with significant difference between both species (Table 2).

In the transverse-section, the lamina wide 281.80 μm in *T. lineata* ssp. *lineata* and 233.86 μm in *T. sylvatica*, with significant differences between both species (Table 2). On both surfaces, the cuticle smooth and thin, with 1.89 μm thickness in *T. lineata* ssp. *lineata* and 1.75 μm in *T sylvatica* on its adaxial surface, and no significant differences (Table 2).
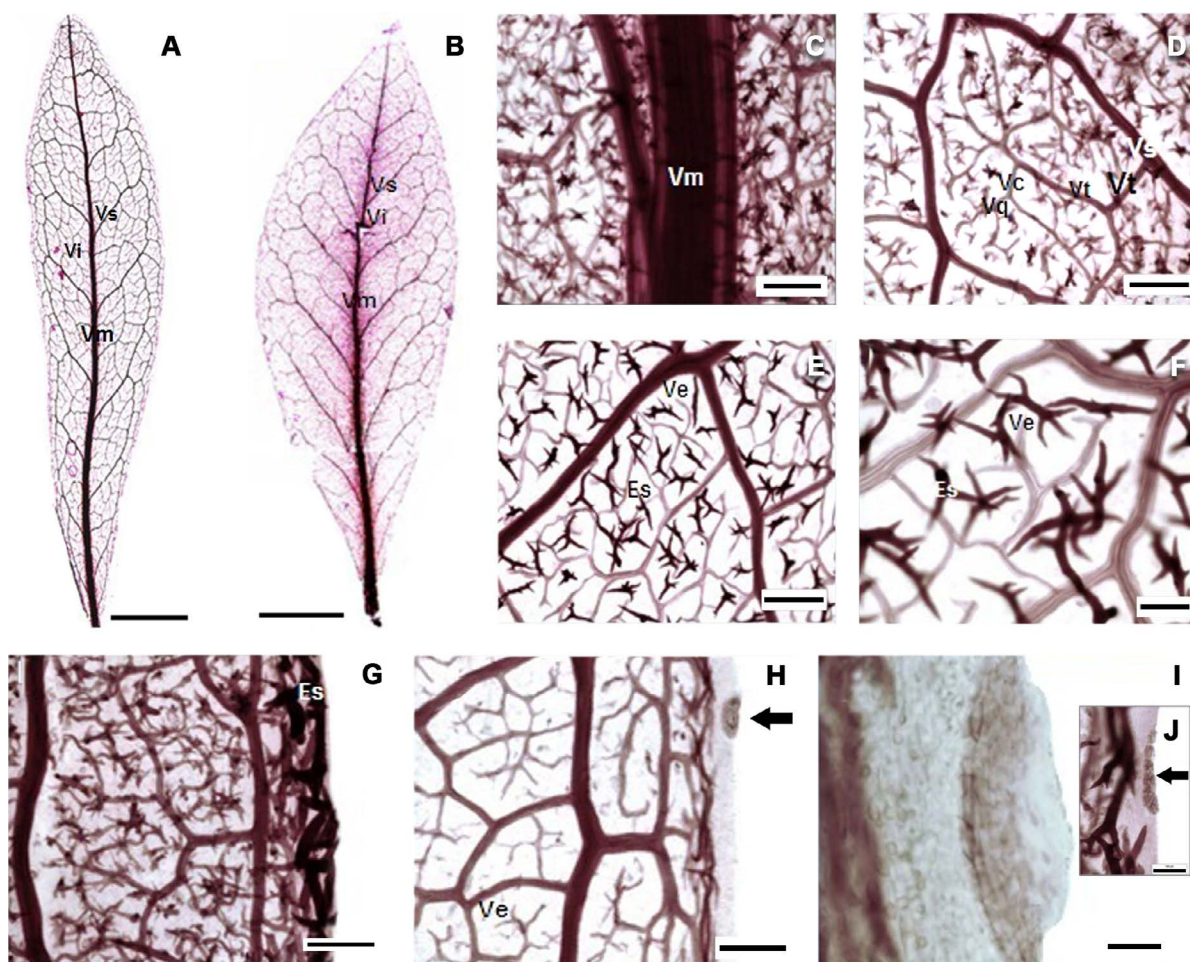
Figure 1. Venation pattern of *Ternstroemia lineata* subsp. *lineata* and *T. sylvatica*, cleared leaves. A, B. Festooned broquidodromous venation pattern, A. *T. lineata* subsp. *lineata*, B. *T. sylvatica*. C. Detail of the midrib, *T. sylvatica*. D. Areoles moderately developed and third, fourth, and fifth-order veins, *T. lineata* subsp. *lineata*. E, F. Veinlets and sclereids, E. *T. lineata* subsp. *lineata*, F. *T. sylvatica*. G, H. Marginal ultimate venation, G. *T. lineata* subsp. *lineata*, H. *T. sylvatica*. I, J. Detail of gland in margin, *T. lineata* subsp. *lineata*. Vm = midrib vein; Vs = secondary veins; Vi = intersecondary vein; Vc = fourth-order veins; Vq = fifth-order veins; Vt = tertiary veins. Es = sclereids; Ve = veinlets. Arrows = glandular scar. Scale bar is 1 cm in A, B; 300 μm in C-E, H; 100 μm in F, G, J; 50 μm in I.

The epidermis simple on both surfaces (Figure 2B-G). In *T. lineata* ssp. *lineata*, the typical cells rectangular with concave and thickened periclinal walls, 18.20 μm length, 6.63 μm wide on the adaxial surface. Epidermal cells on the abaxial surface identical but smaller in size (Figure 2C), with 10.04 μm length and 7.06 μm wide. In *T. sylvatica*, the adaxial epidermal cells rectangular, round-ended with concave-convex outer periclinal walls due to a more frequent huge cellulose deposit at their adaxial end and thin anticlinal walls (Figure 2D), 18.33 μm length, 9.60 μm wide, and with abaxial epidermal cells identical to those of *T. lineata* subsp. *lineata* with 12.49 μm length, 5.74 μm wide (Table 2).

The palisade parenchyma of elongated, tubular cells in *T. lineata* ssp. *lineata* with 57.81 μm length when a single stratum and 105.90 μm length in some individuals with two strata (Figure 2F) and of one stratum in *T. sylvatica* with 40.04 μm length, with differences in the palisade parenchyma length between species (Table 2). The spongy parenchyma is open, having wide, well-developed intercellular spaces, occupying more than two-thirds of the lamina thickness (Table 2, Figure 2C-E, G). Abundant astrosclereids (Figure 2D, E) in the mesophyll, generally long and irregularly distributed. The tannins occluding lumina of the palisade and spongy parenchyma. In addition, suberized

cells (cork cells) in the margin corresponding to deciduous glands (Figure 2G). Vascular bundles collateral closed, surrounded by perivascular fibers thick-walled (Figure 2H, I).

Midrib. In the transverse-section, the midrib on the adaxial surface slightly concave and convex on the abaxial surface (Figure 2J, K). The cuticle very thin and smooth on both surfaces. Epidermis similar to the lamina, but on the abaxial surface, the typical cells rectangular and convex due to cellulose deposits on their outer periclinal wall. In *Ternstroemia lineata* ssp. *lineata* typical cells 20.37 μm length, and 12.21 μm wide and 16.21 μm length and 12.16 μm wide in *T. sylvatica*, with significant differences in the length of the abaxial epidermal cell of the midrib (Table 2). Palisade parenchyma in the adaxial surface similar to that of lamina but decreasing in length as approaching the central region, interrupted by three to four layers of angular collenchyma (Figure 2J, K). Towards the abaxial surface, eight layers of angular to lacunar collenchyma (Figure 2L). Astrosclereids towards the abaxial surface. Prominent collateral closed vascular bundle, arched in both species and surrounded by perivascular fibers (Figure 2K). In the xylem, vessels arranged in radial rows. Sieve tube elements, companion cells, and parenchyma in the phloem.

Table 3. Diacritic morphological and anatomical characters separating *Ternstroemia sylvatica* from *Ternstroemia lineata* ssp. *lineata*.

| Characters	*Ternstroemia sylvatica*	*Ternstroemia lineata* ssp. *lineata*
Leaf morphology	Slightly coriaceous leaves, narrow-elliptic to obovate. Apex slightly acuminate to straight, not retuse ending. Entire margin.	Coriaceous leaves, oblanceolate to obovate. Apex straight to round, retuse ending. Serrate margin minutely.
Number of sclereids/mm²	20.68 ± 10.90 sclereids/mm²	27.77 ± 12.41 sclereids/mm²
Number of stomata/mm² (abaxial)	5.68 ± 1.02 stomata/mm²	10.76 ± 0.34 stomata/mm²
Occlusive cells height	18.58 ± 3.11 μm	20.71 ± 2.41 μm
Epidermic cells at the lamina level (surface area)	Cells of variable size, tetragonal. Anticlinal walls straight or slightly curved, with rounded ends.	Cells of variable size, tetragonal and polygonal, with irregular sides. Anticlinal walls straight or slightly curved, with rounded ends and thickened walls.
Epidermic cells at the lamina level (cross-section)	Adaxial epidermal cells rectangular, elongated, round-ended, with concave-convex periclinal walls, due to an extensive deposit of cellulose more frequent at the adaxial end. Straight and thin anticlinal walls, with abaxial epidermal cells identical to those of *T. lineata* spp. *lineata.*	Typical cells rectangular, elongated, variable in size, with concave anticlinal and periclinal walls, thickened on the adaxial surface. Epidermal cells of the abaxial surface identical, but smaller in size.
Epidermic cells at the midrib level	Adaxial cells similar to those of the lamina. Abaxial cells rectangular and convex due to a cellulose deposit on the outer periclinal wall, absent in the inner and anticlinal walls.	Adaxial cells similar to those of the lamina. Abaxial cells are almost square and convex due to a deposit of cellulose on the outer periclinal wall.
Lamina thickness	233.86 ± 40.73 μm	281.80 ± 73.58 μm
Palisade parenchyma thickness	40.04 ± 2.92 μm	57.81 ± 7.25 μm
Spongy parenchyma thickness	193.82 ± 37.77 μm	223.99 ± 66.33 μm
Frequency of marks corresponding to the serrate margin of the deciduous glands	Infrequent	Frequent
Collenchyma type of the petiole cortex	One to three layers of angular to lacunar collenchyma of thin and elongated cells	Three to five layers of angular collenchyma of isodiametric cells
Petiole area	7.35 ± 0.88 mm²	8.21 ± 0.13 mm²
Petiole protuberances	Absent	Present
Deposit pattern of the epicuticular wax	Granular deposit pattern, evenly distributed.	Scale-like deposit pattern, evenly distributed
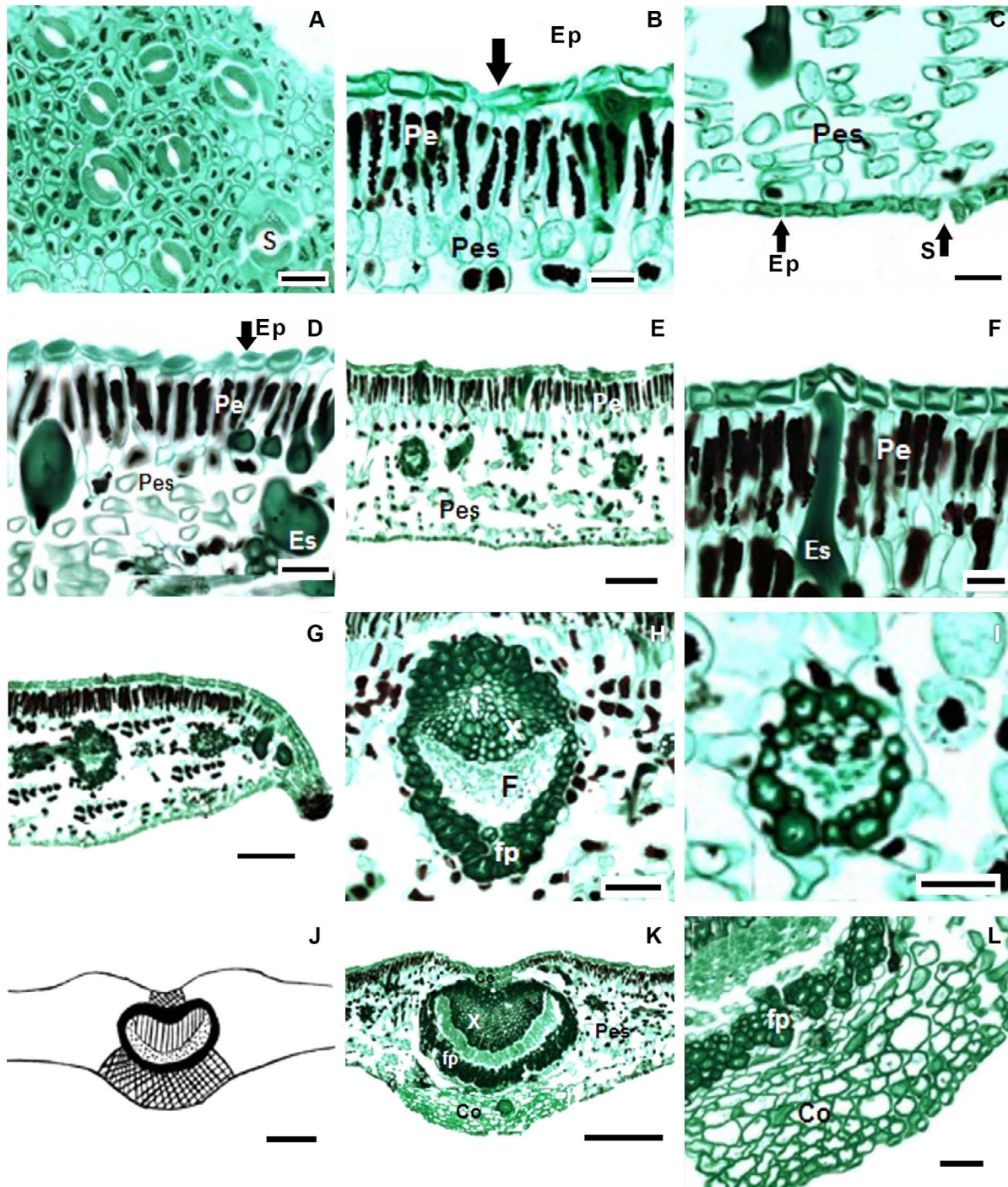
Figure 2. Leaf anatomy of *Ternstroemia lineata* subsp. *lineata* and *T. sylvatica*. A. Tetragonal epidermal cells and stomata, PS, *T. lineata* subsp. *lineata*. B. adaxial and C. abaxial epidermal cells, TS, *T. lineata* subsp. *lineata*. D. Adaxial epidermal cells and sclereids, TS, *T. sylvatica*. E. Bifacial mesophyll, TS, *T. lineata* subsp. *lineata*. F. Double layered palisade parenchyma and sclereid, TS, *T. lineata* subsp. *lineata*. G. Margin, TS, *T. lineata* subsp. *lineata*. H. Collateral vascular bundle in secondary vein, TS, *T. sylvatica*. I. Collateral vascular bundle in tertiary vein, TS, *T. sylvatica*. J. Midrib diagram; black = sclerenchyma, dots = phloem, parallel lines = xylem, cross lines = collenchyma, white = parenchyma. K. Midrib, TS, *T. lineata* subsp. *lineata*. L. Midvein abaxial epidermis, TS, *T. sylvatica*. Co = collenchyma; Ep = epidermis; Pe = palisade parenchyma; Pes = spongy parenchyma; Es = sclereids; S = stoma; X = xylem; F = phloem; fp = Perivascular fibers; PS = paradermal section; CS = transverse section. Scale bar is 20 μm in A-D, F, H, I; 50 μm in L; 100 μm in E, G; 300 μm in J, K.

Petiole. In transverse-section, the petiole broadly obovate (Figure 3A-C) and slightly winged, evident near the base of the lamina in both species, 7.08 mm length and 1.16 mm wide in *T. lineata* ssp*. lineata* with an area of 8.21 mm² and 7.66 mm length and 0.96 mm wide in *T. sylvatica*, with an area of 7.35 mm² (Table 2). The cuticle and epidermis similar to the midrib (Figure 3E). Subepidermally, three to five layers of angular collenchyma in *T. lineata* ssp. *lineata* and one to three continuous layers of angular to lacunar collenchyma in *T. sylvatica*. Both species have more than 10 layers of isodiametric parenchyma cells, with wide intercellular spaces occupying two-thirds of the thickness of the petiole and more reduced intercellular spaces towards the vascular tissue (Figure 3D). Astrosclereids (Figure 3E) and mucilaginous cells common in both species.

The vascular tissue of the distal end is represented by closed, arch-shaped collateral vascular bundle (Figure 3C). The middle region is represented by three vascular bundles surrounded by perivascular fibers with the same arrangement, but the central bundle is arc-shaped and larger than the other two, which are circular and lateral (Figure 3A, B). Near the foliar lamina (proximal end), the petiole is slightly winged and with the same vascular arrangement as the middle region. The xylem and phloem show the same characteristics as the midrib (Figure 3F, G).

Micromorphology of the foliar surface. The epicuticular wax follows a scale-like deposition pattern in *T. lineata* ssp. *lineata* (Figure 4A) and a granular-like pattern in *T. sylvatica* (Figure 4B), in both cases uniformly distributed, being scarce on the abaxial surface of both species. The cuticle with reticulated, striated pattern (Figure 4C). Anomocytic stomata surrounded by grooves arranged in the radial direction of the guard cells, forming extended bands in the larger ones, being more pronounced in *T. lineata* ssp. *lineata* (Figure 4D, E). Dome-shaped scars of the deciduous glands in the margin, with a large depression in the center (Figure 4F, G). Protuberances in the petiole unique in *T. lineata* ssp. *lineata* (Figure 4H, I).

Discussion

A combination of diacritic morpho-anatomical characters was found that allow the distinction of the two species of *Ternstroemia* here studied. The diacritic characters are leaf shape including margin, shape of epidermal cells and anticlinals in surface view, abundance of deciduous glands, length of palisade parenchyma, number of stomata/sq. mm; type and number of collenchyma layers in petiole and petiolar protuberances.

Foliar architecture. Although all the specimens examined were collected in tropical montane cloud forests, the lamina is variable in shape between the individuals of the same population, regardless of the locality where they are found. For this reason, an oblanceolate to obovate lamina was described in *T. lineata* subsp. *lineata* and narrow-elliptical to obovate in *T. sylvatica.* According to Weitzman *et al.* (2004), the most common secondary venation pattern of the Pentaphylacaceae is brochidodromous and sometimes reticulodromous. In *T. lineata* subsp. *lineata* and *T. sylvatica*, a festooned brochidodromous venation pattern was observed as described by Hickey & Wolfe (1975) for the leaves of *Ternstroemia tepezapote* Schltdl. & Cham. from Belize.

It was observed that the leaf texture depends on the number of sclereids/mm², not on the thickness of the cuticle and that the cuticle does not present significant differences between species. The leaves of *T. lineata* subsp. *lineata* presented a coriaceous texture and 27.77 sclereids/mm², while the leaves of *T. sylvatica*, had a slightly coriaceous texture and 20.68 sclereids/mm², showing significant statistical differences in the abundance of sclereids/mm²

Weitzman *et al.* (2004) and Stevens *et al.* (2004) point out that the presence of a "theoid" glandular tooth is characteristic of Pentaphylacaceae and Theaceae; the glands are deciduous in the first and permanent in the second. However, in this study, the presence of a tooth was not observed in any species, as no vascular relationship was found with the projections of the leaf margin. Royer *et al.* (2005) and Ellis *et al.* (2009) point out that the teeth are projections of the leaf margin and have some association with the vascular system. Therefore, we described them as deciduous marginal glands, leaving frequent marks in *T. lineata* subsp. *lineata* and rare in *T. sylvatica*; however developmental studies are required to understand why these deciduous glands appear not show vascular tissue.

Anatomy of the lamina. Dickison (2000) pointed out that the leaves of plants that develop in low-light conditions (as *Ternstroemia* species) show mesomorphic anatomical characters, *e.g.,* thin cuticle, mesophyll with scarce palisade parenchyma, and abundant intercellular spaces in the spongy parenchyma. These traits agree with the characteristics observed in the two species of *Ternstroemia* studied inhabiting TMCF. In addition, the occurrence of sclereids in the mesophyll contributes to light transfer (Karabourniotis *et al*. 2021).

A very thin cuticle was observed on both leaf surfaces, impossible to stain due to its physicochemical properties, contrary to the thick cuticle described by Herat & Theobald (1977) for the adaxial surface of *T. emarginata* (Gardner) Choisy and *T. japonica* (Thunb.) Thunb. by Boeger & Wisniewski (2003) and on both surfaces of the *T. brasiliensis* Cambess. leaf. The thickness of the cuticle is not remarkably different in both species studied. The stomata in both species studied are anomocytic, as cited by various authors (Keng 1962, Herat & Theobald 1977, Weitzman *et al*. 2004, Zhang & Zhuang 2004). Some authors pointed out that in *Ternstroemia*, stomata are confined to one of the surfaces, generally to the abaxial surface. Our study showed stomata on both leaf surfaces (amphistomatic leaf) surrounded by four to five ordinary epidermal cells, as Herat & Theobald (1977) reported for some *Ternstroemia* species.

In the case of the epidermis, Keng (1962) pointed out that the size and shape of the epidermal cells and the nature of their anticlinal walls (straight or wavy) can be of value for the identification of genera and species of the family. In *Ternstroemia lineata* subsp. *lineata* and *T. sylvatica* an unilayered epidermis, was observed on both surfaces, as reported by other genus species (Keng 1962, Herat & Theobald 1977, Boeger & Wisniewski 2003, Zhang & Zhuang 2004). Differences were recorded between the epidermal cells of the two *Ternstroemia* species in both surface and transverse-section views. We did not find statistically significant differences in the epidermal, adaxial and abaxial cells of both *Ternstroemia* species studied. Other studies in *Ternstroemia* reported some similarities and differences regarding the characteristics described for the epidermis. For example, Boeger & Wisniewski (2003) described adaxial epidermal cells of greater size than the abaxial surface, but polygonal in shape, thickened walls, anticlinals wavy, and the outer periclinal convex in *T. brasiliensis*. Herat & Theobald (1977) described in *T. emarginata* square adaxial cells with straight walls, and for *T. japonica* smaller epidermal cells also with straight walls. Weitzman *et al*. (2004) reported the occurrence of hypodermal layers, however they were nor observed in the two species studied.

In *Ternstroemia lineata* subsp. *lineata* and *T. sylvatica*, we found a bifacial mesophyll, differentiated into palisade and spongy parenchyma, similar to other species of the genus (Herat & Theobald 1977, Cao 2000, Boeger & Wisniewski 2003). Weitzman *et al.* (2004) pointed out that the palisade parenchyma consists of a variable number of layers in the members of the family. On the other hand, Metcalfe & Chalk (1972) mentioned that the number of layers of palisade parenchyma is of some value for the identification of genera and species of the family. Herat & Theobald (1977) considered that the number of layers of palisade parenchyma helps differentiate genera, reporting one to four layers in different species of *Ternstroemia*. Cao (2000) reported a palisade parenchyma stratum in *T. aneura* Miq. and Boeger & Wisniewski (2003) two strata in *T. brasiliensis* as do occur in *T. lineata* subsp. *lineata*. Spongy parenchyma is open, with abundant large intercellular spaces, occupying more than two-thirds of the thickness of the lamina, as described by Herat & Theobald (1977) for *T. emarginata* and *T. japonica*.

We found, as earlier authors (Keng 1962, Metcalfe & Chalk 1972, Weitzman *et al.* 2004, Stevens 2021), the occurrence of astrosclereids. In addition, we found that sclereids are concentrated towards the margin or scattered through the lamina but not densely grouped around the veins, as Stevens (2021) mentioned for members of the Ternstroemieae subfamily. As pointed out by Karabourniotis *et al*. (2021), the sclereids may contribute to improving photosynthetic performance and defense protection. Herat & Theobald (1977) observed, as in the two studied Mexican *Ternstroemia*, that the size of the sclereids varies within the same leaf. Keng (1962) suggested that sclereids formed during the early leaf development are small, unlike those formed later; he pointed out that sclereids are of value for delimiting taxa at the generic level. We found particular similarities in *T. lineata* subsp. *lineata* and *T. sylvatica* with other *Ternstroemia* species worldwide. Other authors such as Rao (1951a, b) described the sclereids in *T. japonica*, Herat & Theobald (1977) in *T. emarginata* and *T. japonica*, Cao (2000) in *T. aneura* Miq., and Boeger & Wisniewski (2003) in *T. brasiliensis*. Our observations agree with De Roon (1966) in the presence of highly branched sclereids (astrosclereids) with pointed ends.
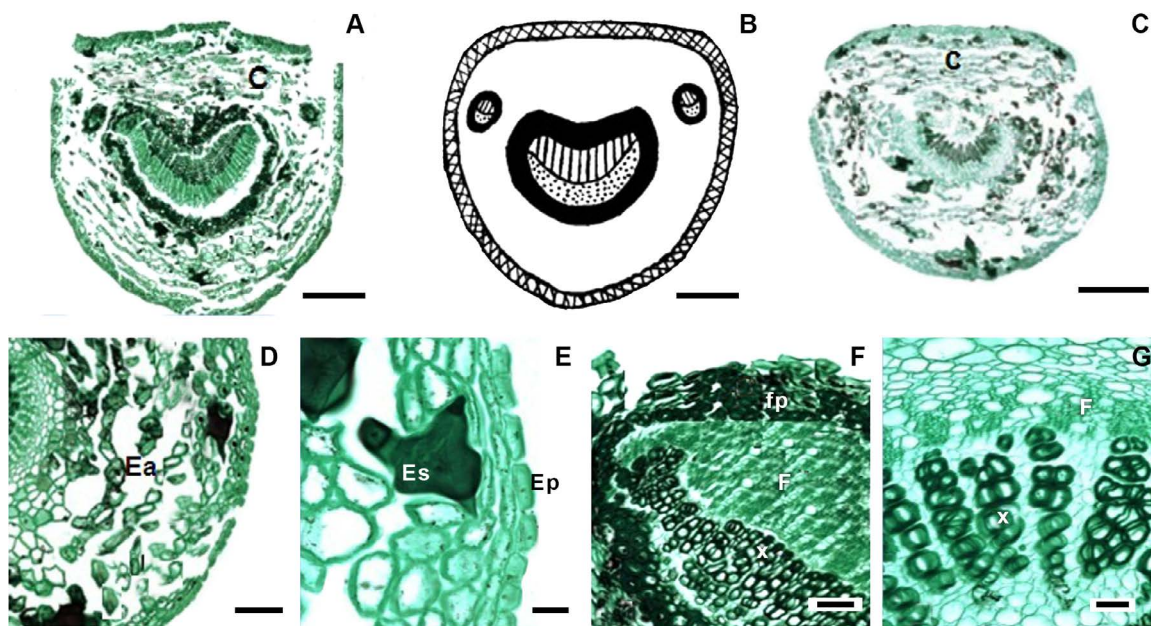
Figure 3. Petiole anatomy of *Ternstroemia lineata* subsp. *lineata* and *T. sylvatica*, transverse sections. A. Petiole at the middle region, *T. lineata* subsp. *lineata*. B. Petiole diagram of the middle region; black = sclerenchyma, dots = phloem, parallel lines = xylem, crossed lines = collenchyma, white = parenchyma. C. Petiole at the distal region, *T. sylvatica*. D. Detail of the open parenchyma, with large intercellular spaces, *T. sylvatica*. E. Detail of the epidermal cells and sclereids, *T. sylvatica*. F. Details of the perivascular fibers, *T. lineata* subsp. *lineata*. G. Detail of vascular tissue in the distal region, *T. sylvatica* C = cortex; Ea = intercellular space; Ep = epidermis; Es = sclereids; F = phloem; fp = perivascular fibres; x = xylem. Bar is 300 µm in A-C; 100 µm in D; 20 µm in E, G; 50 µm F.

Azcárraga-Rossette (2010) mentioned the usual presence in the adaxial epidermis and the mesophyll of pigments that help capture light. We observed the presence of tannins in both species studied, occluding the lumen of the palisade and spongy parenchyma cells, less frequent in the epidermal cells. Tannins protect the protoplast against desiccation and predators; they are essential in the starch metabolism, formation and transport of sugars, as antioxidants, and as colloids maintaining the homogeneity of the cytoplasm (Bonfil-Campos 2010). The presence of tannins was reported in different species of *Ternstroemia* (Metcalfe & Chalk 1972, Herat & Theobald 1977, Boeger & Wisniewski 2003, Stevens 2021). Metcalfe & Chalk (1972) reported cork cells and tannins on the abaxial surface in *Ternstroemia*. In both species, cork cells were observed, corresponding to the deciduous glands leaving frequent scars in the margin of *T. lineata* subsp. *lineata* and rare in *T. sylvatica.*

Mucilage-bearing idioblasts were found among the mesophyll cells of both species; the presence of mucilage in the family was reported earlier by various authors (Keng 1962, Metcalfe & Chalk 1972, Herat & Theobald 1977, Weitzman *et al.* 2004, Stevens 2021). However, we did not find solitary or grouped crystals in the mesophyll despite using polarized light, as mentioned by Metcalfe & Chalk (1972) for the family. The vascular tissue of *T. lineata* subsp. *lineata* and *T. sylvatica* coincide with the description of Metcalfe & Chalk (1972) and Herat & Theobald (1977) for some species of *Ternstroemia*. In both species, perivascular fibers and astrosclereids are clearly related with a "coriaceous" leaf texture.

There are few studies concerning the midrib of *Ternstroemia* subsp., so this study is the first to detail the tissues of this region of the lamina. Herat & Theobald (1977) reported collateral vascular bundles in the midrib of *T. emarginata* and *T. japonica* with a sclerenchyma sheath. These authors pointed out that the shape of the vascular bundles varies within the genus, but they are consistent within the species. They found in *T. emarginata* a U-shaped vascular bundle, while in *T. japonica,* it has a sunken U-shaped one. In *T. lineata* subsp. *lineata* and *T. sylvatica*, the vascular tissue of the midrib, is represented by a prominent closed, arch-shaped bundle in both species, as in *T. emarginata*

and *T. japonica*, surrounded by perivascular fibers. Additionally, Herat & Theobald (1977) observed that xylem vessels are interrupted by rays with cells containing tannins. In addition, they recorded scattered tanniferous parenchyma cells in the phloem of some species. We did not observe tannins either in the xylem and phloem of the studied species.

Petiole anatomy. There are few studies on the petiole of *Ternstroemia* subsp. Herat & Theobald (1977) reported that in *T. emarginata* and *T. japonica,* a relatively thick cuticle and small epidermal cells in the petiole compared to the foliar lamina. In the case of *T. lineata* subsp. *lineata* and *T. sylvatica*, the cuticle and epidermis are similar to the midrib. Esau (1985) pointed out that the collenchyma or sclerenchyma are the supporting tissues of the petiole, arranged similarly to that of the stem. Herat & Theobald (1977) observed parenchyma exclusively in the cortex of the petiole in *Ternstroemia*. In our study, we found angular, 3-5-layered collenchyma in *T. lineata* subsp. *lineata* and angular to lacunar 1-3-layered collenchyma in *T. sylvatica*. Rao (1951a) reported that in other *Ternstroemia* species, there are wide intercellular spaces and astrosclereid in the petiole, as do occur in the species here studied. We did not find crystals in the petiole, as Metcalfe & Chalk (1972) recorded for some family species.

Howard (1979) suggested that the anatomy of the petiole may be helpful as a taxonomic character. In this study, we found differences in the vascular system between both species at different petiole levels (distal end, middle region, and proximal end). Metcalfe & Chalk (1972) commented that "in transverse-section, the petiole of several
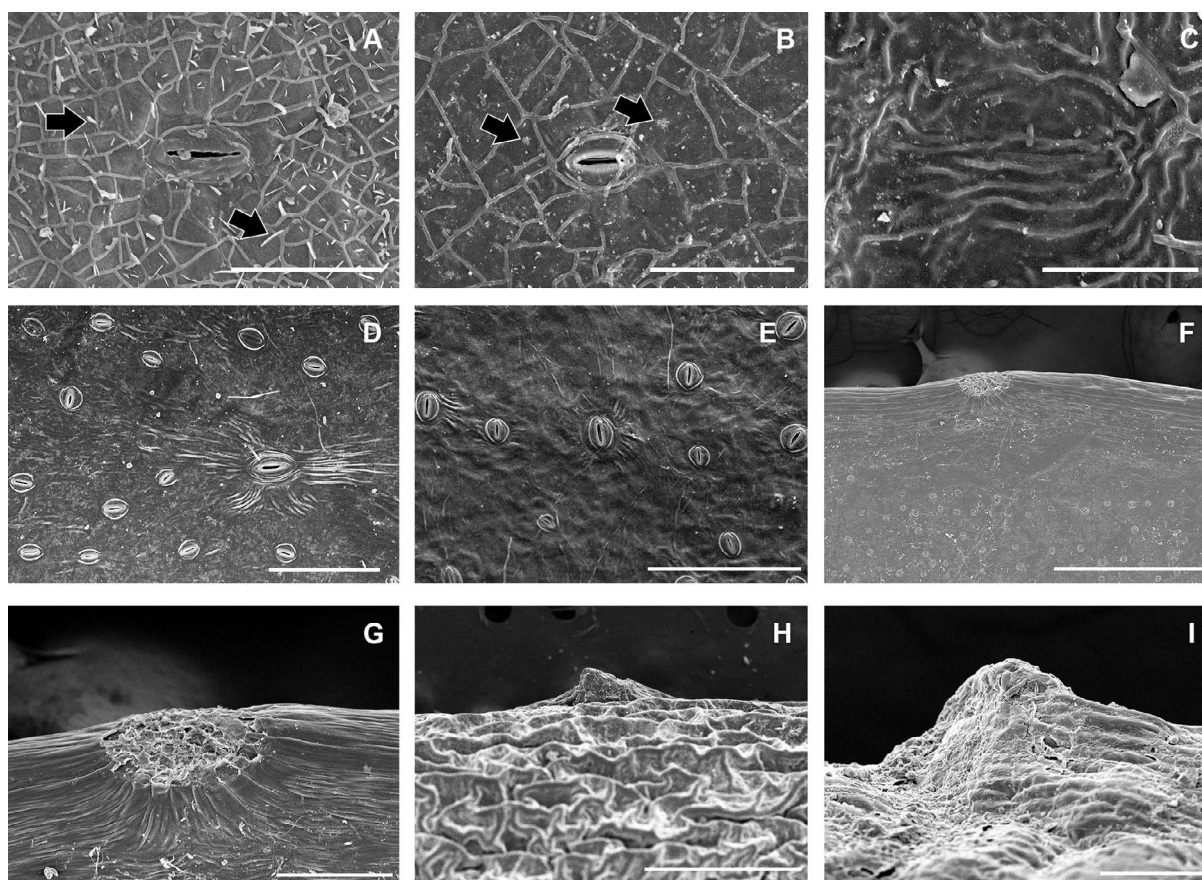

Figure 4. Epidermal micromorphology of *T. lineata* subsp. *lineata* and *T. sylvatica* leaves, SEM. A, B. Epicuticular wax pattern in the abaxial surface, A. *T. lineata* subsp. *lineata*, B. *T. sylvatica*. C. Detail of cuticular striation, *T. sylvatica*. D, E. Anomocytic stomata in the abaxial surface, D. *T. lineata* subsp. *lineata*, E. *T. sylvatica*. F, G. Detail of the scar left by the deciduous gland, F. *T. lineata* subsp. *lineata*, G. *T. sylvatica*. H. View of the striate cuticle of the petiole, *T. lineata* subsp. *lineata*. I. Detail of the protrusion of the petiole, *T. lineata* subsp. *lineata*. Bar is 50 µm in A, B; 100 µm in D, E, G, I; 300 µm in C; 400 µm in F, 500 µm in H.

genera of the Pentaphylacaceae family is characterized by a vascular bundle, but in some cases, small lateral bundles are present". Our study observed that closer to the lamina (proximal end), the petiole is slightly winged and has three vascular bundles. There is a prominent vascular bundle, with the same arrangement as the midrib, surrounded by perivascular fibers and two circular lateral bundles. This arrangement was also recorded in the middle region of the petiole. At the distal end of the petiole, we only observed one vascular bundle. The findings of Keng (1962), Metcalfe & Chalk (1972), and Weitzman *et al*. (2004) coincide with our study, arguing that the distal end of the petiole of some members of Pentaphylacaceae exhibits an arch, U, or V vascular bundle surrounded by fibers. Weitzman *et al*. (2004) pointed out that the lateral bundles of the leaf diverge from the end of the trace, and there are from one to five lateral bundles in the petiole of Pentaphylacaceae. Herat & Theobald (1977) pointed out that in *Ternstroemia emarginata* and *T. japonica*, there is a U-shaped closed collateral vascular bundle surrounded by perivascular fibers.

Micromorphology of the foliar surface. We undertook micromorphology employing scanning electron microscopy (SEM). By this method, we found in *T. sylvatica* and *T. lineata* subsp. *lineata* characters not observed through conventional microscopy. For the first time, we observed anomocytic stomata on the foliar lamina of *T. lineata* subsp. *lineata* and *T. sylvatica*, with a clear pattern of stomatal dimorphism in the same lamina. Stomata have striae arranged radially to the guard cells, forming extended bands in the larger stomata, more visible in *T. lineata* subsp. *lineata*, similar to those described by Wilkinson (1979) for *Nyssa sylvatica* Marshall (Nyssaceae). We observed in *T. lineata* subsp. *lineata* and *T. sylvatica*, a cuticle with a reticulated-striated pattern with thin striae and open valleys. In the case of the petiole, the cuticle had a reticulated pattern of thick striations and very closed valleys.

Epicuticular wax is essential for the functionality and structuring of the cuticle (Koch *et al*. 2009). Therefore, wax morphology is mainly used as an additional diagnostic character *sensu* Wilkinson (1979) for taxonomists. The epicuticular wax *sensu* Barthlott *et al*. (1998) has a short-tubular deposition pattern in *T. lineata* subsp. *lineata* and a granular-like in *T. sylvatica*, uniform and sparsely distributed on the abaxial surface of the lamina of both species. In some parts of the leaf margin, we observed dome-shaped scars of the deciduous glands, with a large depression in the center; these depressions correspond to cork regions in the transversal sections. In the case of the petiole, we found sparse protrusions only in *T. lineata* subsp. *lineata*. These structures are not visible in the petiole cross-sections.

Few studies about the anatomy and foliar architecture of *Ternstroemia* exist worldwide. Our studies on the foliar architecture and anatomy on the leaves of *T. lineata* subsp. *lineata* and *T sylvatica* reveal similarities and differences between them and other species of *Ternstroemia* described elsewhere, which may contribute to the separation of species. Furthermore, the complete micromorphology of the *Ternstroemia* leaf is described for the first time and can contribute to the resolution of taxonomic problems.

We found in both species a glandular margin composed of deciduous glands, a stomatal dimorphism pattern in the size of the guard cells on both sides of the lamina, and adaxial epidermal cells with concave-convex periclinal walls due to a large cellulose deposit, characteristics observed for the first time in these taxa.

Several conclusions are rejected for the genus because we found stomata on both leaf surfaces (amphistomatic leaves). We did not find crystals and hypodermis in the mesophyll, nor was the presence of tannins in the xylem or mucilage in the epidermis. We conclude that the leaf texture (described as leathery for most species) depends on the number of sclereids/mm^2 and the abundance of perivascular fibers. These plants have been described as having thick cuticles, but the reality is that they have very thin cuticles.

Ternstroemia sylvatica and *T. lineata* subsp. *lineata* preferably inhabits the Mexican tropical montane cloud forest. The genus is highly heterogeneous, *e.g.,* the morphology of the leaf varies between individuals of the same population regardless of the locality where it is found. The foliar anatomy of the studied species shares some characteristics with other plants from humid environments, such as thin cuticle, mesophyll with sparse parenchyma palisade and abundant intercellular spaces in the spongy parenchyma, and pigments (tannins) for light capture and protection. Several *Ternstroemia* species developed specific mechanisms to adapt to humidity and shadow-fog conditions. These features are shared with other TMCF diagnostic/characteristic tree species, such as certain species of Clusiaceae Lindl. (*Clusia* L.), Staphyleaceae Martinov (*Turpinia* Vent.), some members of Ericaceae Juss. (*Vaccinium* L.) and

Magnoliaceae Juss. (*Magnolia* L.), Styracaceae DC. & Spreng. (*Styrax* L.), Symplocaceae Desf. (*Symplocos* Jacq.), other Pentaphylacaceae (*Cleyera* Adans., *Freziera* Sw. ex Willd., *Symplococarpon* Airy Shaw), allowing them to flourish in these peculiar environments (Schadel & Dickison 1979, Rodríguez-Ramírez *et al*. 2021).

This study represents the first attempt at the anatomy, foliar architecture, and micromorphology of Pentaphylacaceae in Mexico. It intends to contribute basic information on Mexican tropical montane cloud forest diagnostic taxa. This information is also helpful for taxonomic, ecological, and identity purposes.

Acknowledgments

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