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Taxonomic significance of the epicuticular wax composition in species of the genus *Clusia* from Panama

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Abstract

We attempted to separate species of the genus *Clusia* according to the concentration of linear alkanes (C25 and C35), and the presence and diversity of terpenes in epicuticular wax extracts. We collected leaves of 15 *Clusia* species growing in mountain forests of Panama (Cerro Jefe 1007 m and Altos de Campana 800 m a.s.l.) and from cultivated plants at two lowland sites. Leaf surfaces were washed gently with hexane to extract epicuticular waxes, which were analyzed using gas chromatography and mass spectrometry. The predominant alkanes were C29, C31, and C33. In the extract the ratio C31/C29 was $\leq$1 in 6 of the 15 species analyzed: *Clusia multiflora*, *Clusia peninsulae* (Hammel ined.), *Clusia stenophylla*, *Clusia liesneri*, *Clusia coclensis*, and *Clusia triflora*. The concentrations of C29 and C33 were inversely related, the latter being above 10% in *Clusia divaricata*, *Clusia pratensis*, *Clusia rosea*, *Clusia uvitana*, and *Clusia valerioi*. Proportion of triterpenes was less than 5% in the species *C. minor*, *C. pratensis*, *C. uvitana*, *C. rosea*, *Clusia cylindrica*, *C. divaricata*, and *C. valerioi*. The rest contained squalene, and specific triterpenes such as β-amyrine in *C. liesneri*, betuline in *Clusia osseocarpa*, taraxerol in *C. stenophylla*, and lupeol in *C. multiflora*. The variety of triterpenes was higher in *Clusia liesneri* (5) and *C. multiflora* (3). The results suggest that groups of species can be distinguished within the genus according to the presence of terpenes and ratios of linear alkanes. These groups overlap with those generated by other classifications using morphology and nuclear ribosomal DNA.

Keywords: Alkanes; *Clusia;* Epicuticular wax; Taxonomy; Triterpenes; Panama

1. Introduction

The genus *Clusia* is highly diversified in Central and South America containing around 300 species, depending on taxonomical criteria employed to define genera within the Clusiaceae (Hammel, 1986; Pipoly et al., 1998). Ecological and physiological studies have been conducted on several species of the genus looking into their variety of photosynthetic types: C3, CAM and C3–CAM intermediate species (Lüttge, 1996). Recently, molecular analyses have been conducted to elucidate phylogeny of the Clusiaceae (Gustafsson et al., 2002) and to look into the evolutionary origins

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of crassulacean acid metabolism (CAM) within the genus *Clusia* (Vaasen et al., 2002; Gehrig et al., 2003). *Clusia*'s mostly dioecious nature, usually short-lived flowers, and its frequently resinous character complicate identification of species of this genus (Hammel, 1986). The composition of epicuticular waxes could be a potential marker for species, or species groups, reflecting both ecological and genetic relationships (Barthlott and Wollenweber, 1981; Gülz, 1994). Epicuticular waxes play an important role in restricting cuticular transpiration, of potential significance in habitats receiving large diurnal solar radiation loads (Schreiber and Riederer, 1996; Oliveira et al., 2003). There have been several attempts to use profiles of cuticular waxes as taxonomic criteria to differentiate groups of species within families or genera (among others Maffei, 1996; Mimura et al., 1998). The biosynthesis of epicuticular waxes is under close genetic control, therefore, it could provide insight into genetic differentiation among related group of species (Lemieux, 1996). In this paper we report on the alkane and triterpene profiles of epicuticular waxes of *Clusia* species collected in Panama. The objective was to examine the potential of wax composition to discern among species or groups of species in this genus.

2. Material and methods

The *Clusia* species from Panama analyzed here are shown in Table 1. Species are arranged by the site of their collection and grouped according to Hammel (1986). The Cerro Jefe area is located within the Chagres National Park. The species were collected near the summit (up to 1007 m above sea level), in an open forest dominated by 8 to 15 m

Table 1
Clusia species collected in Panama

Species	Site	Hammel's group	Sample N°
C. divaricata Maguire (904)	Gamboa	2	(55–60)
C. minor L. (228)	Gamboa	2	(1–6)
C. pratensis Seeman	Gamboa	2	(25–30)
C. pratensis (978)	Gamboa	2	(13–18)
C. rosea Jacquin	Gamboa	2	(7–12)
C. rosea	Gamboa	2	(37–42)
C. uvitana Pittier	Gamboa	2	(43–48)
C. valerioi Standley (229)	Gamboa	2	(31–36)
C. valerioi (937)	Gamboa	2	(49–54)
C. valerioi (80)	Gamboa	2	(19–24)
C. rosea	Jardin Summit	2	(67–72)
C. uvitana (fem)	Jardin Summit	2	(73–78)
C. cretosa Hammel ined.*	Campana	3	(91–96)
C. divaricata	Campana	2	(115–120)
C. multiflora Kunth	Campana	3	(109–114)
C. peninsulae Hammel ined.**	Campana	2	(121–126)
C. pratensis	Campana	2	(79–84)
C. rosea	Campana	2	(85–90)
C. stenophylla Standley	Campana	3	(97–102)
C. uvitana	Campana	2	(103–108)
C. coclensis Standley (fem)	Cerro Jefe	3	(133–138)
C. cretosa *	Cerro Jefe	3	(127–132)
C. cylindrica Hammel	Cerro Jefe	1	(163–168)
C. liesneri Maguire (mas)	Cerro Jefe	2	(145–150)
C. multiflora (green leaf)	Cerro Jefe	3	(151–156)
C. multiflora (pink leaf)	Cerro Jefe	3	(157–162)
C. osseocarpa Maguire (fem)	Cerro Jefe	1	(139–144)
C. triflora Cuatrecasas	Cerro Jefe		(169–174)

At the Gamboa site the numbers in parenthesis following species names are laboratory identification numbers. The symbols * and ** indicate unpublished species as registered in the W^{3}Tropicos database of the Missouri Botanical Garden (http://mobot.mobot.org/W3T/). These species were included in Gehrig et al. (2003), as *Clusia* sp. A* and *Clusia* sp. E*. Hammel's groups are 1: *Clusia flava* group; 2: *Clusia minor* group; 3: *Clusia multiflora* group (Hammel, 1986).

tall trees, richly covered by epiphytes (Gentry, 1985; Pierce et al., 2002; Krause et al., 2003). This area has a high floristic endemism (Lewis, 1971). The vegetation at the Altos de Campana National Park is a mountain tropical rain forest, ranging from 600 to 1000 m above sea level on a volcanic substrate. The experimental site at Gamboa is a primary site for ecophysiological projects of STRI in Panama (near sea level). The Summit Botanical Garden is a small municipal garden in the vicinity of Panama City at around 200 m a.s.l. Identification of collected species was conducted by Jorge Aranda based on samples deposited at the Smithsonian Tropical Research Institute in Panama.

Epicuticular waxes were extracted from adaxial and abaxial sides of mature, healthy leaves ($n = 3$). Leaves were drawn on paper for subsequent measurement of area (Licor LI-3100 area meter). Leaves were washed with 50 ml hexane (GC grade) for 90 s using a wash bottle. Extract was evaporated to dryness at room temperature in a ventilated fumehood. This procedure extracts only surface hexane-soluble compounds without disturbing the leaf interior. Dry extract was weighed (10 μg precision), and redissolved in 250 μL hexane. Subsequently, 1 μL was injected into a gas chromatograph (Hewlett-Packard 6890, flame ionization detector, HP5 column 30 m × 0.25 mm × 0.2 μm, using He as carrier gas). Temperature was increased at 10 °C/min from 160 to 240 °C, and then 4 °C/min up to 320 °C and maintained for 10 min. Injector was maintained at 250 °C and the detector at 320 °C. Results are reported as amount of extracted material per unit of leaf area, and as average percentage of chromatogram area for each chemical species. Compound identification was based on co-injection with commercial standards and analysis of a subsample in a GC/MS Varian 3400CX with a Varian Saturn 2000 mass detector with ion trap analyser. The spectrum was a full scan and the mass range was 15 to 650 amu. (Spectrum Libraries: Wiley Registry of Mass Spectral Data and NIST 98 Standard Reference Database.)

Total amount of extracts per leaf side, and species were compared using a one-way ANOVA. The number of replicates per species resulted in heterogeneous variances, therefore we performed a Welch's ANOVA allowing for unequal standard deviations and tested differences between means with Tukey's HSD. All statistical analyses were conducted using JMP Statistics and Graphics Software (2002).

3. Results

3.1. Leaf dimensions

Leaf area of species analyzed here ranged from ≤ 50 cm^2 in the species *Clusia cylindrica*, *Clusia triflora*, *Clusia coclensis*, *Clusia osseocarpa*, *Clusia minor*, *Clusia peninsulae* (Hammel ined.), *Clusia stenophylla* and *Clusia liesneri*, to >75 cm^2 in *Clusia divaricata*, *Clusia valerioi*, *Clusia rosea*, *Clusia multiflora* and *Clusia cretosa* (Hammel ined.). Leaves of *Clusia* species are usually rather thick, the only exception within this group was *C. valerioi* found growing in the understory of a mountain forest in Altos de Campana.

3.2. Wax yield per species

Total wax yield varied by one order of magnitude, from less than 4 to more than 20 μg cm^{-2} (Table 2). Most species yielded similar or only slightly different amounts of wax from both sides (20 out of 27 samples). However, wax yield of the adaxial side of the species *C. stenophylla*, *C. coclensis*, and *C. osseocarpa* was more than twice as much as from the abaxial side, the contrary was observed in *C. peninsulae*. There was no clear relationship between site of collection or species and the wax load measured.

3.3. Alkanes

The alkane composition of epicuticular waxes of *Clusia* was rather homogeneous, but varying in relative abundance (Table 3). The alkanes C28, C29, C30, C31, C32 and C33 were present in different amounts in practically all the samples analyzed. The alkanes C26 or C35 were present in a few species but were mutually exclusive. The most abundant alkanes were C29 and C31, accounting for 30–60% of the total in *C. stenophylla*, *C. multiflora*, and *C. divaricata*, and over 70% in the rest of the species analyzed. The highest C31/C29 ratios were measured in the species *Clusia uvitana* (Campana), *Clusia pratensis* (Gamboa), *C. cylindrica*, *C. valerioi* and *C. divaricata* (Campana and Gamboa). An interesting relationship is the inverse logarithmic correlation between C29 and C33 ($R = 0.91$),

Table 2
Mean amount of hexane-soluble epicuticular waxes ($\mu g\,cm^{-2}$) from leaves of *Clusia* species

Species	Adaxial	Abaxial	Adax.−Abax.	Total
C. divaricata 2	1.4	0.8	+	2.2 f
C. rosea 3	1.6	1.6	0	3.2 f
C. divaricata 1	2.2	1.5	+	3.6 f
C. pratensis 1	1.9	2.0	0	3.9 f
C. valerioi (80)	2.4	2.2	0	4.6 ef
C. rosea 2	2.8	2.7	0	5.6 cdef
C. uvitana 1	3.1	3.0	0	6.1 ef
C. rosea 1	3.0	3.2	0	6.2 ef
C. multiflora 3	2.6	3.7	−	6.3 cdef
C. cretosa 1	3.9	2.4	+	6.3 ef
C. valerioi (229)	3.2	3.2	0	6.5 cdef
C. uvitana 2	3.8	3.4	0	7.2 def
C. rosea 4	5.2	3.9	+	9.1 bcdef
C. stenopylla	7.1	2.3	+++	9.4 bcdef
C. minor	3.9	5.7	−	9.6 bcdef
C. multiflora (pink)	5.5	6.8	−	12.3 bcdef
C. coclensis	9.4	4.6	+++	13.9 abcdef
C. triflora	8.8	6.7	+	15.5 abcdef
C. pratensis 2	8.9	6.9	+	15.9 abcdef
C. liesneri	8.8	7.2	+	16.1 abcdef
C. cylindrica	11.8	8.5	+++	20.3 abcde
C. peninsulae	5.8	16.9	−−−	22.7 abcd
C. valerioi (937)	11.9	11.5	0	23.4 abc
C. multiflora (green)	18.4	6.4	+++	24.8 ab
C. osseocarpa	21.1	8.2	+++	29.3 a

Average of three leaves per species. Number after name indicate that the species was sampled in different sites. Number in parenthesis corresponds to the identification in the Gamboa garden. In the column "Total" numbers followed by the same letter are not statistically different (Tukey HSD test at $p = 0.05$, and Welch's test for means allowing unequal standard deviations $F = 30.78$; $p < 0.0001$). Range of side differences: $0 = \leq 0.5$; + or − = 0.5–2.0; ++ or −− = 2.1–3; +++ or −−− = >3.

because it may indicate a pattern in the biosynthetic pathway of these compounds in *Clusia* (Fig. 1). The C31/C29 ratio and %C33 were also linearly correlated ($R = 0.86$)

We attempted to identify consistent groups of species using alkane relative concentrations and ratios. The first classification used the C31/C29 ratios, and we obtained two groups of species: (a) C31/C29 > 1 ($n = 19$, average 2.91) and (b) C31/C29 < 1 ($n = 12$, average 0.67). Within the first group we separated the set of species with C33 below and above 10%, whereas in the second group C33 was consistently below 10%. Finally we used the sum of C26 to C28 to perform a third separation. *C. stenophylla* and *C. multiflora* from Cerro Jefe showed the larger values of this parameter, and we considered them to constitute a separate group. The *C. multiflora* sample of Altos de Campana differed from those of Cerro Jefe, with less than 50% wax yield, C31/C29 ratio < 1 and a small content of C26 to C28 alkanes.

This analysis allowed us to separate four species groups (Table 4):

Alkane Group I (in parenthesis average value for each parameter): C31/C29 > 1 (1.7), C33 < 10% (6.5), $\sum$(C26 to C28) < 10% (4.0)
Alkane Group II: C31/C29 > 1 (3.4), C33 > 10% (20.3), $\sum$(C26 to C28) < 10% (4.3)
Alkane Group III: C31/C29 < 1 (0.8); C33 < 10% (6.7); $\sum$(C26 to C28) < 10% (4.5)
Alkane Group IV: C31/C29 < 1 (0.6); C33 < 10% (6.0); $\sum$(C26 to C28) > 10% (34.0)

3.4. Triterpenes

The spectra of triterpenes showed marked differences among species (Table 4). We could distinguish clearly three groups of species: A, no triterpene detectable; B, triterpene fraction below 5%; and C, triterpene fraction above 10%.

Triterpenes characterized several species. β-Amyrine occurred only in *C. liesneri*, lupeol was only detected in *C. multiflora*, betuline and sitosterol only in *C. osseocarpa*, taraxerol only in *C. stenophylla*. Compounds grouped under Cholestans were detected only in samples of *C. multiflora* but they could not be thoroughly identified due to

Table 3
Percentages of alkanes, C31/C29 ratios and classification of *Clusia* spp. groups

Species and site	Group	C26	C27	C28	C29	C30	C31	C32	C33	C35	C31/C29	∑(C26 to C28)
C. cretosa CJ	I		0	2	43	3	48	1	3		1.1	2
C. cylindrica CJ	I			4	19	2	57	2	8	9	3.0	4
C. minor (G228)	I		1	4	36	3	47	2	8		1.3	5
C. osseocarpa CJ	I		2	3	38	3	46	1	7		1.2	5
C. divaricata AC	II			4	6	1	45	5	35	3	7.0	4
C. divaricata (G904)	II			2	9	1	51	4	34		5.6	2
C. pratensis (G978)	II			2	11	4	59	5	20		5.2	2
C. pratensis AC	II			1	22	3	61	3	10		2.7	1
C. rosea AC	II	0	3	1	29	2	52	1	10	2	1.8	4
C. rosea JS	II			4	15	2	50	0	24	5	3.3	4
C. rosea G1	II		1	8	17	2	45	3	25		2.6	9
C. rosea G2	II			4	19	2	51	3	21		2.6	4
C. uvitana G	II			5	24	1	59	0	13		2.4	5
C. uvitana JS	II	0	0	2	25	2	51	2	16	2	2.1	2
C. uvitana AC	II			2	20		60		19		3.0	2
C. valerioi (G80)	II	1	2	1	27	2	56	2	13		2.1	4
C. valerioi (G937)	II	1	2	1	13		58	3	25		4.5	4
C. valerioi (G229)	II	4	6	3	25		50	4	19		2.0	13
C. coclensis CJ	III		3	2	43	4	35	14			0.8	5
C. liesneri CJ	III		4	1	42	4	40	3	7		1.0	5
C. peninsulae AC	III			1	51	3	37		8		0.7	1
C. triflora CJ	III			3	57	4	30	1	5		0.5	3
C. stenophylla AC	IV	17		16	22	6	8	5			0.4	33
C. multiflora g CJ	IV	26	31	5	30	2	5	1	0		0.2	62
C. multiflora p CJ	IV	14	14	8	33	2	19	1			0.6	36
C. multiflora AC	IV	1	4		38	3	46		6		1.2	5

CJ: Cerro Jefe, G: Gamboa; AC: Altos de Campana; JS: Summit Garden; p and g: pink and green colored leaves. Zero indicates that the compound is detected but below 1%.

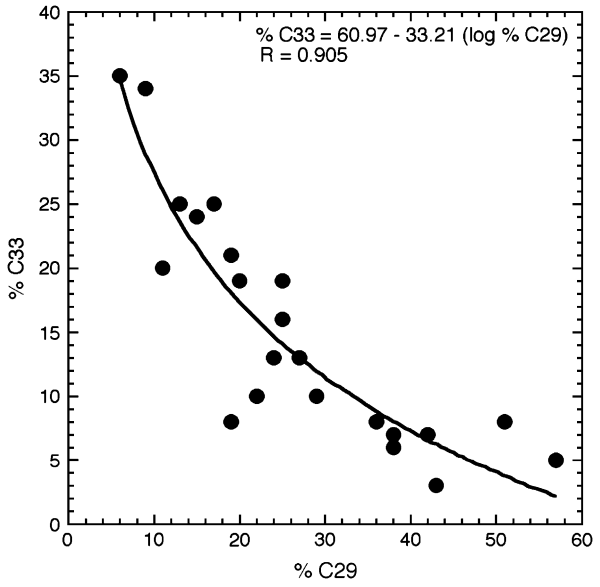

Fig. 1. Inverse logarithmic relationship between the percentages of the alkanes C29 and C33 in the extracts from leaves of *Clusia* species from Panama.

Table 4
Terpene profiles obtained from epicuticular waxes extracted with hexane from *Clusia* plants

Species	Alkanes (%)	Terpenes (%)	Characteristic terpenes
Group A			
C. minor G	67	0	
C. pratensis G	60	0	
C. pratensis AC	88	0	
C. rosea G	73	0	
C. rosea JS	76	0	
C. rosea G	83	0	
C. rosea AC	85	0	
C. uvitana G	58	0	
C. uvitana AC	60	0	
C. uvitana JS	100	0	
Group B			
C. cylindrica CJ	37	1	
C. divaricata AC	43	2	
C. divaricata (G904)	85	2	
C. valerioi (G229)	19	4	
C. valerioi (G80)	40	3	
C. valerioi (G937)	43	2	
Group C			
C. coclensis CJ	25	50	Friedelin (1.0); Friedolane (4.9); Kaurene (41.5); Verticiol (2.2); squalene (0.4)
C. cretosa CJ	16	56	Friedolane (5.2); Kaurene (48.9); Verticiol (2.4)
C. liesneri CJ	46	14	a − AMYRINE (3.9); b − AMYRINE (1.2); Friedelin (6.0); Friedolane (1.6); Squalene (1.7)
C. multiflora CJp	30	26	CHOLESTANS (5.1); Friedelin (2.5); Kaurene (15.4); LUPEOL (2.5); Squalene (0.8)
C. multiflora AC	43	10	CHOLESTANS (3.3); LUPEOL (4.4); Squalene (1.9)
C. multiflora CJg	50	33	CHOLESTANS (4.6); Friedelin (3.5); Kaurene (4.5); LUPEOL (19.2); Squalene (0.7)
C. osseocarpa CJ	41	30	BETULIN (3.7); Friedelin (17.7); SITOSTEROL (1.3); Squalene (0.6)
C. peninsulae AC	58	13	Friedelin (12.3); Squalene (0.6)
C. stenophylla AC	40	29	TARAXEROL (22.1); Squalene (6.7)
C. triflora CJ	27	25	Friedelin (2.0); Kaurene (21.6); Squalene (1.9)

Compounds found only in one species are given in capital letters. Site denominations as in Table 3.

lack of standards. Combinations of triterpenes allowed the characterization of other species such as *C. coclensis* by the presence of Friedolane and Verticiol, and *C. cretosa* by the presence of Friedelin and Verticiol.

4. Discussion

Epicuticular waxes cover the external side of the leaf epidermis of all higher plants (Barthlott, 1989). The composition of epicuticular waxes extracted with a variety of non-polar solvents has been used as a potential tool to differentiate phylogenetically related groups (Barthlott and Wollenweber, 1981; Barthlott et al., 2003; Gülz, 1994; Maffei, 1996).

The *Clusia* species analyzed here had comparatively low wax loads per unit area (2–30 $\mu g\,cm^{-2}$). Amaral et al. (1985) reported chloroform-extracted wax loads ranging from 14 to 700 $\mu g\,cm^{-2}$ for savanna (*Cerrado*) trees and Oliveira and Salatino (2000) reported levels from 60 to 75 $\mu g\,cm^{-2}$ for dry forest (*Caatinga*) trees. However, the loads are equal or higher than those of leaves of *Pisum sativum* extracted with chloroform (Jetter et al., 2000). The relative lower loads measured in *Clusia* species are probably related to the lower extraction power of hexane compared to chloroform. Use of hexane prevented extraction of intracuticular or internal leaf lipids.

Alkane biosynthesis proceeds from very long chain aldehydes through a process of decarbonylation, that yields series of odd carbon molecules (Bianchi, 1987; Lemieux, 1996; Post-Beittenmiller, 1996). The chain length of the precursors available for decarbonylation therefore, determines the alkane profiles. The most abundant, and widely distributed alkanes in higher plants are *n*-nonacosane (C29) and *n*-hentriacontane (C31) (Caldicott and Eglinton, 1973).

Their abundance allowed the identification of alkanes of terrestrial vegetation origin in dust transported by winds from the African continent into mid-Atlantic atmosphere (Schefuß et al., 2003). We cannot explain the biochemical basis for the predominance of C31 and C29, beyond the statement that there is a preferential accumulation of the precursor aldehydes near the epidermal cells where the decarbonylation takes place. We emphasize the finding that C31/C29 ratios change from above to below 1 and they appear to be consistent within species. The proportion of alkanes with chain lengths from C26 to C28 decreases as the proportion of alkanes with chain lengths from C30 to C35 increases, a pattern possibly associated with the sequential biosynthesis of these compounds. This probably explains the inverse logarithmic relationship between percentages of C29 and C33 ($R = 0.905$, see Fig. 1) that allowed the distinction of C33 and C29 accumulating species.

Epicuticular wax composition allowed the differentiation of species groups, according to the proportion of alkanes and the presence of specific terpenes, thus confirming and extending previous findings on *Clusia* species from Venezuela (Medina et al., 2004) including *C. minor* and *C. multiflora*. We distinguished four alkane groups and three terpene groups. Results are consistent within species that were collected at different sites. However, the samples of *C. multiflora* from Cerro Jefe and Campana differed in their C31/C29 ratios. Such difference has been observed between male and female samples of this species (Medina et al., 2004), but we could not explain the result on this basis because the Panama samples were sterile.

Comparing the species composition of these chemical groups with that of the groups defined by Hammel (1986) based on morphology, we found a number of interesting overlaps that deserve further analysis (Table 5). The species belonging to Hammels' *multiflora* group analyzed here (*C. coclensis*, *C. stenophylla*, *C. multiflora*, *C. cretosa*, and possibly *C. triflora*) fall within the triterpene rich group (C) and, except *C. cretosa*, the alkane groups III and IV with C31/C29 ratios < 1, and C33 below 10%. All of Hammels' group *minor* species contain very small amounts of triterpenes in their wax (except *C. liesneri)*, while the Hammels' group *flava* (*C. cylindrica* and *C. osseocarpa*) overlap with the alkane Group I with C31/C29 ratios < 1.

Another emerging pattern is the relationship between the wax groups and those resulting from the genetic analyses conducted by Gehrig et al. (2003). These authors separated a number of species of *Clusia* occurring in Panama and elsewhere using nuclear ribosomal DNA, roughly corresponding with the separation proposed by Hammel (1986) based on morphological characteristics. Group III of Gehrig et al. overlaps with our alkane Groups III, and IV and terpene Group III. The species *C. liesneri* and *C. peninsulae* depart from this relationship.

Our results suggest that wax composition allows the separation of natural groups of *Clusia* species that are comparable to those obtained using morphologic or genetic characteristics. This statement cannot yet be generalized due to the restricted number of species analyzed. We are currently extending this analysis to a larger number of species found in the Guayana region of southern Venezuela.

Table 5
Comparison of species grouping according to chemical, morphological, and genetic markers

Species	Alkanes	Terpenes	Hammel (1986)	Gehrig et al. (2003)
C. cretosa	I	C	*multiflora*	III
C. cylindrica	I	B	*flava*	I
C. minor	I	A	*minor*	II
C. osseocarpa	I	C	*flava*	
C. divaricata	II	B	*minor*	II
C. pratensis	II	A	*minor*	II
C. rosea	II	A	*minor*	I
C. uvitana	II	A	*minor*	II
C. valerioi	II	B	*minor*	I
C. coclensis	III	C	*multiflora*	III
C. liesneri	III	C	*minor*	II
C. peninsulae	III	C	*minor*	II
C. triflora	III	C	*multiflora*	III
C. stenophylla	IV	C	*multiflora*	III
C. multiflora	IV	C	*multiflora*	III

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