

MICROMORPHOLOGICAL AND CHEMICAL CHARACTERISTICS OF CUTICULAR WAXES OF *CLEISTES* (ORCHIDACEAE, POGONIEAE)¹

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Abstract – [Micromorphological and chemical characteristics of cuticular waxes of *Cleistes* (Orchidaceae, Pogonieae)]. Micromorphology of cuticular waxes of leaves of 13 species of South American *Cleistes* Rich. ex Lindl. (Vanilloideae: Pogonieae) were observed by scanning electron microscopy. Chemical constituents of the waxes of eleven species were identified by gas chromatography. The observations were carried out with individuals of native populations occurring in South, Southeastern, Northeastern and Central-Western Brazil. The micromorphology of the cuticular waxes varied from amorphous deposits to parallel stacked platelets. The latter morphological pattern seems to be more common in plants from dryer habitats. Alkyl acids are predominant constituents of waxes of most species. Alkyl alcohols predominate in other species and alkanes are predominant in only one of the studied species. Alkyl esters, monoglycerides and sterols are minor compounds, having not been detected in some species. Either hexadecanoic or octadecanoic are main homologues of alkyl acids; regarding alkyl alcohol homologue distribution, hexacosanol, octacosanol and triacontanol predominates; pentacosane, heptacosane, octacosane, nonacosane and hentriacontane predominate in alkane homologue distributions. No correlation exists between chemical composition and micromorphology of the wax deposits. Stomata distribution restricted to the adaxial or on both foliar surfaces, morphology of wax deposits and chemical composition do not correlate with molecular phylogeny. On the other hand, all these attributes may be useful for species characterization and distinction between species.

Resumo – [Características micromorfológicas e químicas das ceras cuticulares de *Cleistes* (Orchidaceae, Pogonieae)]. A micromorfologia das ceras cuticulares em folhas de 13 espécies de *Cleistes* Rich. ex Lindl. (Vanilloideae: Pogonieae) sul-americanas foram observadas com o auxílio de um microscópio eletrônico de varredura. Os componentes químicos das ceras epicuticulares de 11 espécies foram identificados por cromatografia gasosa. Todas as análises foram feitas com amostras coletadas no campo, a partir de populações nativas, nas regiões sul, sudeste, nordeste e centro-oeste do Brasil. A micromorfologia das ceras epicuticulares variou desde depósitos amorfos até pequenas placas paralelamente orientadas. A presença de ceras epicuticulares formando placas é mais comum em plantas que ocorrem em ambientes secos. Ácidos alquílicos são os principais constituintes das ceras epicuticulares da maioria das espécies do gênero. Álcoois alquílicos predominam em outras espécies e alcanos são os principais constituintes das ceras de uma das espécies. Ésteres alquílicos, mono-glicerídeos e esteróis são encontrados em pequenas quantidades, e não estão presentes em todas as espécies. O ácido hexadecanóico ou o octadecanóico são os homólogos principais de ácidos alquílicos; em relação à distribuição de álcoois alquílicos, predominam octacosanol e triacontanol; pentacosano, heptacosano, octacosano, nonacosano e hentriacontano predominam nas distribuições de homólogos alcanos. De acordo com nossos dados não existe correlação entre a composição química e a micromorfologia das ceras. A posição dos estômatos, distribuídos por toda a face adaxial ou em ambas as superfícies da folha, a micromorfologia e a composição química das ceras epicuticulares não possuem correlação com a filogenia do grupo. Por outro lado, todas essas características podem ser usadas na caracterização e distinção entre as espécies do gênero.

Key words: Orchidaceae, *Cleistes*, wax micromorphology, chemotaxonomy.

Introduction

Vanilloid orchids (i.e., subfamily Vanilloideae; Cameron *et al.* 1999; Freudenstein & Rasmussen 1999; Freudenstein *et al.* 2004; Cameron 2004) comprise tribes Vanilleae and Pogo-

nieae, 15 genera and about 175 species (Cameron *et al.* 1999). Vanilleae is the largest tribe and includes *Vanilla*, a genus with about 90 species. Tribe Pogonieae comprises five genera (Dressler 1993; Cameron 2004). *Cleistes* is the largest genus of the tribe, with about 20 species, the other genera being

small or monotypic (Cameron 1999). According to Hoehne (1940) the center of diversity of *Cleistes* is Central Brazil.

Delimitation is problematic in the *Cleistes* taxonomy. The genus is paraphyletic, with North American *C. divaricata* and *C. bifaria* being more related to North American-Asiatic genera *Isotria* and *Pogonia* than to the South and Central American congeners (Cameron & Chase 1999; Cameron *et al.* 1999; Pansarin 2005; Pansarin *et al.* 2008). The South and Central American species of *Cleistes* is a monophyletic group (Cameron & Chase 1999; Cameron *et al.* 1999; Pansarin 2005; Pansarin *et al.* 2008).

Micromorphology of cuticular waxes has been shown to be useful for taxa delimitation at several taxonomic levels within flowering plants (Jeffree 1986; Barthlott *et al.* 1998; Wang *et al.* 2001). Cuticular waxes are often composed of a complex mixture of different chemical compounds, including cyclic and long-chain aliphatic components, which may further be classified according to functional groups and homologue distribution (Barthlott *et al.* 1998).

Chemical composition data of cuticular waxes sometimes are used for environmental (e.g., Kim *et al.* 2007) and taxonomic considerations (e.g., Eglinton *et al.* 1962; Tulloch 1981; Salatino *et al.* 1989; Avato *et al.* 1990; Vioque *et al.* 1996).

Detailed studies on micromorphology of cuticular waxes have been performed involving monocots, one of the major conclusions being the recognition of two main wax micromorphologies, namely *Strelitzia* and *Convallaria* types, each characterizing groups of monocotyledonous orders (Barthlott & Frolich 1983). Apparently, however, no investigation about orchid wax micromorphology has been published.

The objective of this investigation is to explore the micromorphological (13 species) and chemical (11 species) characteristics of the waxes in the context of *Cleistes* taxonomy and phylogeny.

Material and methods

In this study we report the micromorphology of cuticular waxes of 13 *Cleistes* species, namely *C. aphylla* (Barb. Rodr.) Hoehne, *C. bella* Rchb.f. & Warm., *C. caloptera* Rchb.f. & Warm., *C. castanoides* Hoehne, *C. cipoana* Hoehne, *C. exilis* Hoehne, *C. gracilis* (Barb. Rodr.) Schltr., *C. libonii* (Barb. Rodr.) Schltr., *C. metallina* (Barb. Rodr.) Schltr., *C. moritzii*, *C. paranaensis* (Barb. Rodr.) Schltr., *C. pusilla* Pansarin, *C. tenuis* (Rchb.f. ex Griseb.) Schltr. and *C. ramboi* Pabst. The geographic distribution of *Cleistes* species analyzed covers the South, Southeastern, Central-Western and Northeastern Brazil. Analyzes of chemical composition of the foliar waxes of eleven species were carried out.

Collection sites and plant material

Leaves were collected from dried specimens for scanning electron microscopy (SEM) study and from natural populations for chemical analyzes. Habitats where *Cleistes* specimens grow include, among other biomes, "vereda" (wet savannas), "campos de altitude" (high mountain grasslands), "campo limpo" (grasslands) and "campo sujo" (grasslands with shrubs). Sites of collection, list of species and vouchers are given in table 1. Leaves were taken from the median portion of the aerial stem. Voucher specimens are deposited in the UEC Herbarium (Universidade Estadual de Campinas).

Analysis by scanning electron microscopy (SEM)

Cuticular waxes were examined on both abaxial and adaxial surfaces of the median portion of dried leaves. For each species, at least three analyzes were carried out, using different specimens, preferentially from distinct localities. Small pieces of leaves were fixed on aluminum SEM stubs with double-sided adhesive tape and directly sputter-coated with gold-palladium alloy. Acceleration voltage of 10 kv and average distance of 10-20 mm were used in a Jeol 5800 LV scanning electron microscope. Analyzes of cuticular wax characteristics, measurements and descriptions were based on observations of micrographs, using the corresponding scale bars and magnification value for standardization. Terminology used for characterization of cuticular waxes followed Barthlott *et al.* (1998).

Extraction, purification and chemical analysis of foliar waxes

Fresh leaves were dipped in dichloromethane and shaken at room temperature for 2 min. After solvent evaporation under N₂ flow at room temperature, compounds in the extract were derivatized by treatment with 100 µL BSTFA (Sigma) at 40 °C for 30 min. The solvent was evaporated and the residue dissolved with *n*-heptane. Wax constituents were identified by analyses in a gas chromatograph HP-5890 (Agilent, Palo Alto, CA, USA) coupled to a mass spectrometer HP-5972 (Agilent, Palo Alto, CA, USA), using a capillary column HP-5 (30 m x 0.25 mm x 0.25 µm; Agilent, Palo Alto, CA, USA), injection volume was 1 µL and split 1:20. The temperature program was 40 °C isothermal for 5 min, rising 8 °C/min up to 350 °C and isothermal for 30 min., injector temperature was 310 °C and the interface temperature was 280 °C. Helium was used as carrier gas at 38 cm/s. The mass spectrometer was set to operate with electron impact (EI) at 70 eV and mass range 40 - 700 Daltons. Relative amount of constituents were determined by the areas under the corresponding chromatogram peaks.

Table 1. Specimens of *Cleistes* used for scanning electron microscopy (SEM) and chemical analyses, locations and corresponding geographical coordinates, and voucher numbers.

Species	Location	Coordinates	Analyses performed		Voucher
<i>C. aphylla</i>	Teresina de Goiás - GO	13°36'S 47°13'W	SEM	chemical	Pansarin & Mickeliunas 899 (UEC)
<i>C. bella</i>	Brasília - DF	15°35'S 47°30'W	SEM		Pansarin & Batista 758 (UEC)
<i>C. bella</i>	Serra do Cipó - MG	19°17'S 43°35'W		chemical	Pansarin & Mickeliunas 921 (UEC)
<i>C. bella</i>	Serra do Cipó - MG	19°17'S 43°35'W	SEM		Batista & Bianchetti 887 (CEN)
<i>C. caloptera</i>	Minaçu - GO	13°30'S 48°20'W		chemical	Cavalcanti et al. 1130 (CEN)
<i>C. caloptera</i>	Caldas Novas - GO	17°40'S 48°40'W	SEM		Dias et al. 410 (CEN)
<i>C. castanoides</i>	Cavalcante - GO	13°45'S 47°45'W	SEM		Batista 829 (UEC)
<i>C. castanoides</i>	Teresina de Goiás - GO	13°36'S 47°13'W	SEM	chemical	Pansarin & Mickeliunas 897 (UEC)
<i>C. castanoides</i>	Teresina de Goiás - GO	13°36'S 47°13'W	SEM		Pansarin & Mickeliunas 889 (UEC)
<i>C. cipoana</i>	Serra do Cipó - MG	19°17'S 43°35'W	SEM		Batista & K. Proite 997 (CEN)
<i>C. exilis</i>	Teresina de Goiás - GO	13°36'S 47°13'W		chemical	Pansarin & Mickeliunas 895 (UEC)
<i>C. exilis</i>	Ibicoara - BA	13°30'S 41°25'W	SEM		Koehler 20/00 (UEC)
<i>C. exilis</i>	Diamantina - MG	18°S 43°45'W	SEM		Batista & Proite 1019 (CEN)
<i>C. gracilis</i>	Atibaia - SP	23°13'S 46°35'W	SEM	chemical	Pansarin & Mickeliunas 908 (UEC)
<i>C. libonii</i>	Caraguatatuba - SP	23°45'S 45°30'W	SEM		Pansarin 857 (UEC)
<i>C. metallina</i>	Ouro Branco - MG	20°30'S 43°40'W			Pansarin & Mickeliunas 909 (UEC)
<i>C. moritzii</i>	Rio de Contas - BA	13°45'S 41°45'W	SEM		Aona & Moraes 642 (UEC)
<i>C. moritzii</i>	Rio de Contas - BA	13°45'S 41°45'W		chemical	Pansarin et al. 782 (UEC)
<i>C. paranaensis</i>	Serra do Cipó - MG	19°17'S 43°35'W	SEM	chemical	Pansarin & Mickeliunas 920 (UEC)
<i>C. pusilla</i>	Alto Paraíso de Goiás - GO	14°10'S 47°30'W	SEM		Pansarin & Mickeliunas 898 (UEC)
<i>C. ramboi</i>	Alto Paraíso de Goiás - GO	14°10'S 47°30'W	SEM		Pansarin & Batista s.n. (UEC 117396)
<i>C. ramboi</i>	Teresina de Goiás - GO	13°36'S 47°13'W		chemical	Pansarin & Mickeliunas 872 (UEC)
<i>C. tenuis</i>	Alto Paraíso de Goiás - GO	14°10'S 47°30'W	SEM		Pansarin & Batista 741 (UEC)
<i>C. tenuis</i>	Cavalcante - GO	13°45'S 47°45'W		chemical	Pansarin & Mickeliunas 900 (UEC)

Results

Stomata and micromorphology of cuticular waxes

Species of *Cleistes* present scale-like to evident leaves, spirally arranged along the aerial stem. The leaves are sessile, generally erect and adpressed or patent, and glaucous. The stomata are anomocytic in all species. Leaves of *C. aphylla*, *C. gracilis*, *C. libonii*, *C. moritzii*, *C. pusilla* and *C. tenuis* stomata are seen only on the adaxial surface; in leaves of the other seven *Cleistes* species, stomata occur on both surfaces (Table 2).

Leaf characteristics, stomata distribution and wax ornamentation of the *Cleistes* specimens analyzed are summarized in table 2. The cuticular waxes of *Cleistes* species vary from amorphous films to parallel stacked platelets (Table 2, Figures 1-3). Stomata occur exclusively on the abaxial leaf surface in *C. aphylla*, *C. gracilis*, *C. moritzii*, *C. pusilla* and *C. tenuis*. The other *Cleistes* analyzed species have stomata on both leaf surfaces (Table 2, Figures 1-3). Surfaces with amorphous films and practically with no or only sparse wax crystals characterize the leaf abaxial sides of *C. aphylla*, *C. castanoides*, *C. libonii* (Figures 1A, 1F and 2G, respective-

ly), and both sides of *C. pusilla* (Figures 3D-E). Granules are found on leaf surfaces of *C. aphylla* (Figures 1A-B), *C. bella* (Figure 1D), *C. castanoides* (Figures 1F-G), and *C. cipoana*, *C. exilis*, *C. gracilis* and *C. libonii* (Figures 2A-H). Rugose crystals are observed on leaf surfaces of *C. cipoana* (Figures 2A-B), *C. exilis* (Figure 2C), *C. gracilis* (Figure 2F), *C. libonii* (Figures 2G-H) and *C. pusilla* (Figure 2D). Platelets, often non entire, cover leaf surfaces of most of the studied *Cleistes* species (Figures 1C, 1E, 1H, 2E, 3A-C, 3F-H). Crystal patterns may vary comparing both leaf surfaces of the same species. Such are the cases of *C. bella* (Figures 1C-D), *C. cipoana* (Figures 2A-B), *C. exilis* (Figures 2C-D), *C. gracilis* (Figures 2E-F) and *C. libonii* (Figures 2G-H). Platelets vary in size, being small in *C. bella* (Figure 1C), *C. gracilis* (Figure 2E) and *C. tenuis* (Figure 3G), medium size in *C. paranaensis* (Figure 3B) and large in *C. caloptera* (Figure 1E), *C. castanoides* (Figure 1H), *C. metallina* (Figure 3A), *C. pluriflora* (Figure 3C) and *C. ramboi* (Figure 3H). Crystal sizes, compactness and orientation is different around stomata aperture, comparing with other parts of the leaf surface (see, for example, Figures 1A, 1E, 3A and 3H).

Table 2. Characteristics of habitat, leaf, stomata distribution and wax ornamentation of *Cleisthes* species. Digits inside parenthesis refer to figures 1A to 3H.

Species	Habitat	Leaf characteristics	Stomata	Wax ornamentation
<i>C. aphylla</i>	Wet grasslands	Scale-like, lanceolate to elliptic-lanceolate	hypostomatous	small granules (1A-B)
<i>C. bella</i>	grasslands with shrubs	evident, elliptic to ovate	amphistomatous	large granules, platelets (1C-D)
<i>C. caloptera</i>	wet grasslands	evident, lanceolate to elliptic-lanceolate	amphistomatous	non entire platelets (1E)
<i>C. castanoides</i>	marshy areas	evident, lanceolate to elliptic	amphistomatous	film, granules, non entire platelets (1F-H)
<i>C. cipoana</i>	wet grasslands	scale-like, lanceolate to ovate-lanceolate	amphistomatous	film, rugose, small granules (2A-B)
<i>C. exilis</i>	wet grasslands	scale-like, lanceolate to ovate-lanceolate	amphistomatous	film rugose, small granules (2C-D)
<i>C. gracilis</i>	quartzite islands, disturbed areas	evident, lanceolate to elliptic-lanceolate	hypostomatous	granules, non entire platelets (2E-F)
<i>C. libonii</i>	disturbed areas, "restinga" vegetation	evident, lanceolate to ovate-lanceolate	hypostomatous	film, rugose, small granules (2G-H)
<i>C. metallina</i>	wet grasslands, road margins	evident, lanceolate to elliptic-lanceolate	amphistomatous	non entire platelets (3A)
<i>C. moritzii</i>	wet grasslands	evident, lanceolate to ovate-lanceolate	hypostomatous	non entire platelets (3C)
<i>C. paranaensis</i>	vegetation of rocky outcrops	evident, acute to lanceolate	amphistomatous	non entire platelets (3B)
<i>C. pusilla</i>	high mountain grasslands	scale-like, lanceolate to ovate-lanceolate	hypostomatous	film, rugose (3D-E)
<i>C. ramboi</i>	marshy areas, wet grasslands	evident, lanceolate	amphistomatous	non entire platelets (3H)
<i>C. tenuis</i>	wet grasslands	evident, lanceolate to elliptic-lanceolate	hypostomatous	small non entire platelets (3F-G)

Chemical composition of *Cleisthes* cuticular waxes

Cuticular waxes of the studied *Cleisthes* species are constituted mainly by fatty acids, alkanes and alkyl alcohols (Figure 4). Alkyl esters occur in appreciable amounts in waxes of *C. exilis* and *C. metallina* (Table 3, Figure 4). Other chemical classes, such as alkyl aldehydes, monoglycerides and sterols may be found in *Cleisthes* cuticular waxes as minor constituents, but not in all species (Table 3). In fact, monoglycerides were not detected in *C. exilis*, *C. gracilis* and *C. metallina*. Monoglycerides containing residues of hexadecanoic (palmitic) and octadecanoic (stearic) acids predominate. Sterols were detected in small amounts and only in waxes of *C. castanoides* and *C. tenuis* (Table 3). Alkyl aldehydes are minor constituents of the waxes of *C. aphylla*, *C. bella* and *C. exilis*, hexacosanal and octacosanal predominating (Table 3). The proportion of each constituent class varies widely from one species to another (Table 3, Figure 4). Alkyl alcohols are major constituents of cuticular waxes of *C. aphylla*, *C. bella* and *C. ramboi*. Alkyl acids predominate in cuticular waxes of *C. caloptera*, *C. castanoides*, *C. metallina*, *C. paranaensis*, *C. moritzii* and

C. tenuis (Table 3, Figure 4). Among the cuticular waxes studied, that of *C. gracilis* is the sole example in which neither alkyl alcohols nor alkyl acids predominate, major constituents being alkanes. Alkanes are relatively abundant (>10%) also in the cuticular waxes of *C. exilis*, *C. metallina* and *C. tenuis* (Table 3, Figure 4).

Among the constituent alkyl alcohols, hexacosanol (C_{26}), octacosanol (C_{28}) and triacontanol (C_{30}) are the most important (Table 3). Alkyl alcohol C_{26} is the main homologue of the cuticular waxes of *C. aphylla*, *C. exilis*, *C. paranaensis*, *C. moritzii*, *C. ramboi* and *C. tenuis*, while alkyl alcohol C_{28} is main homologue for *C. bella*, *C. caloptera* and *C. castanoides*, and C_{30} for *C. gracilis* and *C. metallina* (Table 3).

Distribution of main homologues of alkyl acids enables the recognition of two groups of *Cleisthes* species: 1) *C. caloptera*, *C. castanoides*, *C. gracilis*, *C. paranaensis*, *C. moritzii* and *C. tenuis*, with either hexadecanoic (C_{16}) or octadecanoic (C_{18}) as main homologue; 2) *C. aphylla*, *C. bella*, *C. exilis* and *C. ramboi*, with hexacosanoic (C_{26}) or octacosanoic (C_{28}) as main homologues (Table 3).

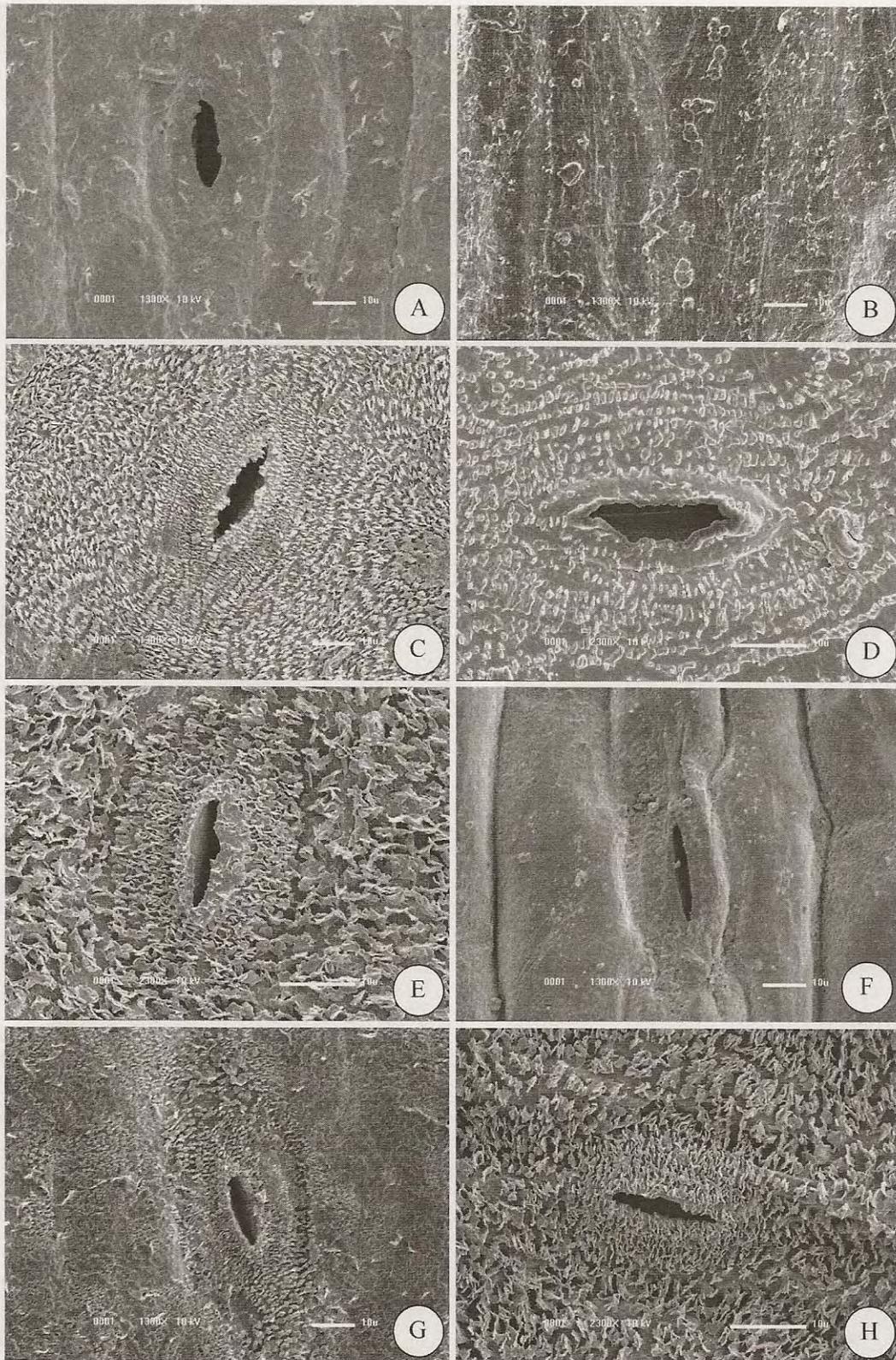


Fig. 1. A-H. Cuticular waxes of leaves of *Cleistes* species as viewed by scanning electron microscopy. Scale bars = 10 μ m. A. *C. aphylla*: abaxial. Coll. Pansarin & Mickeliunas 899 (UEC). B. *C. aphylla*: adaxial. Coll. Pansarin & Mickeliunas 899 (UEC). C. *C. bella*: abaxial. Coll. Pansarin & Batista 758 (UEC). D. *C. bella*: adaxial. Coll. Batista & Bianchetti 887 (UEC). E. *C. aff. caloptera*: adaxial. Coll. Dias et al. 410 (CEN). F. *C. castanoides*: abaxial. Coll. Pansarin & Mickeliunas 889 (UEC). G. *C. castanoides*: abaxial. Coll. Pansarin & Mickeliunas 897 (UEC). H. *C. castanoides*: adaxial. Coll. Batista 829 (UEC).

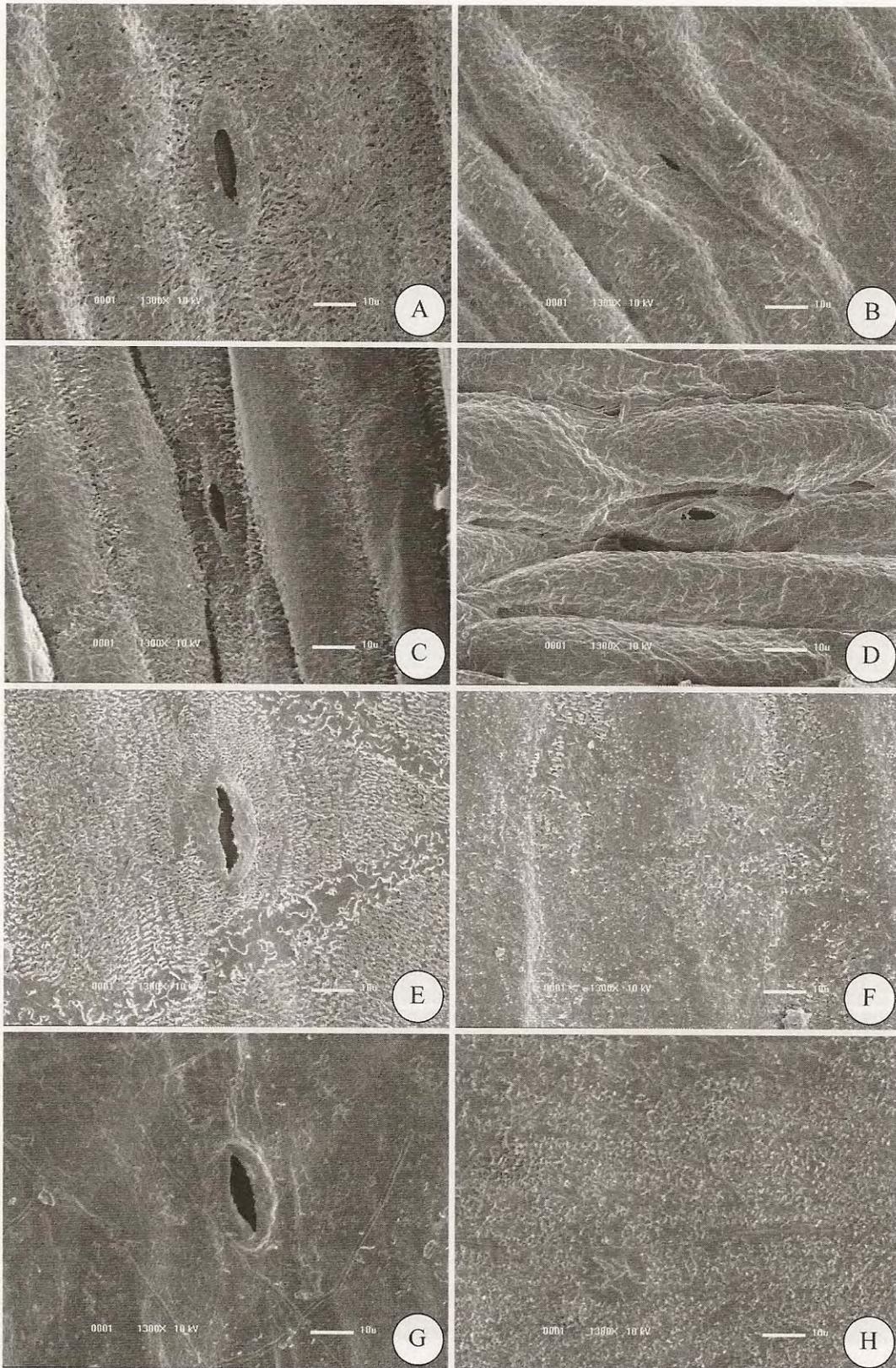


Fig. 2. A-H. Cuticular waxes of leaves of *Cleistes* species under SEM. Scale bars = 10 µm. A. *C. cipoana*: abaxial. Coll. Batista & K. Proite 997 (CEN). B. *C. cipoana*: adaxial. Coll. Batista & K. Proite 997 (CEN). C. *C. exilis*: abaxial. Coll. Batista & Proite 1019 (CEN). D. *C. exilis*: adaxial. Coll. Koehler 20/00 (UEC). E. *C. gracilis*: abaxial. Coll. Pansarin & Mickeliunas 908 (UEC). F. *C. gracilis*: adaxial. Coll. Pansarin & Mickeliunas 908 (UEC). G. *C. libonii*: abaxial. Coll. Pansarin 857 (UEC). H. *C. libonii*: adaxial. Coll. Pansarin 857 (UEC).

Table 3. Contents (%) of chemical constituents of cuticular waxes of *Cleistes* species and corresponding homologues distributions. APH: *C. aphylla*; BL: *C. bella*; CL: *C. caloptera*; CS: *C. castanoides*; EX: *C. exilis*; GR: *C. gracilis*; MT: *C. metallina*; PR: *C. paranaensis*; MR: *C. moritzii*; RM: *C. ramboi*; TN: *C. tenuis*; tr: trace amounts; -: not detected.

Constituents	APH	BL	CL	CS	EX	GR	MT	MR	PR	RM	TN
Alkyl acids											
Total content in waxes	31.4	17.8	88.4	76.0	18.3	15.1	53.5	88.9	75.5	8.3	44.8
Homologues distribution											
Chains <C ₁₆	23.2	5.4	17.8	4.4	13.5	23.5	11.8	15.2	5.9	38.5	3.3
Hexadecanoic (C ₁₆)	17.8	21.6	21.8	29.6	7.5	21.9	22.4	38.2	42.6	6.5	36.7
Heptadecanoic (C ₁₇)	0.8	3.0	3.0	2.7	0.7	tr	1.3	3.3	4.4	-	1.1
Octadecanoic (Stearic, C ₁₈)	8.1	21.4	16.8	27.9	3.0	7.5	26.5	28.9	25.0	3.4	28.5
Octadecenoic (Oleic, C _{18:1})	tr	-	16.8	0.7	-	-	24.2	2.0	tr	-	2.2
Octadecatrienoic (Linolenic, C _{18:3})	-	-	-	9.7	-	-	-	-	-	-	-
Octadecadienoic (Linoleic, C _{18:2})	0.8	5.4	1.1	1.0	-	-	-	2.6	5.9	-	3.3
Nonadecanoic (C ₁₉)	tr	-	0.6	0.3	-	-	-	0.7	-	-	1.1
Nonadecenoic (C _{19:1})	tr	1.2	1.0	2.0	-	-	-	0.7	-	-	2.2
Eicosanoic (C ₂₀)	0.8	3.6	4.5	4.0	0.8	-	0.4	3.8	2.9	2.6	3.3
Heneicosanoic (C ₂₁)	tr	0.1	tr	-	-	-	-	-	-	-	-
Docosanoic (C ₂₂)	2.4	3.0	2.0	2.1	0.7	2.1	4.0	1.3	1.5	2.6	1.7
Tricosanoic (C ₂₃)	tr	1.2	1.0	1.0	tr	-	-	tr	-	-	-
Tetracosanoic (C ₂₄)	0.8	4.2	4.1	9.4	2.4	4.3	0.9	0.6	2.9	-	4.2
Pentacosanoic (C ₂₅)	0.8	1.2	0.6	0.7	-	-	1.3	tr	tr	-	1.1
Hexacosanoic (C ₂₆)	21.9	tr	0.7	0.6	14.4	3.3	0.9	-	1.6	27.4	2.2
Heptacosanoic (C ₂₇)	tr	-	tr	0.3	-	5.4	0.9	1.3	tr	13.9	2.4
Heptacosenoic (C _{27:1})	-	-	1.1	-	-	-	-	-	-	-	-
Octacosanoic (C ₂₈)	13.3	28.2	3.5	1.5	41.4	14.2	1.8	0.7	4.4	3.8	4.5
Nonacosanoic (C ₂₉)	tr	tr	0.2	0.4	0.7	-	-	tr	tr	0.1	Tr
Triacosanoic (C ₃₀)	7.7	0.5	3.5	1.7	14.2	17.8	2.7	0.7	2.9	1.3	2.2
Dotriacosanoic (C ₃₂)	1.6	-	-	tr	0.7	-	0.9	tr	tr	-	-
Alkyl alcohols											
Total content in waxes	56.2	71.3	7.8	17.8	52.3	1.5	5.7	5.3	11.1	89.8	8.9
Homologues distribution											
Phytol (diterpene)	-	-	-	7.4	-	-	-	-	-	-	-
Chains <C ₂₄	0.8	0.2	-	tr	-	33.3	8.1	-	-	tr	-
Tetracosanol (C ₂₄)	0.4	-	-	tr	3.6	11.1	tr	25.0	18.1	1.2	11.1
Pentacosanol (C ₂₅)	tr	0.4	-	tr	1.8	-	4.2	tr	tr	2.6	Tr
Hexacosanol (C ₂₆)	55.8	11.1	16.3	22.0	43.9	-	-	62.5	36.4	88.4	44.4
Heptacosanol (C ₂₇)	0.4	5.9	5.4	1.5	-	-	-	12.5	9.1	0.2	5.6
sec-Heptacosanol (C ₂₇)	-	-	-	-	tr	-	29.1	-	-	0.5	-
Octacosanol (C ₂₈)	22.9	71.8	37.8	60.3	39.8	-	16.7	tr	27.3	1.3	16.7
Octacosenol (C _{28:1})	-	3.3	-	1.5	0.5	-	12.5	-	-	-	-
Nonacosanol (C ₂₉)	-	3.8	8.1	tr	-	-	-	-	-	-	-
Nonacosenol (C _{29:1})	-	-	-	-	1.2	-	4.2	-	-	4.2	-
sec-Nonacosanol (C ₂₉)	-	-	-	-	0.3	-	-	-	-	0.5	-
Triacosanol (C ₃₀)	12.4	3.5	32.4	7.3	8.1	55.6	25.2	-	9.1	1.1	11.1
Dotriacosanol (C ₃₂)	7.3	-	-	-	0.8	-	-	-	-	-	11.1

Table 3 continued

Constituents	APH	BL	CL	CS	EX	GR	MT	MR	PR	RM	TN
Alkyl esters											
Total content in waxes	-	tr	-	-	10.4	-	10.4	-	-	0.8	-
<i>Homologues distribution</i>											
Hexadecanoic octadecyl (C ₁₆ -C ₁₈)	-	-	-	-	-	-	6.8	-	-	-	-
Hexadecanoic tetracosyl (C ₁₆ -C ₂₄)	-	-	-	-	-	-	2.3	-	-	-	-
Octadecanoic hexadecyl (C ₁₈ -C ₁₆)	-	-	-	-	2.6	-	29.5	-	-	-	-
Eicosanoic tetradecyl (C ₂₀ -C ₁₄)	-	-	-	-	6.6	-	4.5	-	-	-	-
Eicosanoic eicosyl (C ₂₀ -C ₂₀)	-	-	-	-	57.9	-	29.6	-	-	-	100
Docosanoic dodecyl (C ₂₀ -C ₁₂)	-	-	-	-	2.6	-	tr	-	-	-	-
Tetracosanoic decyl (C ₂₄ -C ₁₀)	-	-	-	-	1.3	-	-	-	-	-	-
Hexacosanoic hexacosyl (C ₂₆ -C ₂₆)	-	-	-	-	21.1	-	-	-	-	-	Tr
Octacosanoic docosyl (C ₂₈ -C ₂₂)	-	-	-	-	-	-	27.3	-	-	-	-
Octacosanoic tetracosyl (C ₂₈ -C ₂₄)	-	-	-	-	7.9	-	-	-	-	-	-
Alkanes											
Total content in waxes	8.3	6.1	2.0	2.6	15.4	83.4	30.4	2.9	6.7	1.1	44.3
<i>Homologues distribution</i>											
Heneicosane (C ₂₁)	3.0	-	-	-	tr	1.4	-	-	-	4.5	-
Docosane (C ₂₂)	6.0	-	-	-	1.8	3.7	1.6	-	-	2.2	-
Tricosane (C ₂₃)	6.0	6.5	tr	-	8.0	5.9	4.6	10.0	-	4.5	-
Tetracosane (C ₂₄)	6.0	-	-	-	11.6	7.2	6.2	-	-	9.0	-
Pentacosane (C ₂₅)	15.3	14.8	15.0	10.0	19.6	10.1	23.3	30.0	10.0	18.0	10.0
Hexacosane (C ₂₆)	15.3	12.7	-	tr	11.6	14.4	23.2	-	-	20.2	-
Hexacosane (C _{26:1})	-	19.2	-	-	-	-	0.8	-	-	-	-
Heptacosane (C ₂₇)	15.3	33.9	40.0	30.0	12.5	12.7	17.1	30.0	50.0	13.5	30.0
Octacosane (C ₂₈)	9.0	6.5	35.0	10.0	8.9	13.0	11.6	-	tr	13.5	10.0
Nonacosane (C ₂₉)	12.1	6.4	-	40	6.2	11.5	8.5	20.0	30.0	5.6	40.0
Triacontane (C ₃₀)	6.0	-	-	-	1.8	8.6	2.3	-	-	3.4	-
Hentriacontane (C ₃₁)	6.0	-	-	10.0	15.3	6.8	-	10.0	10.0	3.4	10.0
Dotriacontane (C ₃₂)	-	-	-	-	0.9	3.9	0.8	-	-	2.2	-
Tritriacontane (C ₃₃)	-	-	-	-	-	0.6	-	-	-	-	-
Tritriacontene (C _{33:1})	-	-	-	-	1.8	-	-	-	-	tr	-
Tetratriacontane (C ₃₄)	-	-	-	-	-	0.2	-	-	-	-	-
Pentatriacontane (C ₃₅)	-	-	10.0	-	-	-	-	-	-	-	Tr
Alkyl aldehydes											
Total content in waxes	3.8	4.4	-	-	3.6	-	-	-	-	-	-
<i>Homologues distribution</i>											
Hexacosanal (C ₂₆)	100	-	-	-	26.9	-	-	-	-	-	-
Heptacosanal (C ₂₇)	-	3.6	-	-	73.1	-	-	-	-	-	-
Octacosanal (C ₂₈)	tr	96.4	-	-	-	-	-	-	-	-	-
Monoglycerides											
Total content in waxes	0.3	0.4	1.8	2.3	-	-	-	2.9	6.7	-	1.5
<i>Homologues distribution</i>											
1-Hexadecanoic (C ₁₆)	100	tr	100	60.0	-	-	-	-	67.0	67.0	60.0
2-Hexadecanoic (C ₁₆)	-	-	-	10.0	-	-	-	-	-	-	-

Table 3 continued

Constituents	APH	BL	CL	CS	EX	GR	MT	MR	PR	RM	TN
1-Heptadecanoic (C ₁₇)	-	-	-	10.0	-	-	-	-	-	-	-
1-Octadecanoic (C ₁₈)	tr	100	tr	10.0	-	-	-	-	33.0	33.0	40.0
2-Octadecanoic (C ₁₈)	-	-	-	10.0	-	-	-	-	-	-	-
1-Octadecenoic (C _{18:1})	-	-	-	tr	-	-	-	-	-	-	-
Sterols											
Total content in waxes	-	-	-	1.3	-	-	-	-	-	-	0.5
Homologues distribution											
Stigmasterol	-	-	-	0.3	-	-	-	-	-	-	-
Stigmast-4-ene	-	-	-	-	-	-	-	tr	-	-	0.5
Sitosterol	-	-	-	0.7	-	-	-	-	-	-	-
Ergost-5-en-3-ol	-	-	-	0.3	-	-	-	-	-	-	-

Heptacosane (C₂₇) seems to be the main alkane homologue of most species studied, being predominant in the waxes of *C. aphylla*, *C. bella*, *C. caloptera*, *C. moritzii* and *C. paranaensis* (Table 3). Nonacosane (C₂₉) and hentriacontane (C₃₁) predominate among alkanes of the waxes of *C. tenuis* and *C. exilis*, respectively (Table 3).

Discussion

The ultrastructure of cuticular waxes of *Cleistes* species varies from a thin film, often rugose, to platelets, in a similar way as has been reported for species of *Musa* (Freeman & Turner 1985), and other monocotyledonous families (Barthlott *et al.* 1998). The present results suggest that micromorphological characters of leaf surfaces may be used to distinguish among *Cleistes* species. For example, reproductive and vegetative (roots, stem and leaves) characteristics of *C. libonii* and *C. metallina* are very similar in shape and color. However, disposition of stomata and structure of cuticular waxes are sharply distinguishable comparing both species (Table 2). In addition, *Cleistes libonii* is distinct from *C. metallina* by the presence of dark veins on the lip and distribution in Atlantic Forests, mainly along road margins (Pansarin 2003), whereas *C. metallina* occurs in wet fields adjacent to cerrado vegetation.

According to Freeman and Turner (1985), cuticular waxes influence foliar absorption and water loss. Decreasing air relative humidity stimulates wax production (Baker 1974; Grout 1975). Vioque *et al.* (1994) reported that plants growing under low luminosity or in regions with high rainfall have relatively thin deposits of foliar waxes. *C. libonii* presents well-developed leaves and grow exclusively in the Atlantic Rain Forest (Pansarin 2003), where the relative humidity is much higher than in "cerrado" vegetation. In *Cleistes* species growing in grasslands, in "cerrado" vegetation, with high incidence of solar radiation (e.g., *C. castanoides*,

C. bella and *C. paranaensis*), the presence of platelets may be linked to thicker deposits of cuticular waxes, probably contributing to reduce water loss.

Cleistes is a member of tribe Pogonieae, which includes Asiatic, North and South American genera. Molecular analyses (Cameron & Chase 1999; Pansarin *et al.* 2008) recognize two clades, an Asiatic and North American and a South American (see Figure 5 for a summary on this subject). The former includes species of *Isotria*, *Pogonia* and two *Cleistes* species, and the latter exclusively *Cleistes* species. Hence, *Cleistes* is paraphyletic. Patterns of stomata distribution (hypostomatous/amphistomatous) in Pogonieae are possibly linked to the evolutionary history of the group. The North American *Isotria* and North American-Asiatic *Pogonia* have hypostomatous leaves (Cameron & Chase 1999). This character state is assumed as plesiomorphic in the tribe, having been shifted to the amphistomatous condition several times along the evolution of *Cleistes* (Pansarin *et al.* 2008). It is worth mentioning that *C. tenuis* is a basal species in the South American clade (Figure 5) and have hypostomatous leaves. If the hypostomatous condition is really plesiomorphic, then there must have been reversals along the evolution, because a highly derived clade in Pogonieae evolution combines *C. aphylla* and *C. pusila* (Pansarin *et al.* 2008), both with hypostomatous leaves. On the other hand, the sister clade *C. itatiaie* (*C. cipoana*/*C. exilis*) have amphistomatous leaves (Table 2). This character is obviously useful for species characterization, but probably not valuable to indicate kinship among species. For example, a strongly supported clade combines *C. libonii*, *C. caloptera* and *C. metallina* (Figure 5; Pansarin *et al.* 2008), while the first species have hypostomatous, and the other two have amphistomatous leaves (Table 2).

A similar comment holds for the morphology (Figures 1-3) and chemical composition of wax deposits (Table 3, Figure 4). Absence of platelets and film, rugose wax characterize *C. aphylla*, *C. cipoana*, *C. exilis*, *C. libonii* and *C. pusilla*, while platelets characterize *C. bella*, *C. caloptera*, *C. casta-*

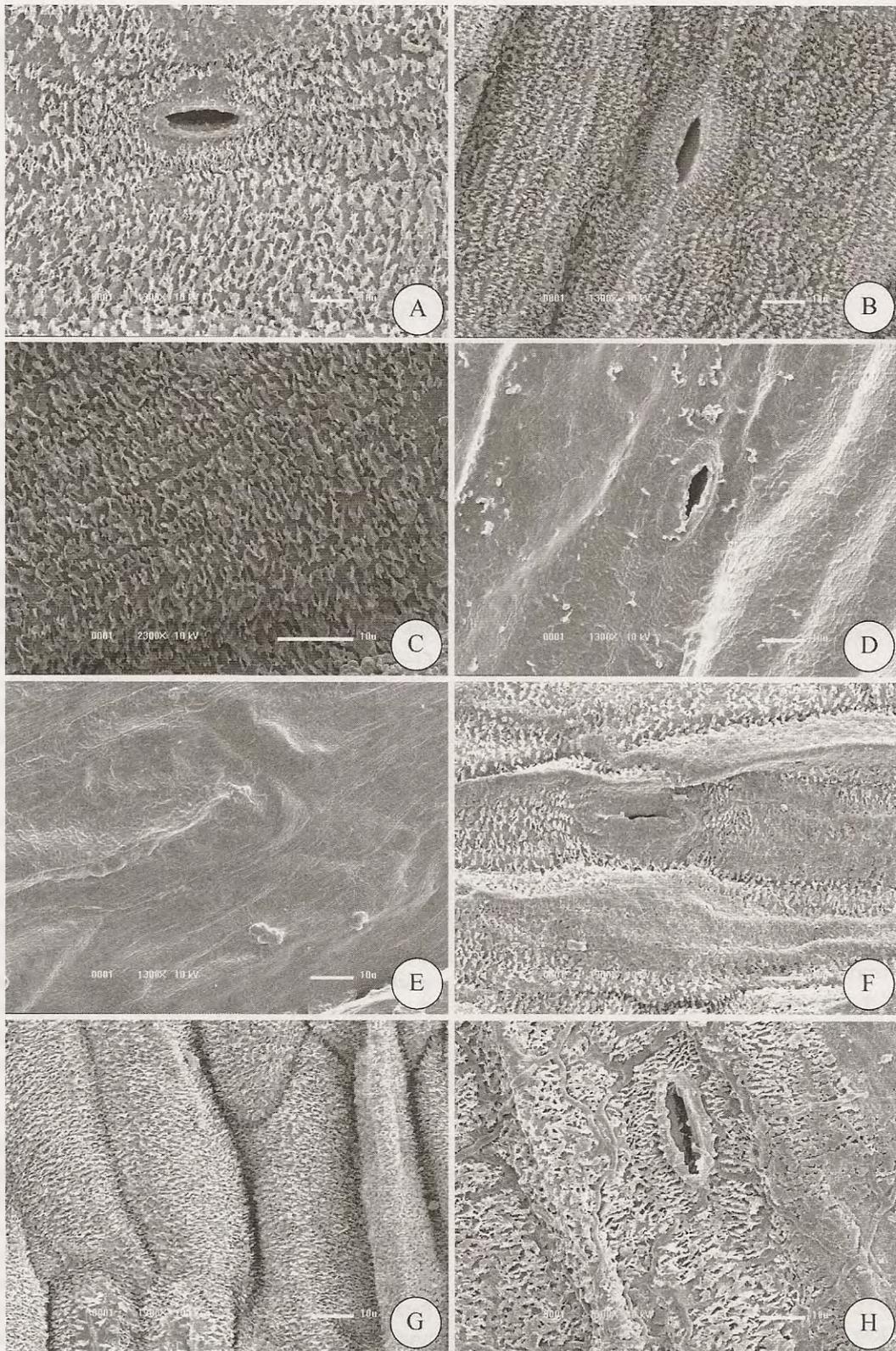


Fig. 3. A-H. Cuticular waxes of leaves of *Cleistes* species under SEM. Scale bars = 10 μ m. A. *C. metallina*: abaxial. Coll. Pansarin & Mickeliunas 909 (UEC). B. *C. paranaensis*: adaxial. Coll. Pansarin & Mickeliunas 920 (UEC). C. *C. pluriflora*: adaxial. Coll. Aona & Moraes 642 (UEC). D. *C. pusilla*: abaxial. Pansarin & Mickeliunas 898 (UEC). E. *C. pusilla*: adaxial. Pansarin & Mickeliunas 898 (UEC). F. *C. tenuis*: abaxial. Coll. Pansarin & Batista 741 (UEC). G. *C. tenuis*: adaxial. Coll. Pansarin & Batista 741 (UEC). H. *C. ramboi*: abaxial. Pansarin & Batista s.n. (UEC 117396).

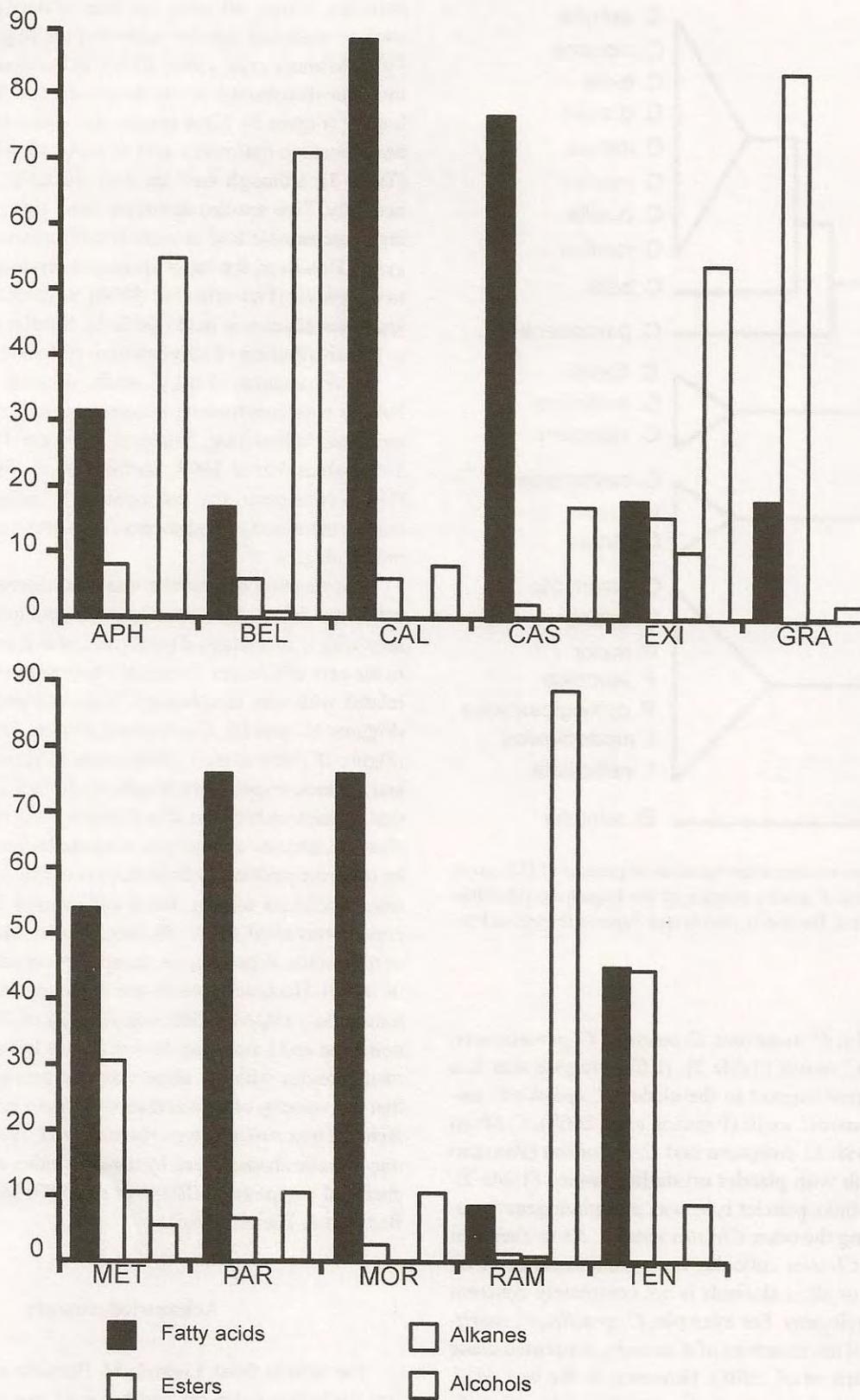


Fig. 4. Chemical classes of constituents and their concentration in cuticular surface waxes of leaves of *Cleistes* species. APH: *C. aphylla*; BEL: *C. bella*; CAL: *C. caloptera*; CAS: *C. castanoides*; EXI: *C. exilis*; GRA: *C. gracilis*; MET: *C. metallina*; PAR: *C. paranaensis*; MOR: *C. moritzii*; RAM: *C. ramboi*; TEN: *C. tenuis*. Ordinate axes: concentration.

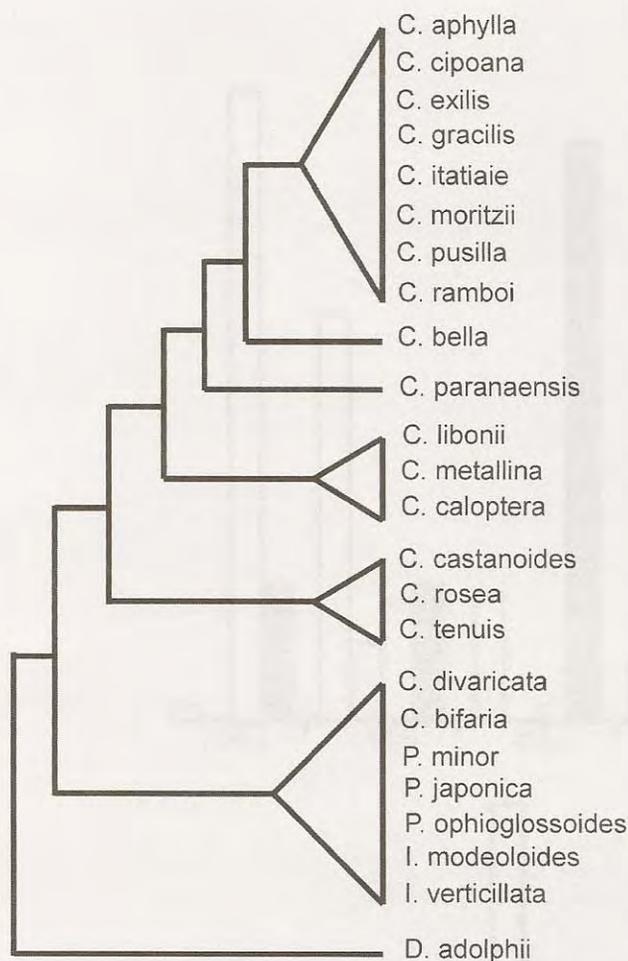


Fig. 5. Phylogenetic relationships based on sequences of ITS, *psaB*, *rbcL*, *rps16* and *trnL-F* among species of the Pogonieae (Orchidaceae) genera *Cleistes*, *Duckeella*, *Isotria* and *Pogonia* (based on Pansarin *et al.* 2008.)

noides, *C. gracilis*, *C. metallina*, *C. moritzii*, *C. paranaensis*, *C. ramboi* and *C. tenuis* (Table 2). If film, rugose wax is a state that may give support to the clades *C. aphylla*/*C. pusilla* and *C. cipoana*/*C. exilis* (Pansarin *et al.* 2008), *C. libonii* shares a clade with *C. caloptera* and *C. metallina* (Pansarin *et al.* 2008), both with platelet crystalline waxes (Table 2). No consistency links platelet type wax and phylogenetic relationships among the other *Cleistes* species. As to chemical composition of *Cleistes* cuticular wax, predominance of either alkyl acids or alkyl alcohols is not completely coherent with *Cleistes* phylogeny. For example, *C. gracilis*, *C. moritzii* and *C. ramboi* are members of a strongly supported clade (Figure 5; Pansarin *et al.* 2008). However, in the wax of *C. gracilis* alkanes predominate, in *C. moritzii* acids and in *C. ramboi* alkyl alcohols (Figure 4). Hence, it is possible that predominance of a certain class of wax constituent may be a useful specific characteristic, but not a reliable criterion for species grouping. Distribution of homologues of wax con-

stituents, mainly alkanes, has been widely and successfully used to establish species relationships (e.g., Salatino *et al.* 1989; Mimura *et al.* 1998; 2004). In the case of *Cleistes*, homologue distribution is also not coherent with the group phylogeny (Figure 5). Most species are characterized by having hexadecanoic (palmitic) acid as main alkyl acid homologue (Table 3), although they are only distantly related phylogenetically. Two species deviating from this pattern and sharing octacosanoic acid as main homologue are *C. bella* and *C. exilis*. However, the latter species is phylogenetically closer to *C. aphylla* (Pansarin *et al.* 2008), which has as main homologue hexadecanoic acid (Table 3). Similar comments apply to the distribution of alkyl alcohol and alkane homologues.

Predominance of alkyl acids, alkanes and alkyl alcohols as wax constituents is common in other groups within monocotyledons (e.g., Freeman & Turner 1985; Avato *et al.* 1990; Meusel *et al.* 1994; Barthlott *et al.* 1998). Jeffrey *et al.* (1975) considered that competition of certain wax mixtures may occur among constituents for expression of their typical morphologies.

The structure of cuticular waxes is dependent on its chemical composition, so that dominant wax compounds often correlates with wax crystalloid types (review in Barthlott *et al.* 1998). In the case of *Cleistes*, chemical composition is clearly not correlated with wax morphology. Wax with platelets of *C. bella* (Figures 1C and D), *C. caloptera* (Figure 1E) and *C. gracilis* (Figure 2E) have as main components alkyl alcohols, alkyl acids and alkanes, respectively (Figure 4). In fact, it has been shown that correlation between wax chemistry and morphology is not absolute, because a same type of crystalloid may be determined by different predominant chemical constituents (as seems is the case of *Cleistes* waxes). Terete rodlets may have as dominant constituents alkyl esters, alkanes, primary alcohols, aldehydes or terpenoids, depending on the species considered (Barthlott *et al.* 1998). Hentriacontan-16-one is an important constituent of transversely ridged rodlets wax deposits of *Aristolochia*, Magnoliaceae and Lauraceae. However, this ketone is absent from many species with the same wax micromorphology. It seems that the velocity of crystallization is also a crucial determinant factor of wax structure type (Meusel *et al.* 1999). Cuticular wax may thus be characterized by mean of either crystalloid type or chemical composition (Baum *et al.* 1989; Meusel *et al.* 1994; Barthlott & Theisen 1995).

Acknowledgements

The authors thank Ludmila M. Pansarin and João A.N. Batista for help in fieldwork and Antonia Lima and Adriane Sprogis for assistance in SEM analyzes. This study is part of a PhD thesis of ERP in the Plant Biology course of the 'Universidade Estadual de Campinas' (São Paulo, Brazil). Financial support provided by FAPESP and CNPq is gratefully acknowledged.

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