



Comparative leaf anatomy of neotropical *Stylogyne* species (Myrsinoideae – Primulaceae)

Anatomia foliar comparada de espécies neotropicais de Stylogyne (Myrsinoideae – Primulaceae)

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Abstract

Anatomical studies were performed here in order to provide diagnostic characteristics to differentiate the species *Stylogyne depauperata*, *S. pauciflora*, *S. sordida* and *S. warmingii*. Fully expanded leaves were processed by the usual techniques of optical microscopy and scanning electron microscopy. Traits common to all species were observed, such as dorsiventral mesophyll, unistratified epidermis, anisocytic stomata, druses and secretory cavities distributed throughout the mesophyll. Cuticular ornamentation, configuration of the vascular system in the petiole and shape of the secretory cavities provide diagnostic characteristics. Variance analysis proved that these characters are potentially efficient to differentiate these species.

Key words: *Ardisia*, secretory structures, taxonomy, Myrsinaceae.

Resumo

Estudos anatômicos foram realizados com o objetivo de buscar caracteres diagnósticos para diferenciar *Stylogyne depauperata*, *S. pauciflora*, *S. sordida* and *S. warmingii*. Folhas totalmente expandidas foram submetidas aos procedimentos usuais em microscopia óptica e eletrônica de varredura. As espécies analisadas apresentam mesofilo dorsiventral, epiderme uniestratificada, estômatos anisocíticos, idioblastos com cristais em drusas e cavidades secretoras dispersas pelo mesofilo. Caracteres diagnósticos são a ornamentação cuticular, disposição do sistema vascular no pecíolo e forma das cavidades secretoras. As análises de variância reforçam a eficiência desses caracteres para a segregação das espécies.

Palavras-chave: *Ardisia*, estruturas secretoras, taxonomia, Myrsinaceae.

Introduction

The Neotropical *Stylogyne* A.DC. comprises 18 species in Brazil (Carrijo *et al.* 2012) distributed in Amazon and Atlantic Rain Forests. The nine members of Atlantic Rain Forest are shrubs with leaves generally punctuated, small 4(5)-merous flowers, and brightly colored fruits (Carrijo & Freitas 2008). The six species with 4-merous flowers seems to be a natural group, characterized by a high frequency of local endemism and low tolerance to environmental disturbance (Carrijo & Freitas 2008, 2009; Carrijo *et al.* 2011). Some of these species are circumscribed by fine characters (e.g. calyx papillose, anthers opening by short or long slits) or by a set of shared features (e.g. inflorescence racemose or fasciculate, petals punctuate), which sometimes makes it difficult

to distinguish related taxa from extremes of infraspecific variation. Anatomical traits proved to be a value tool to delimit taxonomically related species, to provide a consistent foundation for phylogenetic studies, and other ecological applications of species from the Atlantic Rain Forest (Barros & Callado 1997). However, some data on anatomical aspects are available in the literature for Myrsinoideae species, especially for the genus *Stylogyne* (Grose 1908; Otegui 1986; Carrijo *et al.* 2011).

Grose's monograph (1908) provides an overview of the anatomic traits in Myrsinoideae, in order to characterize the genera. More recent contributions have dealt with the description of the trichomes and crystals of *Ardisia* Sw. species (Lersten 1977), and the development of secretory cavities of *Lysimachia nummularia* L. (Lersten 1986). Other

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studies were conducted in order to clarify the limits among species within a genus. By the analysis of features such as venation, trichomes and stomata types, aspects of epidermis and cuticle, and the organization of vascular tissues in the petiole, Otegui (1998) was able to characterize related species of *Myrsine* L. The anatomical aspects of leaves, specifically epidermis and hydathodes, were also useful to delimit a new species from its related taxa (Carrizo et al. 2011). The wood anatomy also proved to be informative for Myrsinoideae systematics by the studies of Lens et al. (2005), in which wood anatomy was used to distinguish families.

Stylogyne depauperata Mez, *S. pauciflora* Mez, *S. sordida* Mez and *S. warmingii* Mez belong to the group of species with 4-merous flowers from Atlantic Rain Forest that is distinguishable by fine traits (see Carrizo et al. 2012). Differences among them are expressed mainly by leaf shape and size, which are attributes known to be influenced by the environment. Based on the premise that anatomy traits are potentially informative for Myrsinoideae systematics, the leaf anatomy of these species was analyzed in order to identify micromorphological characters useful to distinguish and delimit them.

Material and Methods

Fully expanded leaves from the third and fourth node were collected from three individuals of each species in different areas of Atlantic Rain Forest in Rio de Janeiro and Minas Gerais states, Brazil. Parque Estadual da Floresta da Tijuca (22°25'–23°01'S, 43°12'–43°19'W) and Parque Estadual da Pedra Branca (22°50'–23°15'S, 43°20'–43°40'W) are located in Rio de Janeiro state. Parque Nacional da Serra dos Órgãos (22°25'–22°32'S, 42°59'–43°07' W) is located in Teresópolis, Rio de Janeiro state, and Fazenda Fortaleza is a private property in Divino, Carangola, Minas Gerais state. All sites are characterized as ombrophilous forest, according to Veloso et al. (1991) classification.

Collection data of studied material are included in the Herbarium RB (Tab. 1).

For light microscopy (LM), samples from the leaf blades, midribs and distal portion of petioles were fixed in an aqueous solution of 2.5% glutaraldehyde and 4.0% paraformaldehyde in 0.05 M cacodylate buffer at pH 7.2 (Klein et al. 2004) or in formalin-acetic acid-alcohol 70% (FAA) (Johansen 1940) for 48 hours, and then dehydrated in a graded series of alcohol solution (10–100%, 1 hour each). Subsequently, the samples were infiltrated and embedded in methacrylate resin. Transverse sections (4 µm) were made using a rotatory microtome and were stained with toluidine blue (0.1 % aqueous solution) (Pearse 1968). The epidermis was dissociated using the solution of Franklin (1945) stained with 1% safranin and mounted in glycerol 50%. Histochemical tests were performed on fresh leaves sectioned by the free-hand method. Hydrochloric phloroglucin was used to identify secondary lignified walls (Sass 1951), lugol to starch (Sass 1951), Sudan IV to lipophilic substances (Sass 1951), and ferric chloride to phenolic compounds (Johansen 1940). Leaf calcium oxalate crystals were tested by insolubility in acetic acid and solubility in hydrochloric acid (McLean & Cook 1958). The images were obtained by using a digital camera Coolsnap attached to an Olympus BX50 microscope with the aid of the image analysis software Image Pro-Plus (4.0, version for Windows). For scanning electron microscopy (SEM), fragments of leaf blade were fixed in the same way as LM. After that, they were rinsed three times with the buffer and post-fixed for 1 hour with 1.0% osmium tetroxide in 0.05M cacodylate buffer at pH 7.2 (Klein et al. 2004). The post-fixed samples were dehydrated in a cetic series. Subsequently, they were critical-point-dried in CO₂, sputter coated with 20 nm gold, and observed with a scanning electron microscope Zeiss EVO 40. The epidermal microcharacters (cuticular ornamentation, trichomes and stomata) were described following Metcalfe and Chalk (1979).

Table 1 – Species of *Stylogyne* studied and collection data

Species	Collection data	Collector, number and herbarium
<i>Stylogyne depauperata</i> Mez	Parque Nacional da Floresta da Tijuca (22° 25'–23°01' S, 43° 12'–43° 19' W)	T.T. Carrizo 1006 – RB
<i>Stylogyne pauciflora</i> Mez	Teresópolis, Parque Nacional de Serra dos Órgãos (22°25'–22°32'S, 42°59'–43°07' W)	T.T. Carrizo 289 – RB
<i>Stylogyne sellowiana</i> Mez	Grumari, Parque Estadual da Pedra Branca (22°50'–23°15' S, 43°20'–43°40' W)	T.T. Carrizo 136 – RB
<i>Stylogyne warmingii</i> Mez	Divino, Carangola, Minas Gerais (MG). Fazenda Fortaleza	L. S. Leoni 6697 – RB

A binary matrix with the data obtained was done using absence (0) and presence (1). The matrix was subjected to cluster analysis using the Jaccard index as a measure of similarity and the unweighted arithmetic average clustering criterion (UPGMA) between sampling units. This analysis was performed on PAST. A factor analysis by principal components method (PCA) was also performed, which allowed the definition of the characteristics that most influenced the differentiation of species, influencing the spatial model produced. The PCA was performed with eight variables out of 14. The other variables were excluded from analyses because they did not present variation nor had significant values. The eight variables used are: adaxial epidermis with striate surface, circular secretory structure, translucent secretory structure, abaxial epidermis with straight periclinal wall, abaxial epidermis with a convex periclinal wall, nigrescent secretory structure, secretory structures with projections through the mesophyll, brachysclereids in petiole.

Results

The petiole has a uniseriate epidermis, in cross section formed by small, packed, circular shaped cells. Under the epidermis, angular collenchyma formed by 5–6 cell layers was found (Fig. 1a). Starch grains were detected in medulla and cortex (Fig. 1b), except in *S. pauciflora* where a starch sheath surrounding the vascular system was observed (Fig. 1c). Idioblasts containing calcium oxalate prismatic crystals or druses and secretory cavities were observed in the cortex (Fig. 1d). Secretory cavities are formed by epithelial cells filled with phenolic compounds; these cells are responsible for producing the secretion, which is stored in the lumen. The secretion has a brownish color and contains lipophilic compounds (Fig. 1e). In *S. sordida*, isolated or grouped brachysclereids distributed in the cortex were also visualized (Fig. 1g). The vascular system was formed by collateral vascular bundles. In *S. sordida*, they were arc-shaped (Fig. 1f), whereas in *S. depauperata*, *S. warmingii*, and *S. pauciflora*, “V” shaped with convoluted extremities (Fig. 1h). Phloroglucin histochemical test showed that at the distal portion the vascular system are formed by parenchymatic cells in *S. pauciflora* and *S. sordida* (Fig. 1i), while in *S. warmingii* these sheaths are surrounded by sclerenchymatic fiber with thickened walls (Fig. 1j).

The leaf blade of all species, in frontal view presents an epidermis with isodiametric cells with wavy, thin anticlinal cell walls (Fig. 2a). Cross sections of leaf blades showed a uniseriate epidermis (Figs. 2b–c). The abaxial surface of *S. pauciflora* and *S. sordida* had cells with convex outer periclinal cell walls (Fig. 2b), whereas in *S. depauperata* and in *S. warmingii* they were straight (Fig. 2c). Anisocytic stomata were only found on the abaxial surface, at the same level as the other epidermal cells. There are peltate glandular trichomes on both epidermal surfaces, consisting of a basal cell, a short stalk cell, and a large multicellular head (Figs. 2d–e). On the abaxial surface of *S. depauperata* and *S. pauciflora*, the epidermal cells had a cuticular striation parallel to the longest axis of the cell. In *S. sordida* striations were small, less evident, and were restricted to the periphery of the epidermal cells (Figs. 2f–g). Around the stomata, there are epicuticular ornamentations forming concentric rings, being more numerous in *S. depauperata*, *S. warmingii*, and *S. pauciflora*. Surrounding the trichomes, epidermal cells were radially arranged in all species. In all species the mesophyll was dorsiventral (Fig. 3a). Parenchymatic tissue situated near the adaxial epidermis presented small cells with invaginations, characterizing a plicade parenchyma (Fig. 3b). The spongy parenchyma was composed of about six cell layers. In all species, cells in this tissue had different proportions, producing intercellular spaces of different sizes. There were secretory cavities distributed throughout the mesophyll (Fig. 3c). In frontal view, these structures can be elongated or round, and were composed of an epithelial cell layer, bordering a lumen (Fig. 3d). Epithelial cells were elongate and formed a branched structure in *S. warmingii* (Fig. 3e–f). As in the petiole, the secretion is composed of lipophilic and phenolic substances. In fresh leaves, this secretion was translucent, whereas in fixed leaves it had a yellowish color. The only exception is *S. warmingii*, where it was nigrescent in fresh leaves. The presence of idioblasts of druse calcium oxalate crystals in the parenchymatic tissues of the leaf blade is a common character of all analyzed species (Fig. 3g).

Data analysis generated from the binary matrix of presence and absence (Tab. 2) showed the potential value of the anatomical characters to separate the studied species, as is observed in UPGMA cluster analysis, which divided the four

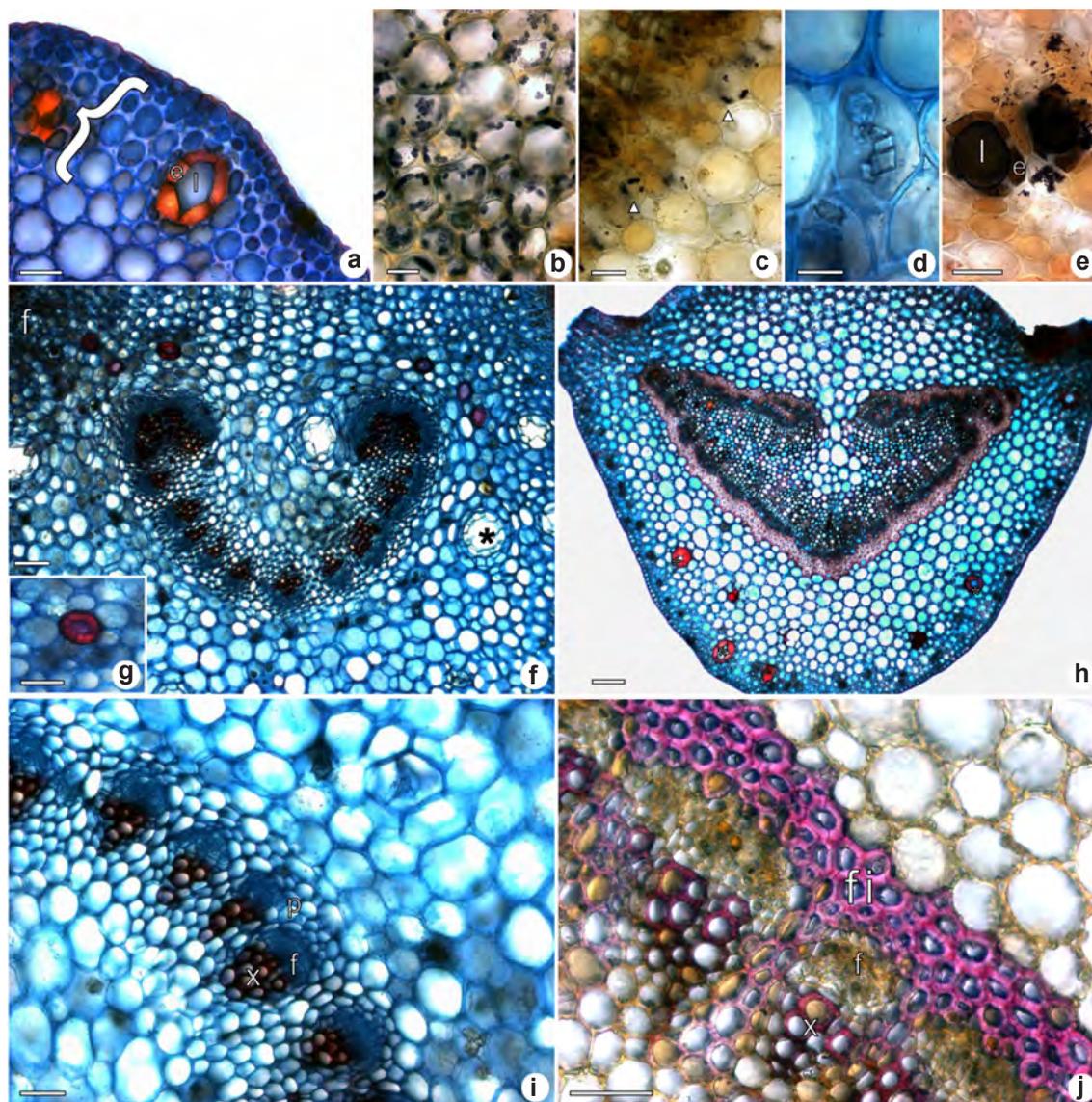


Figure 1 – Anatomical aspects of *Stylogyne* petioles – a. layers of collenchyma and secretory cavity in *S. warmingii* cortex, (e) – epithelial cell, (l) – lumen. Bar = 20µm. b, c. starch grains in amiloplasts in the cortex of *S. sordida* and *S. pauciflora*, respectively. Bar = 50µm. d. prismatic crystals in *S. sordida*. Bar = 50µm. e. secretory cavity, with the secretory content in the lumen (l) surrounded by epithelial cells (e) in *S. sordida* cortex. Bar = 100µm. f. vascular system arc-shaped in *S. sordida* petiole. Bar = 100µm. g. brachysclereids in *S. sordida*. Bar = 100µm. h. vascular system with a “V” configuration in *S. warmingii*, (*) – secretory cavities. Bar = 100µm. i. vascular system in *S. sordida*, with parenchymatic sheath cells (p) and j. in *S. warmingii* with sclerenchymatic sheath cells (fi); (x) – xylem, (f) – phloem. Bar = 50µm.

Stylogyne species in three groups (Fig. 4). The first group was formed by *S. depauperata*, *S. pauciflora*, and *S. sordida* because of the translucent cylindrical or round secretory cavities in the mesophyll. The second group included *S. sordida* and *S. pauciflora* which presented an abaxial epidermis with convex periclinal walls in cross section. The

third group included *S. warmingii*, because of its internal nigrescent branched secretory cavities (Fig. 4). Factor analyses by principal components indicated that the features that influenced the species bidimensional distribution explaining 85% of variation were: adaxial epidermis with striated surface, round secretory cavities, branched

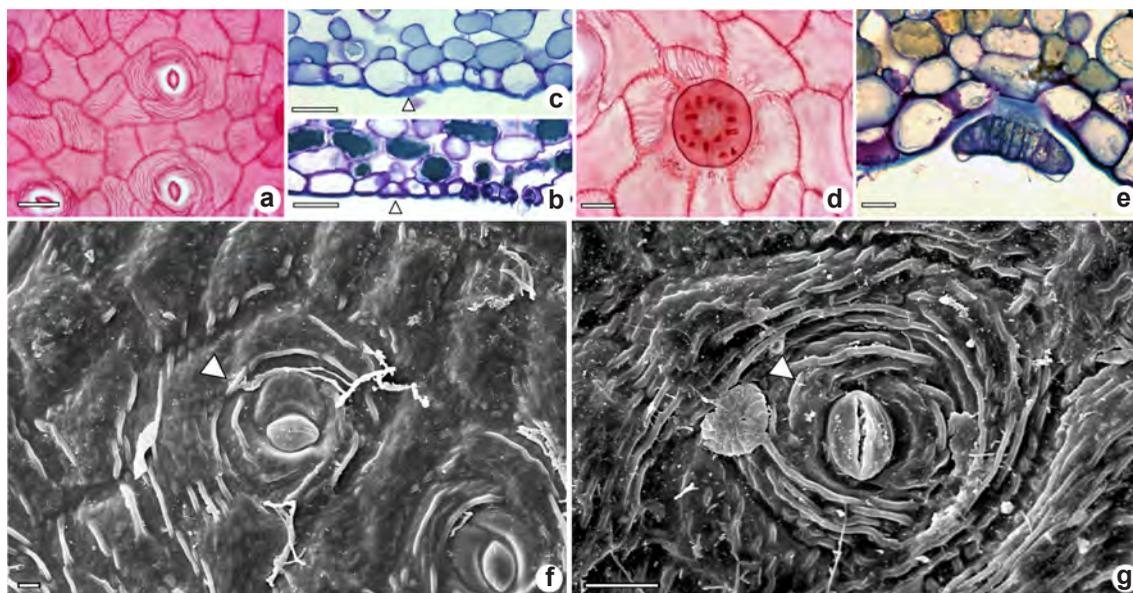


Figure 2 – Epidermal aspects of *Stylogyne* species – a. frontal view of *S. warmingii* abaxial epidermis, with anisocytic stomata. Bar = 50 μm. b, c. transverse section of *S. sordida* and *S. depauperata* leaves, respectively. Bar = 50 μm. b. convex outer periclinal cell walls in the abaxial epidermis. c. straight outer periclinal cell walls in the abaxial epidermis. d, e. frontal view and cross section, respectively, of the peltate trichomes in *S. warmingii*. f, g. frontal view of *S. sordida* and *S. depauperata*, respectively, leaves under scanning electron microscopy, showing cuticle ornamentation. Bars = 10 μm, 20 μm, respectively.

secretory cavities, translucent secretion, nigrescent secretion, abaxial epidermis with straight external periclinal wall, wavy external periclinal wall and isolated sclerenchymatic cells in petiole (Fig. 5).

Discussion

All studied species have an anatomical pattern close to that reported for other Myrsinoideae (Metcalf & Chalk 1979; Otegui & Maldonado 1998; Gostin *et al.* 2011), as the unistratified epidermis with anisocytic stomata placed at the same level of the other cells of this tissue, dorsiventral mesophyll with secretory cavities, and idioblasts containing calcium oxalate crystals.

Although the mesophyll is strongly influenced by environmental variation such as light availability (Rôças *et al.* 1997, 2001), *Stylogyne* species have a dorsiventral mesophyll, formed by plicade cells near the adaxial epidermis. This feature seems to be properly of the genus, being a distinctive character from other Myrsinoideae genera, where it is common to observe the mesophyll formed by common palisade cells (Otegui & Maldonado 1998; Pipoly 1998).

Among Myrsinoideae species, different types of secretory tissues can be found, such as glandular

trichomes, secretory cavities, and hydathodes (Grosse 1908; Solereder 1908; Metcalf & Chalk 1950; Lersten 1977). In *Stylogyne*, secretory cavities and peltate glandular trichomes are found and are described here for the first time. The general characteristic of secretory tissues, such as micromorphology and localization, has been used by many authors in other families as useful features to taxonomy, as in *Lonchocarpus* Kunth – Fabaceae (Teixeira *et al.* 2000), in *Pilocarpus* Vahl – Rutaceae (Muntoreanu *et al.* 2011), where the authors pointed out the secretory cavities as distinctive characters for species segregation, and in *Stachys* L. – Lamiaceae (Salmaki *et al.* 2009) where trichome aspects were useful to segregate species within the genus.

In Myrsinoideae, trichome characteristics have been used by Otegui and Maldonado (1998) where types of trichomes were useful to classify *Myrsine* species, by Fico *et al.* (2007), where trichome types were identified and described to discriminate three *Primula* L. species. Although in *Stylogyne* only one type of trichome, the peltate type with a multicellular head, was found, being of no taxonomic utility within the genus, it is a valuable trait considering the family as a whole.

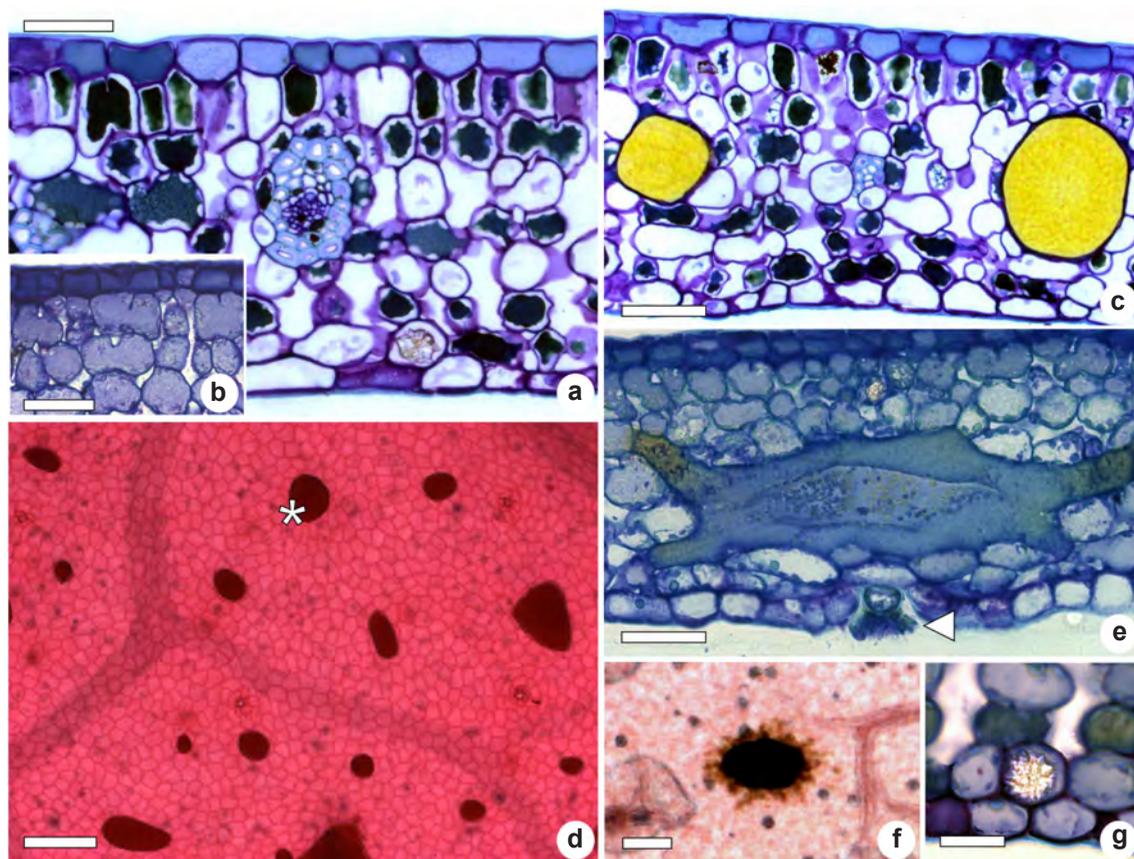


Figure 3 – Mesophyll anatomical aspects of *Stylogyne* – a. cross section of *S. depauperata* leaf, showing the dorsiventral mesophyll and the unistratified epidermis. Bar = 50 μm . b. plicade parenchyma in the mesophyll of *S. warmingii*. Bar = 20 μm . c,d. secretory cavities with lipophilic content in *S. depauperata* mesophyll, c. in cross section and d. in frontal view (*). Bar = 50 μm . e. cross section of *S. warmingii* leaf, showing the branched secretory structure in the mesophyll. Bar = 50 μm . f. frontal view of the branched secretory structure in *S. warmingii*. Bar = 100 μm . g. druse shaped crystal in *S. warmingii* mesophyll. Bar = 20 μm .

Table 2 – Presence (1) and absence (0) matrix of four *Stylogyne* species. FV – Frontal view; TS – transverse section.

	<i>S. depauperata</i>	<i>S. sordida</i>	<i>S. pauciflora</i>	<i>S. warmingii</i>
Epidermal cells with wavy anticlinal walls in the abaxial surface - FV	1	0	0	1
Epidermal cells with wavy periclinal walls in the abaxial surface - TS	0	1	1	0
Epidermal cells with straight periclinal walls in the abaxial surface - TS	1	0	0	1
Adaxial epidermis with striated surface – FV	1	0	1	1
Secretory cavities round shaped or elongated in the mesophyll	1	1	1	0
Branched secretory cavities in the mesophyll	0	0	0	1
Nigrescent secretory cavities in the mesophyll	0	0	0	1
Translucent secretory cavities in the mesophyll	1	1	1	0
Brachysclereids in the petiole	1	1	0	0
Vascular system arranged in arc shape in the petiole	0	1	1	0
Vascular system arranged in a “V” shape in the petiole	1	0	0	1

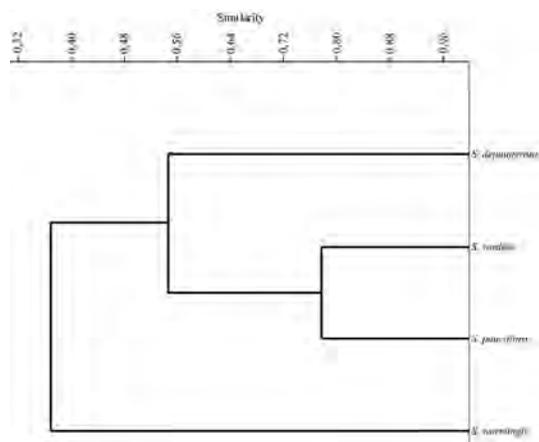


Figure 4 – UPGMA analysis from *Stylogyne* studied species.

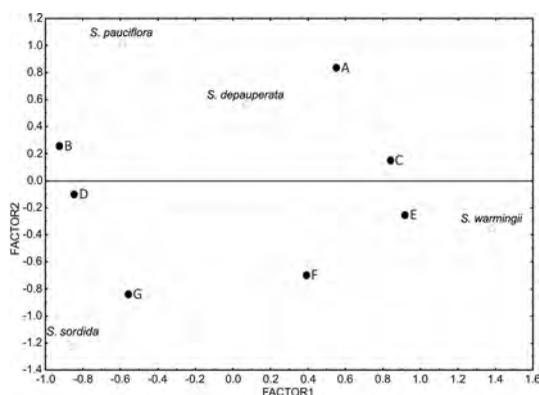


Figure 5 – Biplot principal component analysis (PCA). A: adaxial epidermis with striated surface, B: translucent circular shaped secretory cavities, C: thin wall abaxial epidermis, D: wavy wall abaxial epidermis, E: nigrescent branched secretory cavities, F: isolated sclerenchymatic cells in petiole.

Secretory cavities are an outstanding trait in Primulaceae (Judd *et al.* 2009; Stevens 2001 onwards). They are used, among other features, to segregate the clade formed by *Lysimachia* L., *Myrsine* L., and *Primula* L. into two sub-families: Primuloideae, which is composed of species without secretory cavities, and Myrsinoideae, including species with these cavities (Judd *et al.* 2009). These structures are visible in the leaves as translucent punctuations (Carrijo & Freitas 2008). Among the analyzed *Stylogyne* species, only *S. warmingii* does not present translucent punctuations in leaves. In cross section, secretory cavities have a well delimited shape in all species, except in *S. warmingii*, where they are branched.

Chemical composition of the secretion from secretory cavities is known in *Ardisia* Sw., *Aegiceras* Gaertn., *Embelia* Burm. f., *Myrsine* and *Tapeinosperma* Hook f., where hidroxybenzoquinone derivates and lipophilic substances have been reported. (Tuntiwachwuttikulo *et al.* 1997; Otegui *et al.* 1998). This secretion is associated with different pharmacological activities, such as antioxidant (Sumino *et al.* 2002), antihelminthic (Challam *et al.* 2010) and antileishmania (Germonprez *et al.* 2004; Vermeesch *et al.* 2009). Histochemical test results here performed confirmed the lipophilic composition of the secretion in *Stylogyne*, but further analyses about the nature of this composition are needed.

Cuticular ornamentation is one of the most informative taxonomic features of epidermis in leaves (e.g. Solereder 1908; Metcalfe & Chalk 1979; Barthlott *et al.* 1998), and has been used in species identification, for example in *Trifolium* L.– Fabaceae (Zoric *et al.* 2009) and *Posoqueria* Aubl.– Rubiaceae (Arruda *et al.* 2010). All *Stylogyne* species have a striate epidermis. In *S. depauperata*, *S. pauciflora*, and *S. warmingii* the epidermis is very striated while in *S. sordida* they are few confined to the periphery. Further analysis of the cuticular relief in other *Stylogyne* species has potential to provide attributes for genus taxonomy.

The presence of brachysclereids in the petiole was also an efficient character to distinguish *Stylogyne* species. The presence or absence of this feature in the plant organ has been used by some authors to segregate closely related species, as is shown, for example, in the comparative leaf anatomy of *Macropeplus dentatus* (Perkins) I. Santos & Peixoto and *M. ligustrinus* (Tul.) Perkins (Costa *et al.* 2010) and in some species of *Mirabilis* L. (Nyctaginaceae) (Hernández-Ledesma *et al.* 2011). Besides the taxonomic importance of brachysclereids, Haberlandt (1928) associates this feature with increased organ resistance.

Another relevant trait of species characterization and differentiation is the organization of the vascular system in the petiole (Fahn 1990). While in *S. sordida* and *S. warmingii* it is “V” shaped, in *S. pauciflora* it is arc shaped. Some of these features have been identified in other groups, where they were proved to be useful for taxonomy, as in *Campomanesia* – Myrtaceae (Oliveira *et al.* 2011), Rubiaceae (Martínez-Cabrera 2009) and in Melastomataceae (Reis *et al.* 2004) in which the distinctive characters were, among others, the vascular system pattern in the petiole.

UPGMA clustering analysis and PCA analyses reinforced the value of the anatomical features for species segregation. Anatomical traits that contributed to this conclusion were: shape of secretory cavities in mesophyll, ornamentation of abaxial epidermis, presence of isolated brachisclereids in petiole and the shape of epidermal cells in cross section. Anatomical aspects from all studied species showed a pattern, indicating that they can be grouped in the same genus. Besides, the micromorphological variation was also proved to be efficient for species segregation, as was shown by UPGMA analysis.

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