

Plant anatomy: history and future directions

Do anatomical traits converge on epiphytic plants?

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Abstract

Epiphytes represent approximately 10% of vascular plants, or 25,000 species distributed across 84 families. Water deficit is the greatest challenge faced by epiphytes, resulting in anatomical strategies aimed at maximizing water usage and minimizing water loss. This study aimed to characterize the leaf blade anatomy of 40 species of vascular epiphytes occurring in a cloud forest of the Brazilian Atlantic Forest and assess how leaf anatomy is related to the epiphytic life form. Samples were collected, fixed, dehydrated with ethanol, and embedded in paraffin for sectioning using a microtome or freehand technique. Some samples were dissociated for epidermal observation. A clustering analysis (using UPGMA) was conducted using a presence/absence matrix of 16 anatomical characters. The results showed that species within this functional group exhibit different leaf blade anatomical characteristics, which may or may not be adaptive to epiphytism. However, despite the occurrence of some characteristics such as stomata at the same level as other epidermal cells, thick cuticle, fibers, hypodermis, and homogeneous chlorophyllous parenchyma in 50% or more of the species, there is no set of anatomical characteristics that can be used to define an epiphyte.

Key words: dwarf cloud forest, ecological anatomy, epiphytism, water deficit.

Resumo

As epífitas representam cerca de 10% das plantas vasculares, ou 25.000 espécies distribuídas em 84 famílias. O déficit hídrico é o maior problema enfrentado pelas epífitas, resultando em estratégias anatômicas que buscam maximizar o uso da água, bem como minimizar sua perda. Este estudo teve como objetivo caracterizar a anatomia da lâmina foliar de 40 espécies de epífitas vasculares que ocorrem em uma mini floresta nebulosa da Floresta Atlântica brasileira e avaliar se e de que maneira a anatomia foliar está relacionada à forma de vida epífita. As amostras foram coletadas e fixadas, desidratadas com etanol e incluídas em parafina para seccionamento em micrótomo ou à mão livre. Algumas amostras foram dissociadas para observação da epiderme. Uma análise de agrupamento (através do UPGMA) foi realizada usando uma matriz de presença e ausência de 16 caracteres anatômicos. Os resultados mostraram que as espécies deste grupo funcional apresentam diferentes caracteres anatômicos foliares que podem ou não ser adaptativos ao epifitismo. Entretanto, apesar da ocorrência de alguns caracteres como estômatos no mesmo nível das demais células epidérmicas, cutícula espessa, fibras, hipoderme e parênquima clorofílico homogêneo em 50% ou mais das espécies, não há um conjunto de caracteres anatômicos que possa ser utilizado para definir uma epífita.

Palavras-chave: nanofloresta nebulosa, anatomia ecológica, epifitismo, déficit hídrico.

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Introduction

Epiphytes are plants which directly establish themselves on the trunks or branches of trees, using the supporting plant, called the phorophyte, solely for support. They are important for the biodiversity of tropical forests worldwide, accounting for approximately 10% of vascular plants, with around 25,000 species distributed across 84 families (Zotz 2013). According to Marcusso *et al.* (2022), the great global diversity and richness of epiphytes is found in the Neotropical region, reflecting the great climatic variation and water range of the location.

In terms of species richness, they are mainly represented by monocotyledons due to the significant presence of Orchidaceae, Bromeliaceae, and Araceae, followed by ferns, especially Aspleniaceae, Hymenophyllaceae, and Polypodiaceae. Eudicotyledons are additionally notable, with representatives such as Cactaceae and Gesneriaceae, and one family of magnoliids, Piperaceae (Dubuisson *et al.* 2008).

Regardless of their phylogeny, a group of plants that share similar frequencies of a set of traits and exhibit species with similar adaptations to environmental variables constitutes a functional group, as is the case with epiphytes (Box 1996). The occurrence of a functional type in a vegetation has been used to determine community composition, aiming to assess the influence of edaphic and climatic factors on them (Muller 2005). Plant functional types have become essential for studying and predicting the consequences of global changes on vegetation (Pillar & Debastiani 2012).

The abundance and diversity of epiphytes are strongly influenced by changes in ecological conditions along altitudinal and latitudinal gradients (Gentry & Dodson 1987; Gentry 1988), primarily varying based on light intensity, altitude, characteristics of the host tree bark and water availability (Wolf 1994; Basset *et al.* 2003; Mucunguzi 2007). The longitudinal gradient and the type of suber define a great differentiation of niches and interactions, such as plant-herbivore, plant-pollinators, mycorrhizae, in addition to providing resources and habitat for vertebrates and invertebrates. Furthermore, epiphytes contribute to increased biomass, nutrient cycling and water retention in the ecosystem (Spicer & Woods 2022; Hedges & Thomas 1992; Phillipis *et al.* 2020; Seidel *et al.* 2020).

Water deficit is the major challenge epiphytes face, leading to anatomical strategies that maximize efficient water usage and minimize water loss. However, similar environmental conditions can lead to an evolution of analogous adaptive strategies for common challenges, resulting in different species in similar environments converging in form and function (Cody & Mooney 1978), and explain the emergence of similar characteristics in phylogenetically distant species.

Leaf anatomy is an important tool in understanding the adaptive strategies of plants. Quantitative and qualitative assessments of tissues aim to elucidate the relationship between gas exchange, water absorption, and leaf structure (Abrams *et al.* 1994). Thick cuticle, absorbing scales, stomata in depressions, differentiated mesophyll in hypodermis and chlorophyllous parenchyma, and fibers are common characteristics among various groups of epiphytic plants (Benzing 1990; Zotz 2016).

Although floristic surveys of vascular epiphytes in the Neotropical region have become more common in the last decade (*e.g.*, Nieder *et al.* 2000; Krömer & Gradstein 2003; Arévalo & Betancur 2004; Buzatto *et al.* 2008; Menini Neto *et al.* 2009; Bianchi *et al.* 2012; Zotz 2013; Leitman *et al.* 2014; Mondragón *et al.* 2015; Furtado & Menini Neto 2016), few studies have analyzed the anatomy of multiple species from different families and evolutionary lineages. Most studies are limited to a single genus or a few species within a family (Benzing *et al.* 1978; Silva *et al.* 2006; Rocha *et al.* 2013; Voytena *et al.* 2015).

Therefore, the aim of this study was to assess whether and how the leaf blade anatomy of 40 epiphytic species from dwarf cloud forest in the Atlantic Forest is related to the epiphytic habit in order to address the following question: Do leaf blade anatomical characteristics converge in distinct taxa in response to adaptation to the epiphytic lifestyle?

Materials and Methods

Study area

The specimens used in this study were collected in the Parque Estadual do Ibitipoca (PEIB), located in southeastern Minas Gerais, Brazil, between the coordinates 21°40'–21°44'S and 43°52'–43°55'W, covering approximately 1,488 ha. The area is part of the Serra da Mantiqueira complex, with altitudes ranging from 1,000 to

about 1,800 meters. The park encompasses a mosaic of grassland and forest formations, with a notable presence of dwarf cloud forests occurring at different altitudes. These cloud forests are characterized by a high density of shrubs and small-statured trees (3 to 5 m) and retain moisture from the fog, enabling a high abundance of epiphytes (Oliveira-Filho *et al.* 2013; Furtado & Menini Neto 2018). The climate of the park is classified as Cwb according to the Köppen climate classification system Köppen (1936), meaning it is a humid mesothermal climate with dry winters and mild summers. The average annual precipitation is around 1,532 mm, and the average annual temperature is 18 °C (CETEC 1983). According to Rocha (2013), the average relative humidity remains high throughout the year in the region, with values between 82% and 87% in the hot and rainy season, decreasing by about 5% in the colder period, with values between 76% and 84%.

Analyzed species

The 40 vascular epiphytic species studied were selected among the most frequent species indicated by Furtado & Menini Neto (2018), representing a diversity of genera, families, and phylogenetic lineages. The selection also considered the species richness of each family occurring in the study area (Tab. S1, available on supplementary material <<https://doi.org/10.6084/m9.figshare.25655388.v1>>). Voucher specimens are deposited in the CESJ herbarium at the Universidade Federal de Juiz de Fora.

Sample collection and processing

For each selected species, five individuals from different phorophytes were chosen, and three fully expanded leaves were collected from each of these individuals. The individuals were located at a maximum height of 2 m from the base. The collected samples were fixed in 2.5% glutaraldehyde. The collections were carried out in July 2015 and May 2017.

For the preparation of histological slides, the middle region of the leaves was dehydrated in an ethanol series and embedded in paraffin (Johansen 1940). The samples were then sectioned using a rotary microtome (10 µm), deparaffinized, and stained with Astra Blue and Basic Fuchsin (modified Roeser 1972 by Luque *et al.* 1996). Afterward, they were dehydrated again in an ethanol series and mounted in clear vitreous varnish 500® (Paiva *et al.* 2006). Some of the materials that did not respond

well to embedding were sectioned freehand using a table microtome. These sections were later clarified in 20% sodium hypochlorite, washed with distilled water, stained with Astra Blue and Basic Fuchsin, and mounted in glycerinated water. Samples from the leaf blade slides were either rendered transparent (Johansen 1940) or dissociated (Franklin 1945) and stained with Basic Fuchsin to highlight stomata and trichomes in a paradermal view.

Thirty fields (cross-sections) were photographed from each of the five individuals of each of the 40 species, totaling 150 fields per species, from which micromorphometric measurements were taken. Stomata and trichome density (number of stomata or trichomes/unit area) was calculated based on dissociation or clearing, analyzing 20 fields from the median region of each of the five individuals of each species, totaling 100 fields per species. Micromorphometric analysis were performed using the ZEN 2 software (blue edition).

Next, the samples were sectioned freehand and subjected to the following histochemical tests: 4% formalin with ferrous sulfate and potassium dichromate for general phenolic compounds (Gabe 1968), phenol for silica identification (Johansen 1940), glacial acetic acid to check for the presence of calcium carbonate, 10% hydrochloric acid for calcium oxalate detection (Chamberlain 1932), and acidified phloroglucinol for lignin identification (Johansen 1940).

The observations and images were captured using a light microscope (Zeiss-model AX10) with an attached AxioCam ERc 5s digital camera at the Plant Anatomy Laboratory of UFJF.

Multivariate analyses

A cluster analysis of the species was performed using the unweighted pair group method using arithmetic averages (UPGMA) and the Jaccard similarity index. To do so, a matrix of presence (1) or absence (0) of 16 qualitative leaf blade anatomical characters related to environmental adaptation (described in Tab. 1) was prepared to investigate convergence among the different evaluated species.

Only quantitative data were used for the PCA analysis, including the cuticle thickness on the adaxial and abaxial epidermal surfaces, stomatal density, and the palisade/spongy tissue ratio. For these characters, the overall mean (n = 30 measurements) of each character was calculated for five individuals of the 40 species. Values

Table 1 – Anatomical characteristics of the evaluated species of vascular epiphytes occurring in the dwarf cloud forests of Parque Estadual do Ibitipoca, Minas Gerais, Brazil.

1 - Epidermal cells with sinuous anticlinal walls	9 - Parenchyma with large isodiametric cells
2 - Epidermal cells with straight anticlinal walls	10 - Parenchyma with large intercellular spaces
3 - Stomata at the same level as other epidermal cells	11 - Phenolic content
4 - Stomata under the other epidermal cells	12 - Silica body
5 - Hypostomatic leaf	13 - Crystals
6 - Anfistomatic leaf	14 - Hypodermis with thickening bars
7 - Trichomes	15 - Perivascular fibers
8 - Aquifer hypodermis	16 - Extravascular fibers

below of the overall mean were defined as thin and values above the overall mean were defined as thick, or low and high to stomatal density. The cuticle thickness measurements took into account the actual cuticle layer, including the cell wall, as it was not possible to identify the different layers in many species. The total leaf blade thickness was evaluated by creating a box plot due to the large variability in leaf blade thickness, which would otherwise disproportionately influence the PCA results. Trichome density was also calculated; however, this character was not included in the PCA analysis as it had only taxonomic value in many species. The analyses were performed using the Past 3.0 software program (Hammer *et al.* 2001).

Results

Anatomical traits

There was significant variation among the 40 analyzed species in both the quantitative and qualitative characteristics evaluated. In relation to the thickness of the cuticle layer and cell wall, there is a great variation between species from different groups and even within species from the same group. The overall mean values used as reference were: adaxial side - 3,26 μm and abaxial side - 2,76 μm . Among the nine species of Bromeliaceae, *Tillandsia usneoides* (L.) L., *Tillandsia gardneri* Lindl., *Nidularium ferdinandocoburgii* Wawra, *Vriesea penduliflora* L.B. Sm. and *Vriesea friburgensis* Mez. have a thin cuticle, while in *Tillandsia stricta* Sol. and *Tillandsia geminiflora* Brongn. the cuticle is thick. Only *Billbergia distachia* (Vell.) Mez. has a difference in cuticular thickness between the two sides of the leaf, with a thick cuticle on the adaxial side and a thin one on the abaxial side. Among the Orchidaceae, *Stelis caespitosa* Lindl., *Octomeria crassifolia* Lindl., *Maxillaria subulata* Lindl. and *Bifrenaria vitellina* (Lindl.) Lindl. have a thick cuticle, with

flanges on the abaxial face of *O. crassifolia* and *M. subulata*, and wavy ornamentation in *Bulbophyllum micropetaliforme* J. E. Leite. In *Epidendrum paranaense* Barb. Rodr. and *Scaphyglottis modesta* (Rchb.f.) Schltr., the adaxial cuticle is thick and the abaxial cuticle is thin. In other orchid species, the cuticle is thin, without ornamentation or flanges.

Serpocaulon catharinae (Langsd. & Fisch) A.R. Kaulf., *Cochlidium punctatum* (Raddi) L.E. Bishop, *Microgramma squamulosa* (Kaulf.) de la Sota, *Pleopeltis macrocarpa* (Bory ex Willd.) Kaulf., *Griselinia ruscifolia* (Clos) Taub. and the three species of Araceae have a thick cuticle, with ornamentation on the abaxial surface of *Anthurium minarum* Sakur. & Mayo and flanges in *Anthurium scandens* (Aubl.) Engl.; in other fern species, as well as in *Hillia parasitica* Jacq. and in Piperaceae, the cuticle is thin, without ornamentation. *Nematanthus strigillosus* (Mart.) H.E. Moore presents differentiation in cuticular thickness between the sides of the leaf, in which the adaxial cuticle is thin and the abaxial cuticle thick.

Except for *Peperomia*, all species had a uniseriate epidermis, and only fern species showed chloroplasts in the epidermal cells. The anticlinal walls of the epidermal cells in species of Orchidaceae, Piperaceae, Araceae, *Nematanthus strigillosus*, *Griselinia ruscifolia*, *Hillia parasitica* Jacq. and *Hymenophyllum polyanthos* (S.) Sw. were straight (Fig. 1a), while these walls were sinuous in Bromeliaceae and other ferns (Fig. 1b). In the 3 species of *Vriesea*, in *Tillandsia stricta*, in *Billbergia distachia* and *Nidularium ferdinandocoburgii*, the epidermal cells have a greatly reduced lumen due to the thickening of the anticlinal and internal periclinal walls, confining the protoplasm to 10% of the cell volume.

The presence of stomata on both surfaces of the epidermis (amphistomatic character) was observed in *Tillandsia stricta* and *Tillandsia gardneri* Lindl.

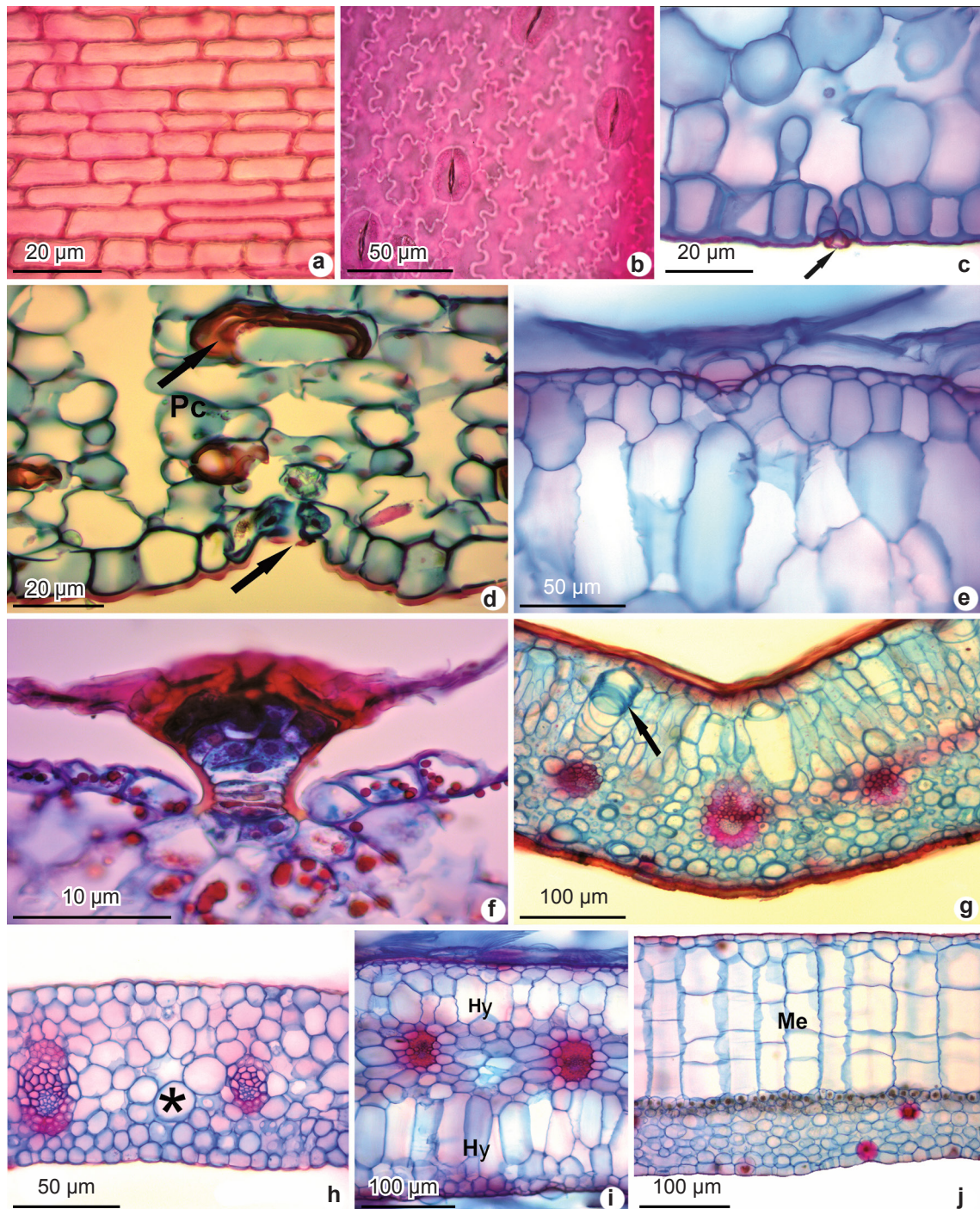


Figure 1 – a-j. Leaf blade anatomy of the Parque Estadual do Ibitipoca epiphytic species – a-b. epidermal dissociations – a. rectilinear anticlinal walls in *Maxillaria subulata*; b. sinuous anticlinal walls in *Cochlidium punctatum*; c-j. cross sections – c. stomata at the same level as epidermal cells in *Epidendrum pseudodiforme*; d. sunken stomata in *Anthurium minarum*; e. scale in *Tillandsia gardneri*; f. scale in *Pleopeltis hirsutissima*; g. helical thickening in the wall of hypodermal cell of *Bulbophyllum micropetaliforme*; h. aquiferous parenchyma in *Dichaea cogniauxiana*; i. aquiferous hypodermis on both sides of the leaf blade in *Tillandsia gardneri*; j. multiple epidermis in *Peperomia mandioccana*. (Pc = phenolic compounds; Hy = hypodermis; Me = multiple epidermis; * = aquiferous parenchyma).

The other species were hypostomatic, except for *Hymenophyllum polyanthos*, which did not have stomata. Stomata at the same level as the other epidermal cells were observed in ferns, *Nematanthus strigillosus*, *Griselinia ruscifolia*, *Hillia parasitica*, Orchidaceae, Piperaceae, *Vriesea* species, and *Nidularium ferdinandocoburgii* (Fig. 1c). Stomata in other species occurred below the other epidermal cells (Fig. 1d).

Trichomes were observed in 26 analyzed species. Among these, the Orchidaceae species *Epidendrum pseudodiforme* Hoehne & Schltr., *Epidendrum paranaense* Barb. Rodr., *Bulbophyllum micropetaliforme*, *Maxillaria subulata*, *Bifrenaria vitellina*, *Pleurothallis rubens* Lindl., *Promenaea xanthina* (Lindl.) Lindl. and *Stelis caespitosa* had only glandular trichomes. *Peperomia mandioccana* Miq., *Nematanthus strigillosus*, and *Pecluma pectinatiformis* (Lindm.) M.G. Price had both glandular and non-glandular trichomes. Only in *Peperomia diaphanoides* Dahlst. were observed glandular trichomes, distinguishing it from *P. mandioccana*. Scales were also observed in all Bromeliaceae species (Fig. 1e) and the two species of *Pleopeltis* (Fig. 1f). Star-shaped non-glandular trichomes were observed in *Hymenophyllum polyanthos*.

Most species had low trichome density according to the micro morphometric analyses. However, *Tillandsia* and *Vriesea friburgensis* Mez species exhibited high scale density on both epidermal surfaces among Bromeliaceae. *Peperomia mandioccana* had high density of both glandular and non-glandular trichomes on both epidermis surfaces, while *P. diaphanoides* had a high density of glandular trichomes only on the abaxial surface. *Nematanthus strigillosus* showed a high density of glandular trichomes on the adaxial surface and a high density of both glandular and non-glandular trichomes on the abaxial epidermis surface.

Epidermal cellular contents were detected in 12 species. *Anthurium minarum*, *A. scandens*, *Philodendron appendiculatum*, *Cochlidium punctatum* and *Vriesea penduliflora* had phenolic compounds in the cells. *Anthurium minarum* and *A. scandens* also had calcium oxalate crystals, as well as *Griselinia ruscifolia*, *Philodendron appendiculatum* Nadrz & Mayo, *Dichaea cogniauxiana* Schltr., and *Promenaea xanthina*. Druses were observed in *Anthurium*, *P. appendiculatum*, and *G. ruscifolia* species, while raphides were observed in the epidermal cells

of *D. cogniauxiana*, *P. xanthina* and *Billbergia distachia*. Silica bodies were found in the cells on both epidermis surfaces in *Vriesea* and *B. distachia*.

Water storage tissue was found in 29 species, differentiated into multiseriate water-storing epidermis, aquiferous hypodermis (Fig. 1i), or in a parenchyma with larger isodiametric cells (Fig. 1h). Large parenchymatic cells with thin walls suitable for water storage were observed in *Prosthechea pachysepala* (Klotzsch) Chiron & V.P. Castro, *Dichaea cogniauxiana*, *Promenaea xanthina* and *Bifrenaria vitellina* but this does not characterize a hypodermis. In *Prosthechea allemanoides* (Hoehne) W.E. Higgins, *Scaphyglottis modesta*, *Gomesa glaziovii*, *Hadrolaelia coccinea*, *Bulbophyllum micropetaliforme*, *Stelis caespitosa*, *Maxillaria subulata*, *Octomeria crassifolia*, *Hillia parasitica*, *Microgramma squamulosa* and the eight species of Bromeliaceae an aquiferous hypodermis was observed (Fig. 2a). *Tillandsia usneoides* is the only Bromeliaceae with an aquiferous parenchyma. Helical thickenings (Fig. 1g) were found in the cell walls of the typical aquiferous hypodermis in *Bulbophyllum micropetaliforme*, *Stelis caespitosa*, *Maxillaria subulata*, *Octomeria crassifolia*, and *Pleurothallis rubens*. The four or five layers of epidermal cells (multiseriate epidermis) in the two species of *Peperomia* serve as water storage cells, with thin concertina-like walls (Fig. 1j). Adjacent to the epidermis, in the three species of *Vriesea*, in *Billbergia distachia* and in *Hadrolaelia coccinea*, one or more layers of cells with thickened walls are observed, which constitute a mechanical hypodermis (Fig. 2b).

In *Maxillaria subulata*, *Scaphyglottis modesta*, *Bifrenaria vitellina*, *Gomesa glaziovii*, *Epidendrum paranaense*, *Prosthechea pachysepala* and in the medial region of the three species of *Vriesea*, fibers or groups of fibers were observed dispersed in the hypodermis or water-storing parenchyma (Fig. 2c). In *Maxillaria subulata*, in addition to the occurrence of fibers in the hypodermis and mesophyll, a string of fibers can be observed subepidermally throughout the leaf blade.

In *Billbergia distachia*, *Prosthechea*, *Epidendrum*, *Scaphyglottis modesta*, *Gomesa glaziovii*, *Hadrolaelia coccinea* and *Bifrenaria vitellina*, groups of fibers can be observed in the mesophyll. Perivascular fibers were observed in almost all species studied (Fig. 2d), with the exception of *Microgramma squamulosa*, *Asplenium auritum*, *Pleopeltis hirsutissima*, *Hillia parasitica*, *Nematanthus strigillosus* and *Peperomia*.

The Orchidaceae, Piperaceae, *Tillandsia* species, as well as *Billbergia distachia*, *Nidularium ferdinandocoburgii*, *Pecluma pectinatifomis*, *Asplenium auritum*, *Serpocaulon catharinae* and *Cochlidium punctatum*, exhibited a homogeneous chlorophyllous parenchyma in their mesophyll. *Cochlidium punctatum*, *N. ferdinandocoburgii*, *A. auritum*, *Anthurium minarum*, *Serpocaulon catharinae* (Langsd. & Fisch) A.R. Sm. and *T. gardneri* stood out for having large intercellular spaces in the mesophyll. The chlorophyllous parenchyma in the Araceae (Fig. 2e) species, *Nematanthus strigillosus*, *Griselinia ruscifolia*,

Hillia parasitica and in the two *Pleopeltis* species was dorsiventral, with one to three layers of palisade parenchyma. The intercellular spaces of the spongy parenchyma were reduced in *Anthurium scandens* and *P. hirsutissima* (Fig. 2f). In addition, the chlorophyllous parenchyma in *Vriesea friburgensis* and *V. penduliflora* was also dorsiventral, consisting of short cells in the palisade parenchyma and brachiform cells in the spongy parenchyma, with large intercellular spaces (Fig. 2g). On the other hand, *Vriesea bituminosa* Wawra had a homogeneous chlorophyllous parenchyma composed only of brachiform cells,

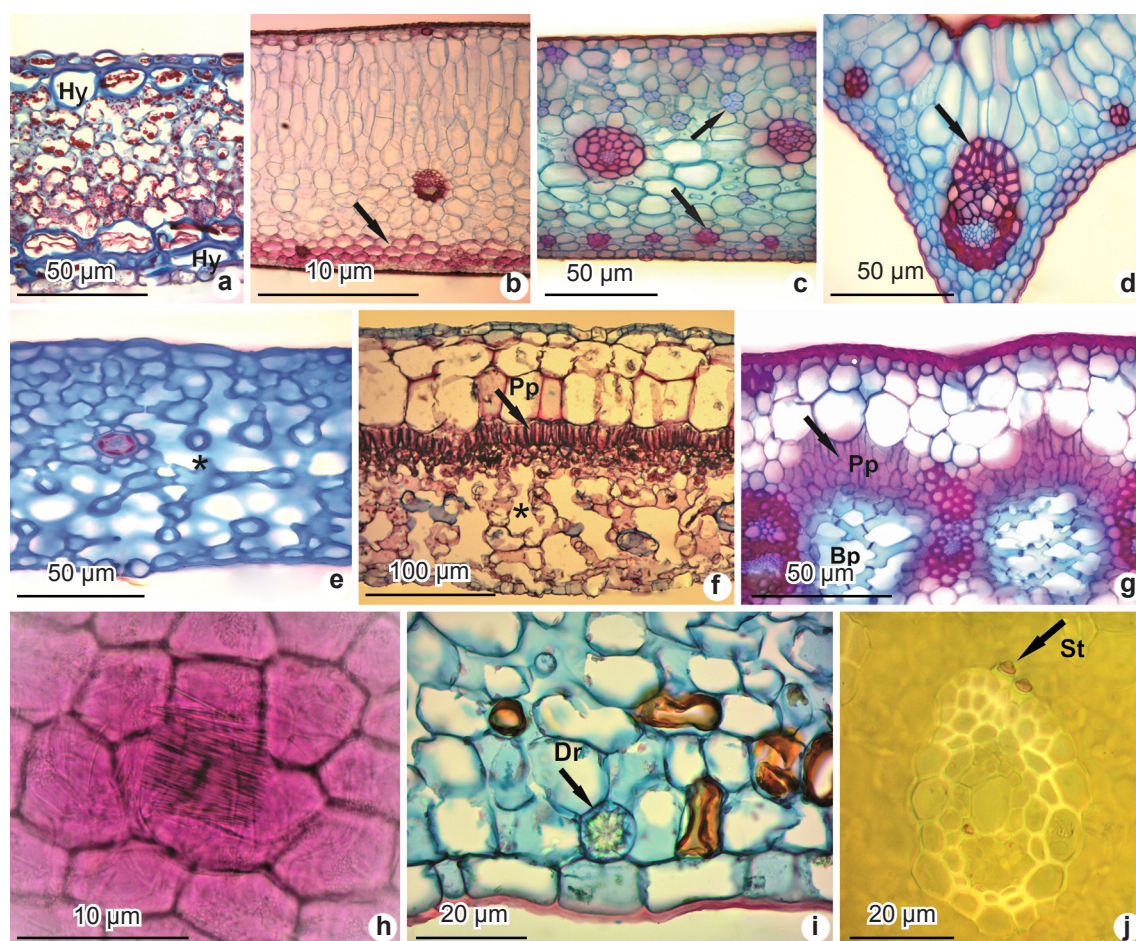


Figure 2 – a-j. Leaf blade anatomy of the Parque Estadual do Ibitipoca epiphytic species – a-i. cross sections – a. hypodermis on both sides of the leaf blade in *Microgramma squamulosa*; b. mechanical hypodermis in *Hadrolaelia coccinea*; c. extravascular fibers in *Scaphyglottis modesta*; d. perivascular fibers in *Gomesa glaziovii*; e. lacunar chlorophyll parenchyma in *Cochlidium punctatum*; f. dorsiventral chlorophyll parenchyma in *Nematanthus strigillosus*; g. dorsiventral chlorophyll parenchyma in *Vriesea penduliflora*; h. raphides in *Dichaea cogniauxiana*; i. druse in *Anthurium minarum*; j. stegmata em *Prosthechea allemanoides*. (Hy = hypodermis; Pp = palisade parenchyma; Bp = brachiform parenchyma; Dr = druse; St = stegmata; * = homogeneous chlorophyll parenchyma).

resulting in the presence of large intercellular spaces.

In Orchidaceae, Bromeliaceae, Araceae, Piperaceae and Eudicots the vascular bundles are collaterals. In ferns, the bundles are amphicribral, with the exception of *Hymenophyllum polyanthos*, which has a collateral bundle.

Calcium oxalate crystals were identified in the mesophyll of certain species, appearing as raphides in *Anthurium*, *Tillandsia geminiflora*, *T. stricta*, *Dichaea cogniauxiana*, and *Promenaea xanthina* (Fig. 2h), and as druses in *Philodendron appendiculatum*, *Griselinia ruscifolia*, *Hillia parasitica*, *Anthurium*, and *Peperomia* (Fig. 2i).

Silica bodies (stegmata)(Fig. 2j), associated with perivascular fibers, were observed in *Dichaea cogniauxiana*, *Epidendrum paranaense*, *Gomesa glaziovii*, *Maxillaria subulata*, *Octomeria crassifolia*, *Promenaea xanthina*, *Prosthechea* and *Scaphyglottis modesta*.

Multivariate analyses

The UPGMA analysis (Fig. 3) revealed the formation of three groups. Group A consisted of four species (*Hymenophyllum polyanthos* and Araceae), Group B consisted of 20 species (Orchidaceae and Piperaceae), and Group C consisted of 16 species, comprising Bromeliaceae and ferns. The distinguishing character between the two larger groups is the sinuosity of the cell walls in the epidermis, with straight walls in Group B and sinuous walls in Group C. Group A also consists of species with epidermal cells exhibiting straight walls, but Araceae has a dorsiventral mesophyll and *H. polyanthos* lacks chlorophyllous parenchyma, which placed these species in a separate group.

According to the micro morphometric analyses, the cuticle in 19 species was considered thin on both leaf blade surfaces, while the cuticle in *Billbergia distachia*, *Epidendrum paranaense*, and *Scaphyglottis modesta* is thick on the adaxial surface and thin on the abaxial surface, and the reverse occurs in *Nematanthus strigillosus*. The chlorophyllous/non-chlorophyllous tissue ratio and stomatal density were high in 19 and 13 species, respectively. A total of 20 species were considered to have high total leaf blade thickness, including eight out of the nine Bromeliaceae species analyzed.

From the PCA results (Fig. 4), it can be determined that the main characteristic supporting separation of the groups is stomatal density, which

is high in groups 1, 2, and 5, and low in the other species. The cuticle thickness, especially on the adaxial leaf blade surface, has little impact on the group separation, as observed in groups 2, 4, 5, and 6. On the other hand, the PC/PNC ratio varied widely within each group, and therefore it is not considered a decisive characteristic in grouping the analyzed epiphytic species.

Leaf blade thickness was not evaluated in the PCA analysis due to the significant variability among the species (Fig. 5), which would have disproportionately influenced the results. The lowest thickness was observed in *Hymenophyllum polyanthos*, while the highest was in *Epidendrum pseudodiforme*. The wide range of variation within each of the analyzed epiphyte groups (Ferns, Monocots, Magnoliids, and Eudicots, identified by colors in Fig. 5), particularly among the Orchidaceae, indicates that leaf blade thickness is an unreliable characteristic for analyzing the epiphytic condition.

Figures with cross-sections and dissociation of the 40 species studied can be seen in the supplementary material (Figs. S1-S8, available at <<https://doi.org/10.6084/m9.figshare.25655388.v1>>).

Discussion

The results demonstrated that the vascular epiphytes analyzed exhibit different leaf blade anatomical characteristics which may or may not be adaptive to the epiphytic habit. In cloud forests, the constant occurrence of clouds maintains air humidity, so that vertical and horizontal precipitation reaches the trunks of phorophytes, providing a microenvironment that explains anatomical characteristics determining the establishment and survival of these species (Kersten 2010; Sultan 1967).

Among these characteristics, thick cuticle, water storage tissue, stomata below the other epidermal cells, and mechanical tissue were observed in the studied groups and are described in the literature for species exposed to high light intensity, water and nutrient deficits, as well as for epiphytes (Kessler & Siorak 2007; Dubuisson *et al.* 2008; Gurevitch *et al.* 2009).

Approximately 50% of the species studied had thin cuticles, while the other 50% had thick cuticles, varying widely within the major analyzed groups (Bromeliaceae, Orchidaceae, and ferns). The primary ecological role of the cuticle is to reduce water loss, primarily due to the hydrophobic

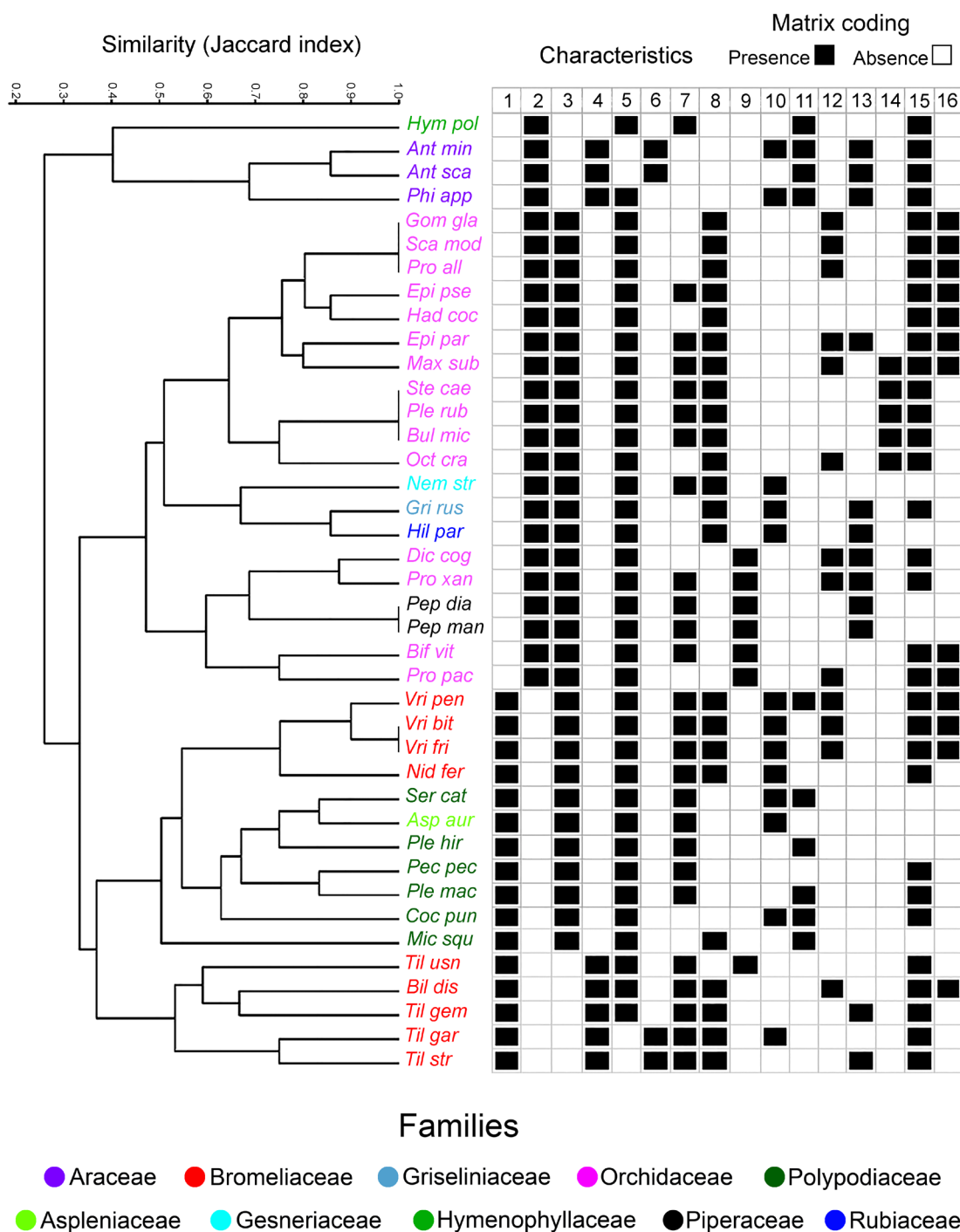


Figure 3 – Dendrogram resulting from the UPGMA analysis (Jaccard index) with qualitative characteristics of 40 species of vascular epiphytes in the dwarf cloud forests in Parque Estadual do Ibitipoca. The names of the species are composed of the first three letters of the genera and the first three letters of the epithet. Cophenetic coefficient = 0.81.

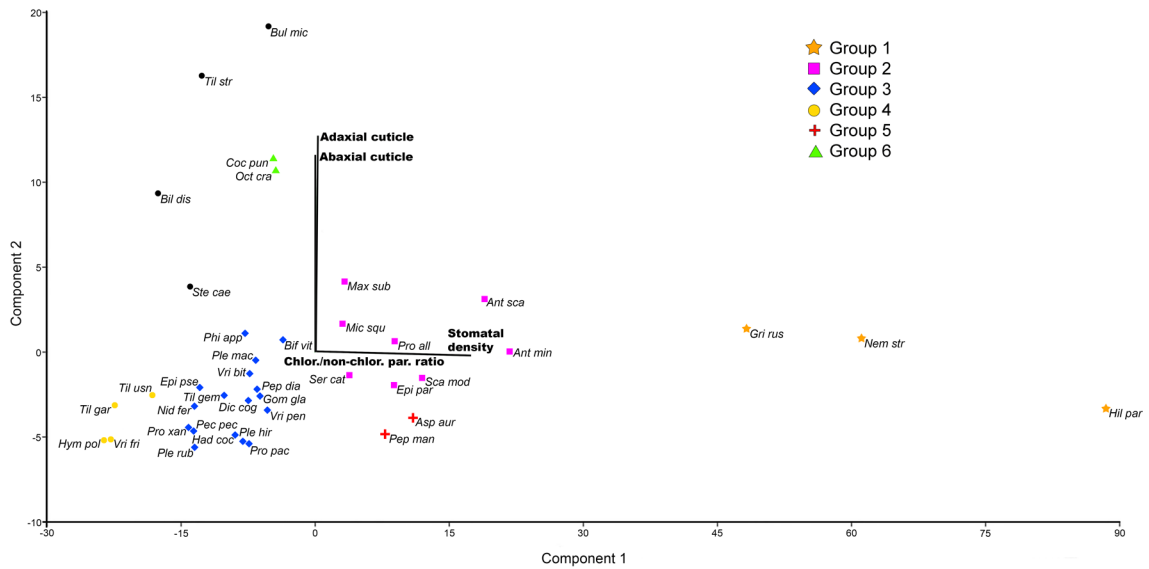


Figure 4 – PCA analysis. The names of the species are composed of the first three letters of the genera and the first three letters of the epithet. Analyzed quantitative characteristics: cuticle thickness of the adaxial and abaxial faces of the leaves, chlorophyll/non-chlorophyll parenchyma ratio, and stomatal density. The colors used in the species names are the same as those used in Figure 3.

properties of waxes (Yeats & Rose 2013), thus (along with stomatal closure) contributing to maintaining the plant’s water balance. According to Riederer & Schreiber (2001), there is no correlation between water permeability and cuticle thickness, indicating that the chemical composition

of the waxes, relative humidity, and temperature, which influence water molecule diffusion and partition coefficients, are responsible for varying the cuticle’s permeability, increasing transpiration at higher temperatures. This finding justifies the variability in cuticle thickness within the functional

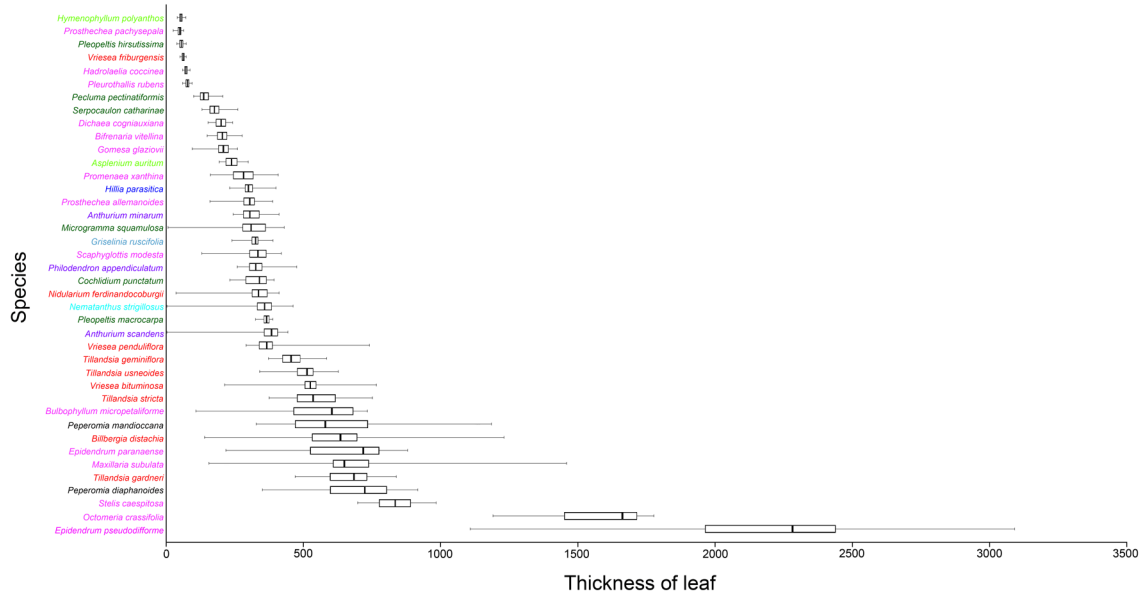


Figure 5 – Boxplot of total leaf blade thickness of the analyzed species in Parque Estadual de Ibitipoca (µm). The colors used in the species names are the same as those used in Figures 3 and 4.

group of epiphytes and explains the low expression of this trait in the quantitative analysis.

The sinuosity of the cell walls is highly pronounced in ferns, especially in *Cochlidium punctatum*, *Serpocaulon catharinae*, and *Pecluma pectinatiformis*, while the walls are straight in *Peperomia*, *Nematanthus strigillosus*, *Hillia parasitica*, *Griselinia ruscifolia*, *Hymenophyllum polyanthos*, and in all Araceae and Orchidaceae species. Some authors have discussed the adaptive value of sinuous cell walls. Watson (1942) explains that the cuticle and cell walls in leaves exposed to the sun become rigid more quickly compared to shaded leaves, where the walls remain plastic for a longer period, thus favoring undulations to develop. This observation contradicts what was observed in *H. polyanthos* collected in full shade. According to Krauss (1949), undulations increase the contact surface between cells, providing greater resistance to the epidermal tissue during leaf expansions and contractions resulting from turgor variation.

Sunken stomata, often covered by trichomes, also reflect an important adaptation to the low water availability of the epiphytic environment (Pereira *et al.* 2011). However, stomata at the same level as the other epidermal cells were observed in 31 out of the 40 species analyzed. The moisture brought by fog during certain periods of the day is a prominent characteristic of cloud forests (Hietz 2010b), enabling the occurrence of species whose leaves do not have sunken stomata. On the other hand, despite the adaptive value of this trait, the occurrence of stomata at the same level as the other epidermal cells is widely reported by various authors for ferns and orchids occurring in different environments (Bonates 1993; Silva & Milaneze-Gutierrez 2004; Silva *et al.* 2006; Noguera-Savelli & Jáuregui 2011; Arévalo *et al.* 2011; Moraes *et al.* 2011), suggesting a strong taxonomic significance of this trait, not only in terms of topography, but also stomatal type.

The presence of a water-storage hypodermis can be considered highly adaptive. In addition to storing water for photosynthetic processes during water deficits, the hypodermis acts as a light filter and provides mechanical support (Fahn & Cutler 1992; Scatena & Segecin 2005). The occurrence of multiple epidermal layers in *Peperomia* derived from periclinal divisions of the protoderm is a common feature already described in ontogenetic studies (Takemori 2002; Takemori *et al.* 2003; Souza *et al.* 2004; Evert 2006), primarily serving as a water storage tissue, as observed in

P. mandioccana and *P. diaphanoides*. Pleated cells were observed in these species, providing greater flexibility for water accumulation and loss depending on environmental conditions, indicating an important water reserve strategy (Kaul 1977; Takemori *et al.* 2003). *Octomeria crassifolia*, *Stelis caespitosa*, *Pleurothallis rubens*, *Bulbophyllum micropetaliforme*, and *Maxillaria subulata* stand out with thickening bars in their hypodermal cells. Pirwitz (1931) previously described the water storage function of these structures based on the cellulose composition of the bars and their ability to hydrate hypodermal cells. Additionally, the bars provide mechanical support for the turgor variation of the hypodermal cells and adjacent tissues (Aybeke *et al.* 2010). Stern *et al.* (2004) described thickening bars for some species of *Dichaea*, *Bifrenaria*, and *Promenaea xanthina*, although they were not observed in the individuals evaluated in the present study.

The absorptive scales observed in *Pleopeltis* and all Bromeliaceae species have different morphological types between the two groups, but coincide in terms of the water absorption mechanism through wings and the base (Tomlinson 1969; Hietz 2010a; Lagoria *et al.* 2018). The widespread distribution and morphological specialization of scales in Bromeliaceae, especially in *Tillandsia*, not only have a strong taxonomic character (Tomlinson 1969; Benzing 2000), but are also extensively related to extreme epiphytism, with functions such as water and nutrient absorption, photoprotection, and transpiration reduction (Pierce *et al.* 2001). Benz & Martin (2005) also discuss the contribution of the extensive indumentum to moisture retention on the leaf blade surface and reduced gas diffusion in epiphytic *Tillandsia* species.

Glandular trichomes were observed in seven of the 13 Orchidaceae genera studied. Benzing & Pridgeon (1983) compared the functional aspects of trichomes in Orchidaceae and Bromeliaceae species and concluded that water and mineral absorption in orchids is not as specialized and efficient as in bromeliads, suggesting that the velamen plays a primary role in water absorption in epiphytic orchids. Furthermore, all Orchidaceae species exhibited low trichome density, indicating other functions of these structures within the group.

Despite the xeromorphic characteristics attributed to epiphytes (Benzing 1990; Zotz 2016; Dias-Pereira *et al.* 2022), thin leaves with homogeneous chlorophyllous parenchyma were

observed in 50% of the analyzed species. According to Dickson (2000), light levels determine the internal leaf structure, resulting in thicker leaves with dorsiventral chlorophyllous parenchyma in species exposed to high light intensities, while shaded environments, such as epiphytic habitats, have thinner leaves with homogeneous mesophyll and abundant intercellular spaces. Among the various analyzed species, *Peperomia diaphanoides*, *Octomeria crassifolia*, and *Stelis caespitosa*, which were collected in shaded areas, have fleshy leaves with thick leaf blades. However, this greater thickness is due to the voluminous water storage tissue in these species and not to the presence of well-developed dorsiventral chlorophyllous parenchyma. According to Lambers *et al.* (1988), leaf structure plays an important role in the adaptation of these plants to shading, providing better light capture and photosynthetic efficiency.

The presence of extensive lacunar parenchyma was generally observed in species with dorsiventral mesophyll. According to Ivanova & P'yankov (2002), a higher proportion of lacunar parenchyma represents an acclimation form to shading, as these cells are adapted to absorb diffuse light, which is more prevalent in shaded conditions (Healey *et al.* 1998).

Based on the micromorphometric analysis, the chlorophyllous/non-chlorophyllous tissue ratio was found to be high in 19 out of 40 species, indicating a low investment in chlorophyllous tissue. This result demonstrates the priority of these species in developing support, storage, or water loss protection tissues rather than optimizing light capture. Therefore, it can be concluded that epiphytic species exhibit morphological and primarily physiological strategies to adapt to shading, such as rosette phyllotaxis in Bromeliaceae and CAM metabolism (Benzing 1990; Lüttge 1989).

Phenolic compounds were observed in nine of the evaluated species, including seven fern species. Some authors (Mole *et al.* 1988; Larson 1988; Mole & Waterman 1988; Dudt & Shure 1994; Ghasemzadeh *et al.* 2010) suggest that excess light intensity is the main factor for accumulating phenolic compounds, contradicting the observed presence of phenolic compounds in fern species collected in full shade in this study. It is likely that the occurrence of these compounds is also related to predation, as the fern leaves collected exhibited low predation levels compared to orchids (personal observation).

Stegmata, described by Benzing (2000) and found in nine Orchidaceae species in this study, were also reported by Sandoval-Zapotitla *et al.* (2010) in 140 species of the Oncidiinae subtribe and by Zanenga-Godoy & Costa (2003) in *Cattleya*. However, the adaptive significance as a specialization for low water availability environments and consequently epiphytism has never been elucidated, with only suggestions of water absorption capacity through silica (Kaufman *et al.* 1970; Khattab 2017), assistance in leaf support (Zanenga-Godoy & Costa 2003), and silicification in tissues with a high transpiration rate (Bennett 1982), as silica is transported via transpiration stream (Moller & Hanne Rasmussen 1984). In fact, Rasmussen (1985) questions the high occurrence of stegmata in epiphytic orchid species compared to terrestrial ones, as the stems of phorophytes are low in silica compared to the soil.

Supporting tissues such as perivascular and extravascular fibers and mechanical hypodermis were observed in most species. According to Turner (1994), sclerophylly is associated with species with long-lived leaves and low mineral concentrations, mainly N, P, and Ca, which supports the epiphytic habit since many Orchidaceae and Bromeliaceae species experience long leaf lifespans and nutrient deficits. The mechanical hypodermis observed in *Vriesea*, *Billbergia distachia*, and *Hadrolaelia coccinea* helps minimize water loss through transpiration. Furthermore, these tissues are related to support and maintenance of leaf architecture, especially when exposed to water fluctuations, as is the case with epiphytes (Lüttge 1989; Fahn & Cutler 1992).

Summing up, the answer to the title of this article is no, leaf anatomical traits do not converge in epiphytic plants. Epiphytes exhibit various adaptations related to water and nutrient economy and absorption. In comparison to terrestrial flora, they possess anatomical peculiarities that qualify them as a functional group. However, based on the observations in this study, despite the occurrence of certain features such as thick cuticle, fibers, hypodermis, and homogeneous chlorophyllous parenchyma in 50% or more species, there is no set of anatomical attributes that characterize epiphytes as a whole. In other words, the convergence of anatomical characteristics is low within the analyzed group of epiphytes, even among species of the same genus, as evidenced by the clustering analyses. This indicates that the development of adaptive strategies compatible with the epiphytic habit is

specific to each group. Many highly successful species do not exhibit typical anatomical strategies to cope with the water and nutritional conditions of the epiphytic environment. Morphological characteristics, phenology, physiological and reproductive mechanisms, in conjunction with anatomical features, can thus be identified as possible determining factors for the occurrence and adaptation of these species to the epiphytic environment.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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