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Original Paper

Leaf anatomy for delimiting Atlantic Forest species of *Psidium* (Myrtaceae)

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Abstract

Leaf anatomical features are widely used to better understand angiosperm taxonomy. However, such information is scarce for the family Myrtaceae. Aiming to fill this knowledge gap, we studied anatomical and micromorphological leaf characters of ten species of *Psidium*: *P. cattleyanum*, *P. cauliflorum*, *P. corynanthum*, *P. guajava*, *P. guineense*, *P. macahense*, *P. myrtoides*, *P. oligospermum*, *P. ovale* and *P. sartorianum*. Uniseriate epidermis, paracytic stomata, secretory cavities, and adaxial hypodermis are common among the studied species and are typical characteristics of Myrtaceae. The presence of three or more layers of palisade parenchyma and the absence of sclerenchyma are diagnostic characters of *P. guajava*. The abaxial epidermis of *Psidium cattleyanum* and *P. macahense* possess curved walls. *Psidium corynanthum* and *P. macahense* are the only species with sinuous anticlinal walls on both sides of the epidermis, with all other species having straight or curved walls. Open bicollateral vascular bundles are present in all the studied species except *P. guineense*. With the exception of *P. cauliflorum*, all the studied species possess druses. The absence of collenchyma in the adaxial face is exclusive to *P. ovale*. The leaf anatomy of *P. macahense*, *P. corynanthum*, *P. oligospermum* and *P. ovale* are described for the first time here.

Key words: anatomical features, angiosperm, quantitative characters, UPGMA, taxonomy.

Resumo

As características anatômicas foliares são amplamente utilizadas para melhor compreender a taxonomia entre as angiospermas. No entanto, tais informação são escassas para a família Myrtaceae. Com o objetivo de preencher esta lacuna de conhecimento, estudamos a anatomia foliar de dez espécies do gênero *Psidium*: *P. cattleyanum*, *P. cauliflorum*, *P. corynanthum*, *P. guajava*, *P. guineens*e, *P. macahense*, *P. myrtoides*, *P. oligospermum*, *P. ovale* e *P. sartorianum*. A epiderme unisseriada, os estômatos paracíticos, as cavidades secretoras e a hipoderme adaxial são comuns entre as espécies estudadas e são características típicas de Myrtaceae. A presença de três ou mais camadas de parênquima paliçádico e a ausência de esclerênquima são caracteres diagnósticos de *P. guajava*. A epiderme abaxial de *Psidium cattleyanum* e *P. macahense* possuem paredes curvas. Psidium corynanthum e P. macahense são as únicas espécies com paredes anticlinais sinuosas em ambos os lados da epiderme, sendo que todas as outras espécies têm paredes retas ou curvas. Os feixes vasculares bicolaterais abertos estão presentes em todas as espécies estudadas, exceto em *P. guineense*. Com exceção de *P. cauliflorum*, todas as espécies estudadas possuem drusas. A ausência de colênquima na face adaxial é exclusiva de *P. ovale*. A anatomia foliar de *P. macahense*, *P. corynanthum*, *P. oligospermum* e *P. ovale* é descrita pela primeira vez neste trabalho.

Palavras-chave: características anatômicas, angiospermas, caracteres quantitativos, UPGMA, taxonomia.

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Introduction

Psidium is the third largest genus of the angiosperm family Myrtaceae in Brazil, comprising about 65 species (Tuler *et al.* 2020). Recent phylogenetic studies have demonstrated the genus to be monophyletic (Costa 2009; Vasconcelos *et al*. 2017; Tuler *et al*. 2019), while recent taxonomic studies have improved species circumscription (*e.g.*, Landrum & Sobral 2006; Landrum & Funch 2008; Soares-Silva & Proença 2011; Landrum & Proença 2015; Landrum 2017; Tuler *et al*. 2016, 2018, 2019, 2020). However, many nomenclatural issues among species of the genus remain unclear. Although anatomical features are highly informative to taxonomic decisions among angiosperms (Metcalfe & Chalk 1979; Dickison 2000; Olson 2005), there exists little such information in the literature for species of *Psidium* (Gomes *et al*. 2009; Al-Edany *et al*. 2012; Oliveira *et al*. 2017), especially considering the species richness of the genus.

Taxonomic revisions of Neotropical *Eugenia* (Hussin *et al.* 1992; Cardoso & Sajo 2004), *Campomanesia* (Oliveira *et al*. 2011), and *Myrcia*, *Myrceugenia* and *Plinia* (González 2011), briefly assessed leaf anatomical characters and indicated their significance for the systematics and taxonomy of these genera. The characteristics found to be most useful in distinguishing species of Myrtaceae are leaf venation pattern, vascular arch shape, presence and types of trichomes, form and arrangement of epidermal cells, and presence of secretory cavities.

Relatively few leaf anatomical studies have included species of *Psidium* (see Soares-Silva & Proença 2008; Gomes *et al*. 2009; Al-Edany *et al*. 2012; Oliveira *et al*. 2017). Amongst leaf anatomical characters common within the genus are uniseriate epidermis; hypostomatic leaves (amphistomatic in *P. ratterianum*; Proença *et al*. 2010), paracytic stomata and abundant tector trichomes on the abaxial surface and rare on the adaxial surface. Furthermore, the mesophyll is dorsiventral with one to three layers of palisade parenchyma and a compact arrangement of spongy parenchyma; the hypodermis is adaxial and formed by two continuous layers; the vascular bundle of the midvein is bicollateral and protected by lignified fibers; and subepidermal secretory cavities and prismatic crystals are present throughout the mesophyll.

Herein, we describe anatomical and micromorphological leaf characters of ten species of *Psidium* found in the Atlantic Forest of Brazil to determine whether anatomy can provide useful information for species taxonomy. Based on previous anatomical studies within Myrtaceae, we hypothesized that the arrangement of epidermal cells, vascular arch shape and the presence and type of trichomes to be the most distinctive characters among, but invariable within the studied species. Finally, we submitted the data to multivariate analysis to better determine those features that explain differences among species.

Material and Methods

The taxonomic sampling of this study included ten species of *Psidium* that occur in the Atlantic Forest of Brazil: *P. cattleyanum* Sabine, *P. cauliflorum* Landrum & Sobral, *P. corynanthum* (Kiaersk.) Burret, *P. guajava* L., *P. guineense* Sw., *P. macahense* O. Berg, *P. myrtoides* O. *Berg*, *P. oligospermum* Mart. *ex* DC., *P. ovale* (Spreng.) Burret., and *P. sartorianum* (O.Berg) Nied. (Tab. 1).

Study material was obtained from herbarium samples (VIES). Analyses were performed with completely expanded leaves of three different individuals per species (except to *Psidium cauliflorum*, *P. oligospermum*, *P. sartorianum* and *P. corynanthum*). The leaf samples were submitted to the reverse herborization process (Smith & Smith 1942) and boiled in distilled water for (ca.) 5 minutes. After cooling down to room temperature, the materials were distended in a 2% potassium hydroxide (KOH) solution (Smith & Smith 1942) for 2 hours, then washed 3 times in distilled water for 50 minutes, and stored in 70% ethanol (Johansen 1940).

For analysis of the type of epidermis and stomata, the samples underwent diaphanization, for which they were clarified with 50% bleach and stained with alcoholic safranin (Johansen 1940). The slides were mounted with glycerinated gelatin (Kaiser 1880) and sealed with colorless enamels (Kraus & Arduin 1997).

For the mesophyll analysis, transverse free-hand sections were cut from the apex, middle, and basal regions of the leaf blade, which were clarified in 50% sodium hypochlorite and stained with astrablue/safranin (9:1%) for 5 seconds. The sections were then mounted on slides using

Table 1 – The studied species of *Psidium* used in the epidermis analyses, and their respective collectors and collection numbers for the herbarium samples from which study material was obtained.

Species	Collector / collector number
Psidium macahense O. Berg	*Souza PF 192*
	Gomes JML 1224
	Thomaz LD 697
Psidium cattleyanum Sabine	*Vervloet RR 1413*
	Thomaz LD 1442
	Pereira OJ 2888
Psidium cauliflorum Landrum & Sobral	*Tuler AC 485*
	Gomes JML 3198
Psidium guajava L.	*Tuler AC 428*
	Couto DR 1007
	Thomaz LD 1894
Psidium guineense Sw.	*Silva LA 288*
	Tuler AC 548
	Dutra VF 812
Psidium myrtoides O.Berg	*Tuler AC 451*
	Kollmann LJC 5118
	Kollmann LJC 776
Psidium oligospermum Mart. *ex* DC.	*Silva LA 106*
Psidium ovale (Spreng.) Burret	*Demuner V 920*
	Fernandes T 398
	Kollmann LJC 5338
Psidium sartorianum (O.Berg) Nied	*Folli DA 376*
Psidium corynanthum (Kiaersk.) Burret	*Tuler AC 507*

glycerinated gelatin and sealed with colorless nail polish (Kraus & Arduin 1997). Morpho-anatomical analysis was performed using Leica DM2500 equipment with Leica MC170 HD image capture attached. The classification of structures followed the terminology of Metcalfe & Chalk (1979).

Data analysis

A total of 31 leaf anatomical characters were used for statistical analysis (Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.22253401.v1>). A matrix was constructed using qualitative morphological (n = 23) and quantitative leaf characters (n = 8). Qualitative morphological character states were coded as 0 or 1 for absence and presence, respectively, since individuals of same species can possess different states. Each quantitative character represented the average of five measurements were expressed as recorded (Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.22253401.v1>).

The analysis was divided into three approaches. (1) Gower's dissimilarity coefficient (Legendre & Legendre 2012) is used for mixed data and was applied here since it does not consider the absence of data as evidence of dissimilarity between taxa. (2) Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA), with the Mantel test being used to compare the relationship between the original matrix and the distance matrix (Borcard *et al*. 2018; Legendre & Legendre 2012). (3) A heat map (Hummel *et al*. 2017) was generated to represent clusters through the association between variables and species, represented on a numerical scale ranging from 0 (no association) to 1 (association), with stronger associations having darker colors. The heat map was produced using the distance matrix obtained with the Gower method and the morphological data matrix. All data analyses were developed in the R environment (R Core Team 2020), using the packages Vegan (Oksanen *et al*. 2020) and Cluster (Maechler *et al*. 2020).

Results

The matrix of morphological character states (Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.22253401.v1>) is descriptive and provides a summary of the examined characters.

Leaf morphoanatomy

Psidium guajava, *P. guineense* and *P. myrtoides* have non-ramified unicellular tector trichomes (Fig. 1a) on both sides of the leaf (Fig. 1a); *P. cattleyanum*, *P. cauliflorum* and *P. sartorianum* only have non-ramified unicellular tector trichomes on the adaxial surface; *P. oligospermum*, *P. corynanthum*, *P. ovale* and *P. macahense* did not present trichomes or are glabrescent.

In adaxial view, the epidermal cells of *P. guajava* and *P. guineense* have straight walls on both sides (Fig. 1b); *P. cattleyanum* has straight to curved walls on the adaxial surface and straight walls on the abaxial surface (Fig. 1c); *P. sartorianum* and *P. ovale* have straight walls on the adaxial surface (Fig. 1d-e) and straight slightly sinuous to sinuous walls on the abaxial surface; *P. oligospermum* and *P. myrtoides* have straight walls on the adaxial surface, and straight to slightly sinuous walls on the abaxial surface; *P. corynanthum* has slightly sinuous and sinuous anticlinal walls on both sides of the epidermis; *P. cauliflorum* has straight sinuous walls on the adaxial surface and slightly sinuous to sinuous walls on the abaxial surface; *P. macahense* has sinuous walls on the adaxial surface and curved to sinuous walls on the abaxial surface. All the analyzed leaves are hypostomatic and have paracytic stomata (Fig. 1f).

Mesophyll and midrib region

In transverse section, all the studied species have a uniseriate epidermis (Fig. 2). Below the epidermis on the adaxial face are two to three layers of cells with anticlinal walls that are higher than the other cells in the epidermis (Fig. 2a-d). The mesophyll is dorsiventral with one to three layers of palisade parenchyma and a compact arrangement of spongy parenchyma (Fig. 2a-d). *Psidium oligospermum* and *P. myrtoides* have one layer of palisade parenchyma (Fig. 2c); *P. ovale* varies between one and two layers (Fig. 2d); *P. corynanthum*, *P. guineense*, *P. cauliflorum* and *P. sartorianum* have two layers; and *P. cattleyanum*, *P. guajava* and *P. macahense* have three (Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.22253401.v1>).

All the studied species have secretory cavities in the epidermis (Fig. 2a-b,e,g) and prismatic crystals associated with the vascular bundle. Druses are present in the mesophyll of all species (Fig. 2a-b) except for *P. cauliflorum*.

The shape of the midrib of all the studied species is flat-convex (Fig. 2e). Some layers of angular collenchyma are found in both faces of all species (Fig. 2f) except for *P. ovale*, for which they are on the abaxial face. The collenchyma thickness varies among species (Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.22253401.v1>). Open bicollateral vascular bundle are present in all species (Fig. 2e) except for *P. guineense* it is closed (Fig. 2f).

Cluster analysis (UPGMA) and heat map

Cluster analysis based on presence/absence data (Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.22253401.v1>), followed by the partition test, revealed two distinct groups. Group 1, the larger group, includes *P. cattleyanum*, *P. macahense*, *P. corynanthum*, *P. cauliflorum*, *P. sartorianum*, *P.ovale*, *P. oligospermum* and *P. myrtoides*, whereas Group 2 includes *P. guajava* and *P. guineense* (Fig. 3).

A binary data matrix of the qualitative and quantitative leaf characters was constructed and submitted to grouping analysis for the ten species (horizontal grouping) and for the characters (vertical grouping) in the heat map (Fig. 4). The resulting grouping of 31 characters (Fig. 4, vertical) revealed a group of characters conserved in all or almost all species.

The more variable characters were quantitative (epidermis, palisade parenchyma, spongy parenchyma, collenchyma, and hypoderm thickness), as represented in the heat map (Fig. 4).

The heat map showed identical profiles among the qualitative characters. Thus, no significant differences were found among species (number of layers of epidermis, presence of cuticle, presence of prismatic crystals, presence of secretory cavities, midrib flat-convex, bicollateral vascular bundles, paracytic stomata and hypostomatic leaves), while others have almost identical profiles but with exclusive characteristics in relation to the other species. *Psidium guajava*, *P. cattleyanum* and *P. macahense* are the

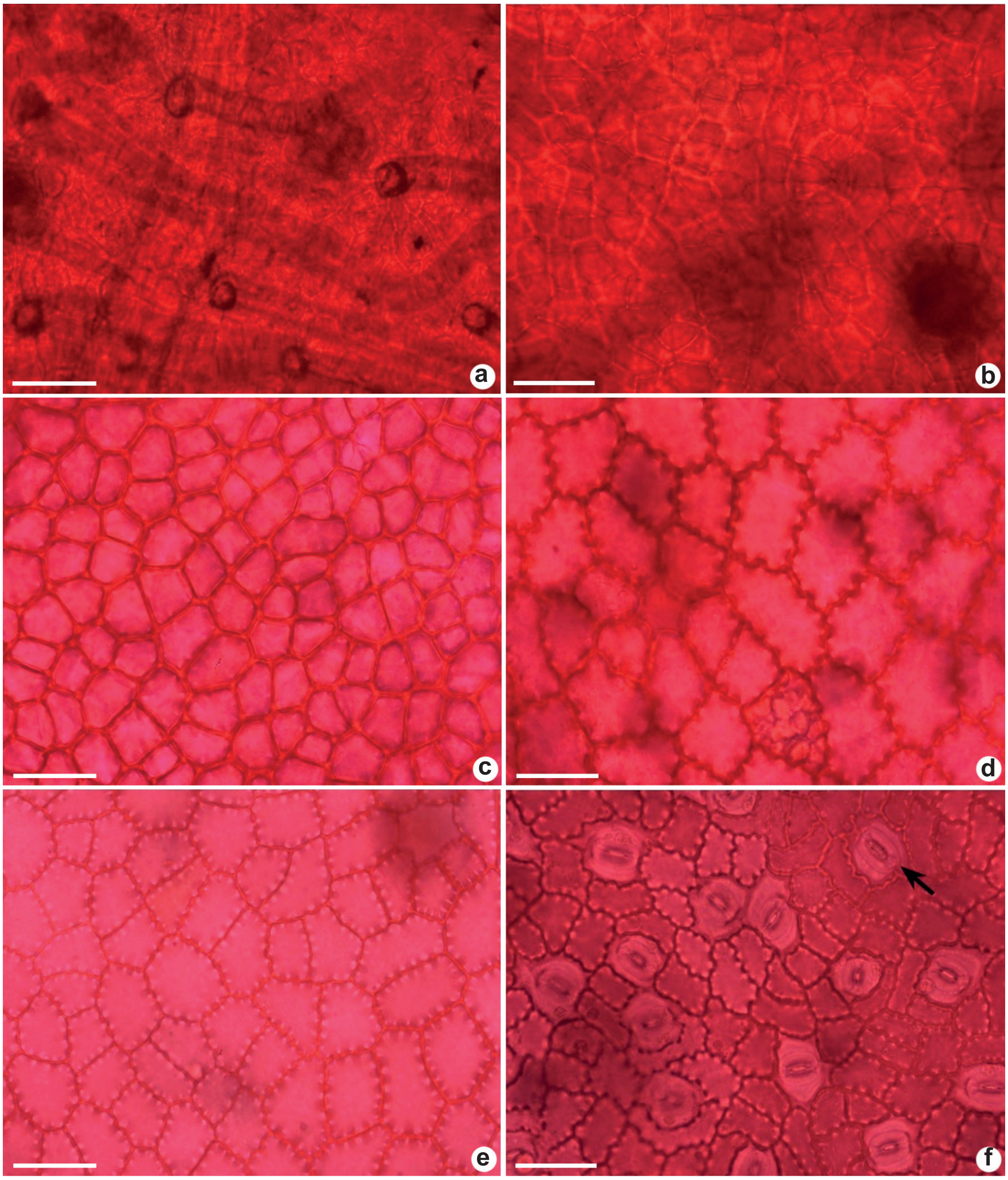

Figure 1 – a-f. Epidermal cells (front view) of species of *Psidium* – a-b. *Psidium guajava* – a. abaxial surface with non-ramified unicellular tector trichomes and straight anticlinal cell walls; b. adaxial surface with non-ramified unicellular tector trichomes and straight anticlinal cell walls; c. *P. cattleyanum* – adaxial surface with straight anticlinal cell walls; d-e. *P. ovale* – adaxial surface with sinuous walls; f. *P. myrtoides* – abaxial surface with paracytic stomata (arrow) and sinuous walls, adaxial surface with straight walls. Scale bars = 50 µm.

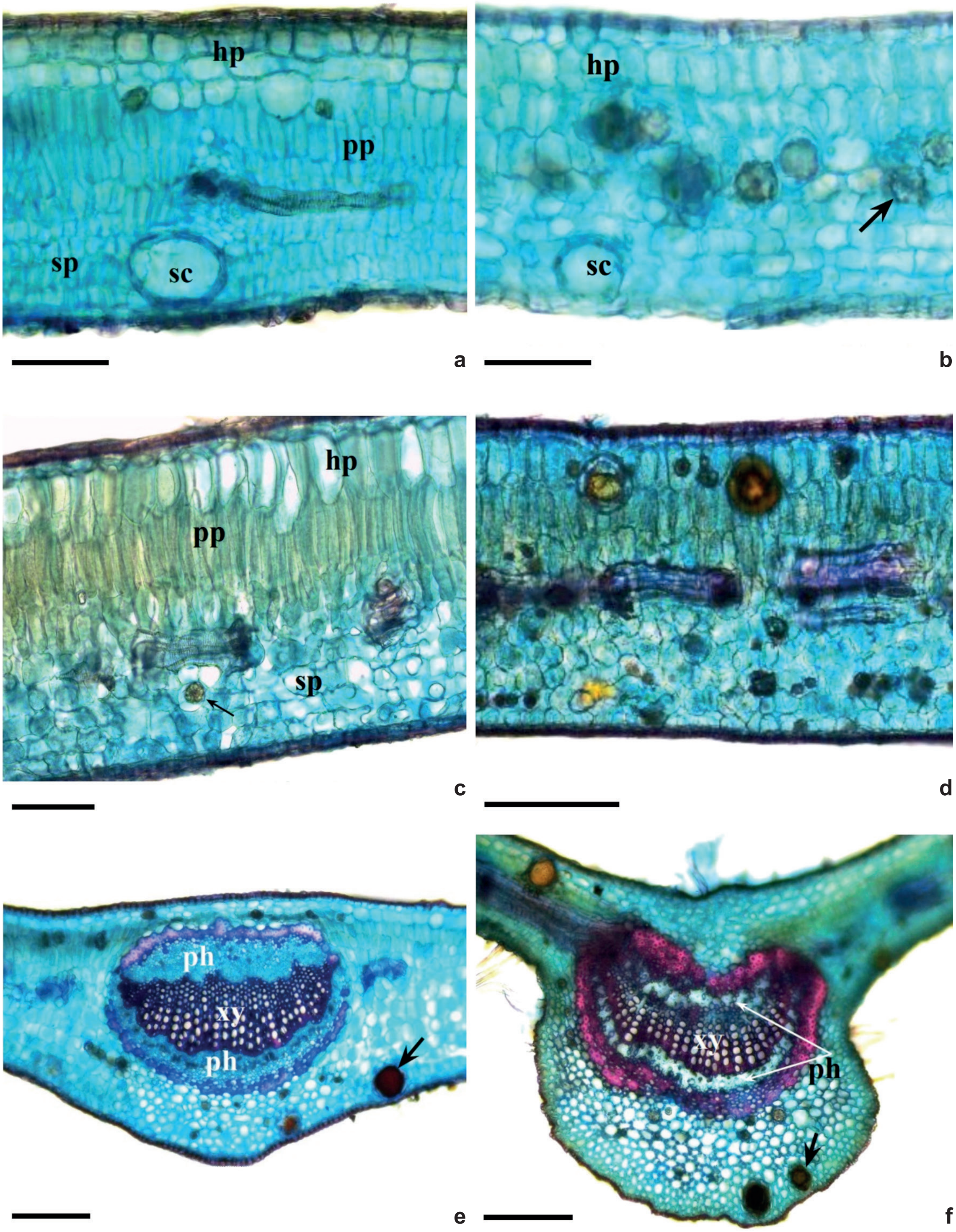

Figure 2 – a-f. Cross sections of leaf blade – a. *Psidium guajava* – three subepidermal layers (hypodermis) and three layers of palisade parenchyma and spongy parenchyma, secretory cavity in the abaxial face; b. *P. sartorianum* – two subepidermal layers (hypodermis), druse (arrow) and prismatic crystals; c. *P. oligospermum* – uniseriate hypodermis, druse (arrow); d. *P. ovale* – dorsiventral mesophyll, secretory cavity (arrow); e. *P. myrtoides* – vascular cylinder in the midrib region; f. *P. guineense* – detail of closed bicollateral vascular bundle (arrows). (hp = hypodermis; sp = spongy parenchyma; ph = phloem; pp = palisade parenchyma; sc = secretory cavity; xy = xylem. Scale bars: a-c = 100 µm; d, e, f = 200 µm.

only species to have three or more layers of palisade parenchyma. The absence of sclerenchyma is an exclusive characteristic of *P. guajava*.

Psidium cattleyanum, *P. guajava* and *P. guineense* have two or more layers of hypodermis on the adaxial face, whereas all other species have a single layer of hypodermis. *Psidium cattleyanum* and *P. macahense* have curved walls on the abaxial epidermis (Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.22253401.v1>).

Psidium oligospermum, *P. corynanthum*, *P. ovale* and *P. macahense* lack non-ramified unicellular tector trichomes in the adaxial epidermis, whereas all other species have them present. *Psidium corynanthum* and *P. macahense* are the only species that have slightly sinuous and sinuous anticlinal walls on both sides of the epidermis, with all other species having straight or curved walls.

Open bicollateral vascular bundle is present for all species except for *P. guineense*. All the studied species possess druses except *P. cauliflorum*. All species also have bicollateral collenchyma except *Psidium ovale*, which lacks it adaxially (Tab. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.22253401.v1>).

Discussion

Uniseriate epidermis, cuticle, hypodermis in the adaxial side, paracytic stomata and secretory cavities were found in all species of *Psidium* studied here and are common within other genera of Myrtaceae (Machado *et al*. 1987; Medeiros 2000; Donato & Morretes 2009, 2011, 2013; Pacheco-Silva & Donato 2016; Sá *et al*. 2016).

Hypostomatic leaves (amphistomatic in *P. ratterianum*; Proença *et al*. 2010), and paracytic stomata are common within Myrtaceae, having been reported in *Eugenia*, (Behar 1971; Palhares 2003; Alves *et al*. 2008; Donato & Morretes 2009), *Myrcia* (Gomes *et al*. 2009; Donato & Morretes 2011) and *Plinia* (Donato & Morretes 2013) and all the species of *Psidium* studied here. Our results confirm literature records of hypostomatic leaves, thus increasing the number of studied species. This trait was also found in species adapted to the interior of the Atlantic Forest (Barros *et al*. 1997) and is recognized as a strategy related to luminosity and periods of water scarcity by reducing transpiration rates (Dickison 2000).

Dorsiventral mesophyll is typical of the family Myrtaceae (Keating 1984) and was found in most of the species of *Psidium* studied here. Duarte & Paula (2005) and Gomes *et al.* (2009) observed this same pattern of subepidermal cells in *P. guajava* and other species of Myrtaceae, respectively, and

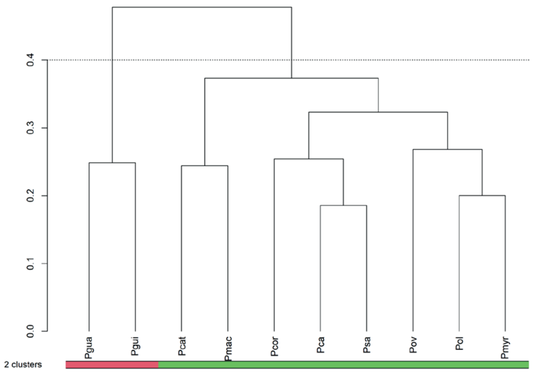

Figure 3 – Grouping of species of *Psidium* based on 31 leaf anatomical characters. (Pca = *P. cauliflorum*; Pcat = *Psidium cattleyanum*; Pcor = *P. corynanthum*; Pgua = *P. guajava*; Pgui = *P. guineense*; Pmac = *P. macahense*; Pmyr = *P. myrtoides*; Pol = *P. oligospermum*; Pov = *P. ovale*; Psa = *P. sartorianum.*

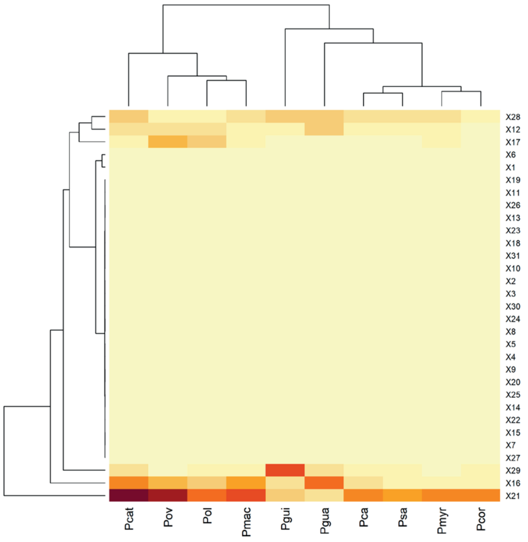

Figure 4 – Heat map of 31 leaf anatomical characters of *Psidium.* The lighter colors (orange) represent weaker associations, darker colors (edge) represent stronger associations. (Pca = *P. cauliflorum*; Pcat = *Psidium cattleyanum*; Pcor = *P. corynanthum*; Pgua = *P. guajava*; Pgui = *P. guineense*; Pmac = *P. macahense*; Pmyr = *P. myrtoides*; Pol = *P. oligospermum*; Pov = *P. ovale*; Psa = *P. sartorianum.*

characterized them as hypodermis. However, the authors cite the need for ontogenetic study of this tissue to know its origin since, unlike epidermal cells, which originate from the protoderm, cells of the hypodermis originate from ground tissue (Esau 1965).

Secretory cavities are abundant in all tissues and have variable dimensions. Volatile oils produced by secretory cavities in Myrtaceae have been identified as flavonoids and terpenoids (Wollenweber *et al*. 2000; Díaz-de-Cerio *et al*. 2017). They are important in the interaction between plants and their biotic environment, as they are involved in defense against herbivory (Van Poecke *et al*. 2001; Matsuki *et al*. 2011) and pathogens (Levin 1976), in addition to attracting pollinators (Pichersky & Gershenzon 2002).

Druses occur throughout the palisade and spongy parenchyma of the studied species except for *P. cauliforum*. Druses are widely present in a diverse array of vegetative and reproductive structures in several genera of Myrtaceae, (Donato & Morretes 2007; Alves *et al*. 2008; Cardoso *et al*. 2009; Gomes *et al*. 2009). The presence of small crystals in vascular plants is related to the removal of oxalate from the metabolic system and the storage of calcium (Franceschi & Nakata 2005; Korth *et al*. 2006).

Multivariate analysis revealed quantitative micromorphological leaf characteristics that were informative in differentiating almost all the studied species.

Group 1 gathered the largest number of species and although they share many characteristics they still have unique features that can be used for identification. Group 2 includes *Psidium guajava* and *P. guineense*, which are characterized by the combination of three layers of hypodermis on the adaxial face, tector trichomes on the abaxial face and the absence of an open vascular bundle. In addition, *P. guajava* is the only species that does not possess sclerenchyma, while the vascular bundles of *P. guineense* are completely circled by sclerenchyma.

The quantitative anatomical characters studied here varied more among species than the qualitative characters, making them more useful delimiting the genus. Phenotypic plasticity may have contributed to the variation found in tissue thickness, although this cannot be stated with certainty as no studies have evaluated this factor.

Quantitative leaf anatomical characters showed potential to segregate species of *Psidium*. Incorporating these morpho-anatomical findings into future studies, particularly phylogenetic analyses, may provide a better understanding of the evolution of the genus. Further studies, including a broader sampling of species, are needed towards this possibility.

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