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Identification of volatile compounds from three species of *Cyathodium* (Marchantiophyta: Cyathodiaceae) and *Leiosporoceros dussii* (Anthocerotophyta: Leiosporocerotaceae) from Panama, and *C. foetidissimum* from Costa Rica

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Summary: *Cyathodium* is a thalloid marchantialean liverwort with five species reported for the Neotropics. Three species that occur in Panama (*C. bischlerianum*, *C. spruceanum*, *C. cavernarum*) and one from Costa Rica (*C. foetidissimum*) were studied chemically. Female and male plants of the dioecious *C. spruceanum* were very similar in their chemical composition except for two compounds that were found only in female plants. All samples of *C. spruceanum* and *C. bischlerianum* contained, in less than three percent, the sesquiterpenes germacrene D and bicyclogermacrene. The presence of these compounds suggests a close affinity between these two species. *Cyathodium bischlerianum* contained mainly aromatic monoterpenes with nerolidol as the main compound. *Cyathodium cavernarum* also had a very distinct chemical composition with an octane derivative as its major compound. Indole compounds were found only in *C. foetidissimum*. The presence of these compounds in plants from Costa Rica and Tahiti suggests that they could be considered as potential chemosystematic markers for the species. Based on their chemical composition there is a clear distinction between the four species of *Cyathodium* studied. The chemistry of these species supports previous morphological and genetic studies. Only two compounds could be identified in *Leiosporoceros dussii*. There is a need for additional genetic and chemical studies on neotropical *Cyathodium* and *Leiosporoceros*.

Key words: Costa Rica, diterpenes, hornwort, Neotropical liverworts, Panama, sesquiterpenes, skatole.

Resumen: Identificación de compuestos volátiles de tres especies de *Cyathodium* (Marchantiophyta: Cyathodiaceae) y *Leiosporoceros dussii* (Anthocerotophyta: Leiosporocerotaceae) de Panama y *C. foetidissimum* de Costa Rica. *Cyathodium* es una hepática marchantial con cinco especies comunicadas para el Neotrópico. Se estudió la composición química de tres especies que crecen en Panamá (*C. bischlerianum*, *C. spruceanum*, *C. cavernarum*) y, una que crece en Costa Rica (*C. foetidissimum*). Plantas femeninas y masculinas del dioico *C. spruceanum* fueron muy similares en su composición química excepto por dos compuestos que se encontraron solo en plantas femeninas. Todas las muestras de *Cyathodium spruceanum* y *C. bischlerianum* contenían, en un porcentaje de menos del tres por ciento, los sesquiterpenos germacreno D y biciclogermacreno. La presencia de estos compuestos sugiere una afinidad muy cercana entre las dos especies. *Cyathodium bischlerianum* contiene principalmente monoterpenos aromáticos con nerolidol como el compuesto principal. *Cyathodium cavernarum* también tuvo una composición química muy distintiva con un derivado del octano como su compuesto principal. Compuestos de indol fueron encontrados solo en *C. foetidissimum*. La presencia de estos compuestos en plantas de Costa Rica y Tahiti sugiere que puedan ser marcadores quimosistemáticos para esta especie.

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Las cuatro especies de *Cyathodium* estudiadas se pueden distinguir de acuerdo con su composición química. La química de estas especies apoya estudios genéticos y morfológicos previos. Solo dos compuestos pudieron ser identificados en *Leiosporoceros dussii.* Se necesitan estudios genéticos y químicos adicionales para los *Cyathodium* y *Leiosporoceros* neotropicales.

Palabras claves: Antocerote, Costa Rica, diterpenos, escatol, hepáticas neotropicales, Panamá, sesquiterpenos.

Introduction

Bryophytes are a major group of land plants that occur in most ecosystems and substrates from the Arctic to the Antarctic, except in the sea. Taxonomically, they are placed between the green algae and the vascular plants (ferns and flowering plants). There are, ca. 22,000-25,000 species of bryophytes in three lineages (Magill, 2010; Villarreal *et al.*, 2010; von Konrat *et al.,* 2010), the liverworts (Marchantiophyta), the hornworts (Anthocerotophyta) and the mosses (Bryophyta). They are considered the closest modern relatives of the ancestors of the first terrestrial plants (Renzaglia *et al.*, 2007). The liverworts have been one of the most chemically studied of all bryophytes. This is due to the presence of oil bodies (membrane-bound organelles) in the cells of most liverworts that contain terpenoids suspended in a carbohydrate and/or protein-rich matrix (Vanderpoorten & Goffinet, 2009). Spörle *et al*. (1991a, 1991b, 1991c) and Asakawa *et al.* (2013) reported, in Panamanian bryophytes, the presence of spiroterpenoids in *Plagiochila moritziana* Gottsche & Lindenb. ex Hampe, (-)-geosmin and other terpenoids in *Sympyogyna brongniartii* Mont. and lipophilic constituents from *Monoclea gottschei* Lindb. subsp. *elongata* Gradst. & Mues (published as *M. gottschei* Lindb. subsp. *neotropica*).

Cyathodium (Marchantiophyta: Cyathodiaceae) is a pantropical thalloid liverwort comprising 12 species distributed worldwide (Srivastava & Dixit, 1996; Salazar *et al.,* 2004). Five of the species occur in the Neotropics, three of these are endemic to the New World: *C. bischlerianum* Salazar Allen, (until now endemic to Panama), *C. spruceanum* Prosk. and *C. steerei* Hässel, while two occur also in the Paleotropics, *C. cavernarum* Kunze and *C. foetidissimum* Schiffn. The center of diversity of the genus appears to be India, with eight species (Srivastava & Dixit, 1996). The plants are relatively simple in structure with the thallus composed of a central layer of air chambers covered by a dorsal and a ventral layer of cells. The air chambers are separated by uniseriate vertical rows of cells and are open to the upper surface by distinct pores flanked by narrow, elongated botuliform cells (Fig. 1). In some species, *C. foetidissimum*, the center part of the thallus has a multistratose area. In *C. steerei* the multistratose area is tuberculate and it is located at the base of the thallus (Hässel de Menéndez, 1961, 1962).

Cyathodium is poorly known in the Neotropics mainly by few herbarium collections (Salazar Allen *et al.,* 2004). The paucity of collections may be related to the seasonal growth of these plants. They grow during the rainy season and start dying out at the onset of the dry season. Nevertheless, they can persist under very humid conditions on banks of creeks and rivers and in terraria for most of the year and in axenic agar cultures under controlled environmental conditions. In the field, only seasonal plants appear to produce sporophytes (Salazar Allen *et al.,* 2004). Of the five Neotropical species three are monoecious (*C. bischlerianum*, *C. cavernarum*, *C. foetidissimum*) and two (*C. spruceanum, C. steerei*) are dioecious.

Each neotropical species is morphologically and genetically distinct (Salazar Allen *et al.,* 2004; Salazar Allen, 2005; Salazar Allen & Korpelainen, 2006). Genetic variations in nucleotide sequences in the nuclear ribosomal DNA region, ITS1-5.8S rRNA-ITS2 were analyzed for three species (*C. cavernarum*, *C. spruceanum* and *C. foetidissimum*). Sequences for *C. bischlerianum* failed and were not included in the genetic analysis (Salazar Allen & Korpelainen, 2006). The largest genetic differences were found between *C. foetidissimum* and *C. spruceanum*. Samples from *C. cavernarum* and *C. spruceanum* from nearby geographical areas were shown to be genetically more closely related than those of geographical distant areas. In *Cyathodium* the oil bodies occur in specialized cells (idioblasts) of the thallus devoid of chloroplasts (*C. cavernarum, C. bischlerianum, C. foetidissimum*

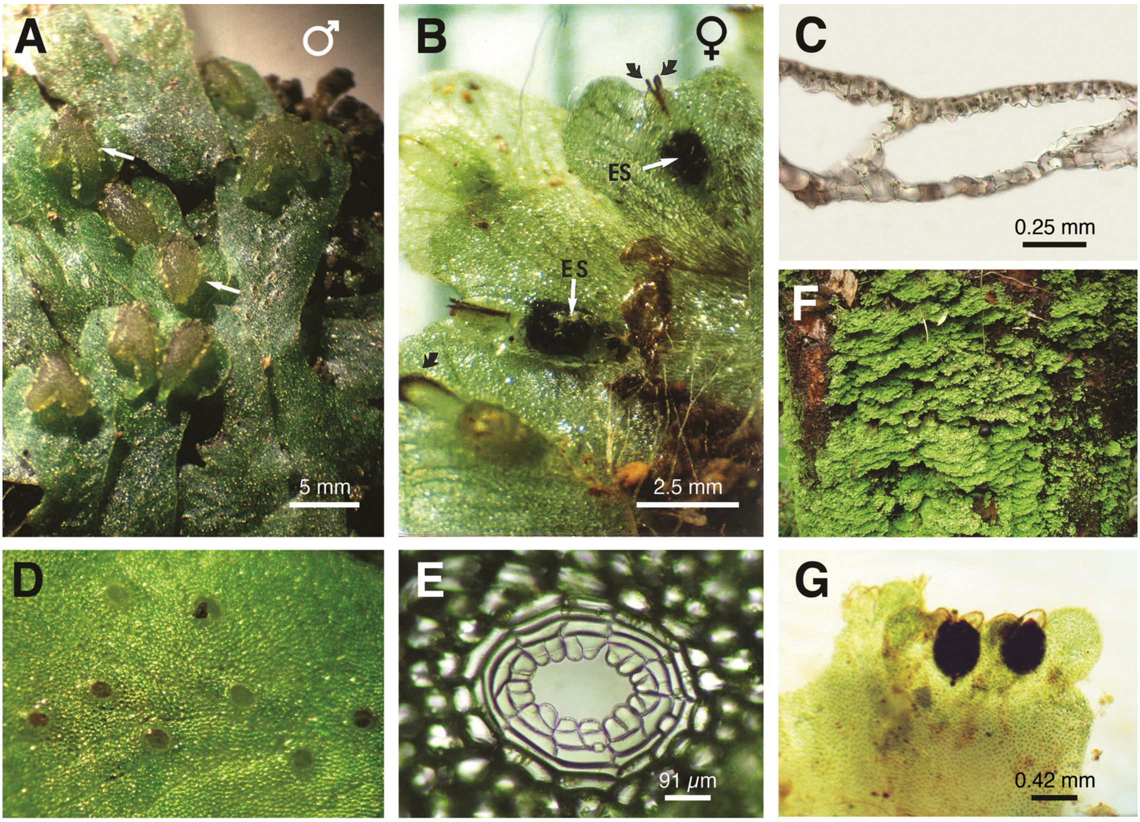

Fig. 1. A-E. *Cyathodium spruceanum*. A. Male plant with antheridial receptacles (arrows). B. Female plants, ES = Sporophytes, Arrows = apex of involucre. C. Transverse section of thallus showing air cavities. D. Upper surface of thallus with pores. E. Pore with 3(-4) rings of cells. F-G. *Cyathodium bischlerianum*. F. Growing on trunk of tree. G. Dorsal view of plant with sporophytes (dark areas). (A-B from *Salazar Allen 16700*; G from *Salazar Allen 16765*, C from *Gudiño 398*, D- E from *Gudiño 337*, F from *Gudiño 340*) (Photos A-B, G, Salazar Allen; C-E, F, J.A. Gudiño)

and, in *C. spruceanum* only in the border cells). In *C. spruceanum* the oil bodies are present in all cells with the chloroplasts (Fig. 2). Substances in the oil bodies confer a distinctive odor to some of the species, *e.g.*, an unpleasant odor in *C. foetidissimum* (Ludwiczuk *et al.,* 2009; Salazar Allen *Pers. comm*.), and a cedar oil smell in *C. steerei* (Hässel de Menéndez, 1962). Nevertheless, only one species, *C. foetidissimum*, has been chemically investigated. Skatole, which is responsible for the very intense unpleasant odor of the ether extract of *C. foetidissimum* (Ludwiczuk *et al.,* 2009), is a well-known compound produced by biodegradation of tryptophan that is responsible for the fecal odor of this liverwort. This is the second record of skatole in the Marchantiophyta. Previously, this compound was detected in an *Asterella*-like liverwort collected in Malaysia (Askawa *et al.,* 1995). *Cyathodium foetidissimum* also elaborates isolepidozene and lunularin. Isolepidozene is known as the main volatile component of *Concephalum japonicum* (L.) Dum. and *Marchantia emarginata* subsp. *tosana* (Stephani) Bischl. (*as Marchantia tosana* Stephani). Lunularin was previously isolated from or detected in *Dumortiera hirsuta* (Sw.) Nees, *Marchantia polymorpha* L., *M. chenopoda* L., *M. berteroana* Lehm. & Lindenb., *M. paleacea* var. *diptera* (Nees & Mont.) Inoue, and *Ricciocarpos natans* (L.) Corda. All these species are thallose liverworts in the order Marchantiales of the Marchantiophyta. *Cyathodium foetidissimum* is closely related chemically to the Marchantiopsida (Asakawa *et al*., 2013).

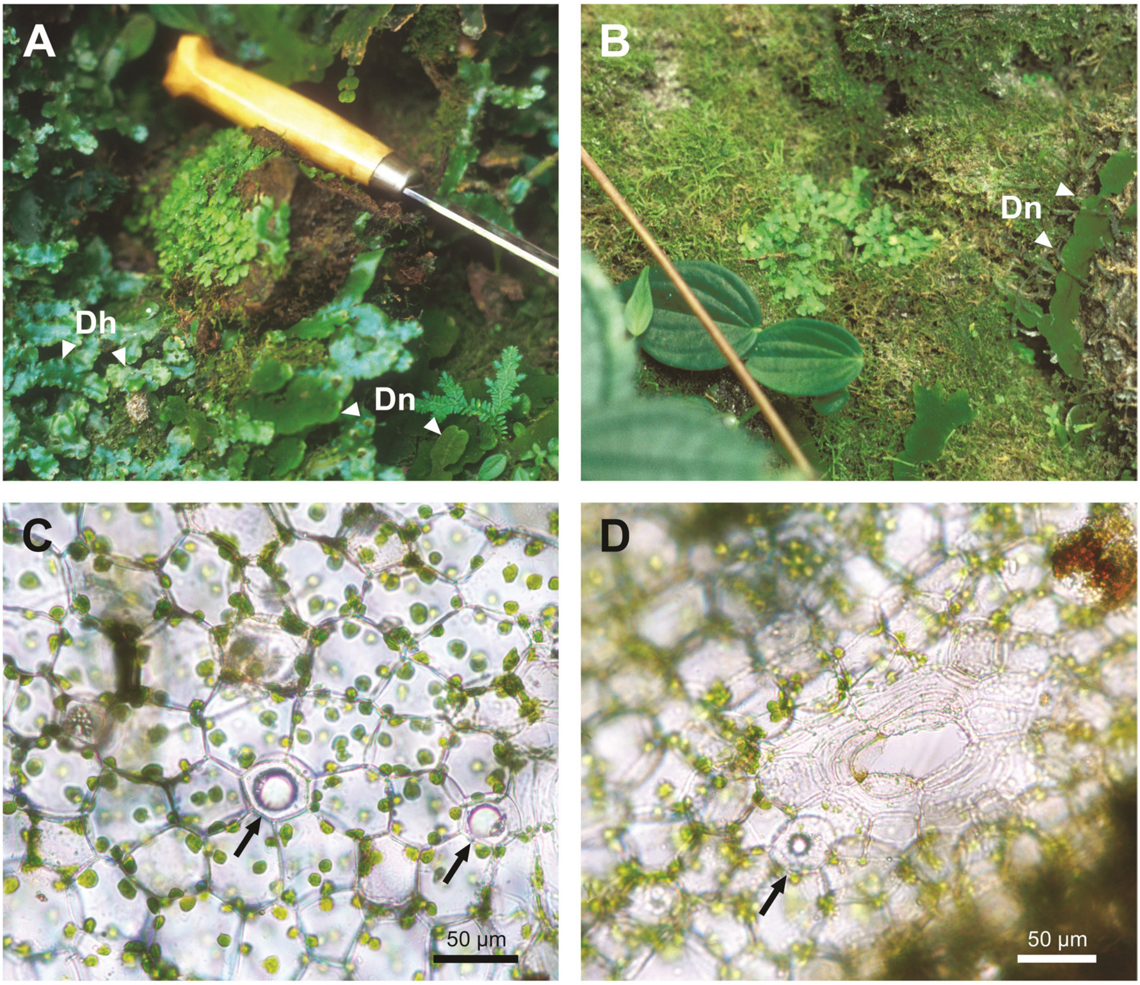

Fig. 2. *Cyathodium foetidissimum.* A-B. Plants in their natural habitat. Dh = *Dumortiera hirsuta*, Dn = *Dumortiera hirsuta* subsp. *nepalensis* (Tayl.) Schust. C. Idioblasts (arrows) on cells of thallus. D. Pore on upper surface of thallus. (A-B from *Salazar Allen et al. 17047*; C-D from *Salazar Allen 20627*). (Photos by

Phytochemical analyses of polar and non-polar compounds were pursued by our group in 2004 to determine 1) if morphological and genetic differences that distinguish the species were reflected in their chemistry, and 2) if there were chemical variations related to the sexual state of the plants, particularly in the dioecious *C. spruceanum* (young or old males and females with sporophytes).

Leiosporoceros dussii Hässel (Anthocerotophyta, Leiosporocerotaceae) is a hornwort that, unlike other hornworts, has its associated cyanobacteria in longitudinal thallus channels (Villarreal & Renzaglia, 2006) (Fig. 3). It grows on rocks and in volcanic and sandy soils near creeks or in roadway ditches (Villarreal, 2009). It has been reported as occurring in Mexico, Costa Rica, Panama, the Caribbean region, Colombia and Ecuador (Villarreal, 2009). Morphological and phylogenetic studies have revealed that it is the most genetically and morphologically divergent hornwort (Duff *et al.*, 2004; Villarreal *et al.*, 2010). It is also the most basal of all hornworts (Villarreal *et al.*, 2010). Nevertheless, there is a lack of information on its chemical composition with or without its symbiont cyanobacteria. The aim of this study was to

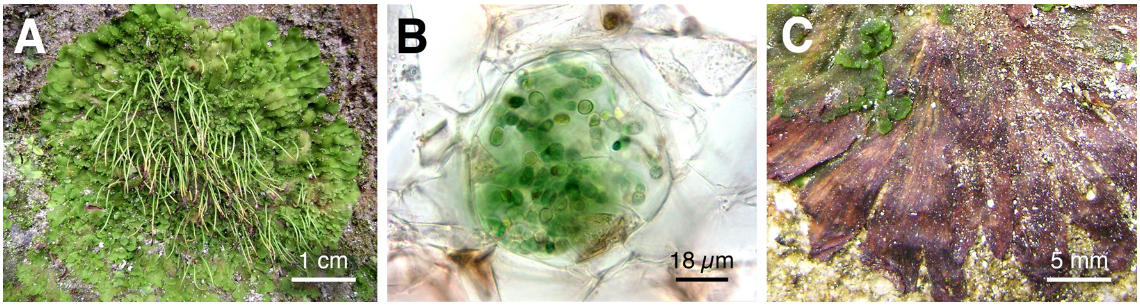

Fig. 3. *Leiosporoceros dussii*. A. Female plant in its natural environment. B. Transverse section of *Nostoc* canal. C. Decomposing thallus. (B from *Gudiño 492*; C from *Salazar Allen 21368*) (Photos A, M. Moya; B, J.A. Gudiño; C, Salazar Allen).

determine the chemical differences in fresh samples of male and female plants and decomposing ones of *Leiosporoceros dussii*, collected from its natural environment.

Materials and Methods

Forty milligrams of fresh plants of *C. bischlerianum*, *C. cavernarum* and *C. spruceanum* (female with young sporophytes, young male plants and senescence males) were selected from populations of Panama. Young male plants were those with their receptacles light green colored; senescence males had their male receptacles brown-colored with empty sperm cavities. Only plants collected in Panama were used for *Leiosporoceros dussii* and sterile young plants from axenic cultures (Salazar Allen & Korpelainen, 2006) for *C. foetidissimum* from Costa Rica. Using sterile tweezers, the samples were placed in sterile glass vials to which a 0.75 ml of n-hexane and n-octadecane (50 µg/ml of n-octadecane and n-hexadecanol as internal standards) was added to the samples to arrive at a final concentration of 20 µg/mg. The closed vials were placed in a rack and submerged in a 250 ml beaker containing 50 ml of water. The beaker was heated in a microwave at high power (700-800 W) for one minute. The vials were cooled at room temperature and the supernatant was transferred to clean sterile glass vials. The closed vials were stored at -18^{o} C. Separation, quantification and determination of compounds were done using an Agilent Technologies, Model 6890N gas chromatograph, fitted with a Model 5973 mass selective detector (MSD) and managed by an Agilent Chem Station Data system. An HP-5MS (5% phenyl methylpolysiloxane) fused silica capillary column (30 mm x 0,25 mm id., film thickness 0,25 µm) was used. Helium at a flow rate of 1.0 ml /min was used as carrier gas. An MS interface temperature of 270^{o} C, electron impact (EI) ionization mode, an ionization voltage of 1,300 V; and a scanning range *m/z* 40-400 at 1 scan/s was used.

The MS spectra obtained were compared to the spectral fragmentation patterns available from spectral libraries (AdamsWiley and NIST data base) (McLafferty, 1993; Adams, 1995; Vila *et al*. 2004; V*ila et al.* 2010).

The percentage represented by each of the identified compounds was obtained using the peak area normalization method. Not all compounds were identified. For example, from a sample analyzed by GC-MS, 100 peaks were produced but only 20 compounds could be identified representing 78% of the total peak area obtained.

Specimens studied. *Cyathodium bischlerianum*. PANAMA. *Prov. Panama: Dtto. Panama*, Parque Nacional Soberanía, Sendero El Charco, 1-XII-2004, 70 m. Salazar Allen, Chung & De Gracia 20993 (PMA).

Cyathodium cavernarum. PANAMA. *Prov. Coclé: Dtto. Antón*, El Valle de Antón, cerca del Mirador, 21-VII-2004, ±736 m, Salazar Allen, Chung, De Gracia & Ramírez 20926, 20927 (PMA). PANAMA. *Prov. Coclé: Dtto. Antón*, El Valle de Antón, sobre pared del puente sobre el río Guayabo, 21-VII-2004, 624 m, Salazar Allen, Chung, De Gracia & Ramírez 20939 (PMA).

Cyathodium spruceanum. PANAMA. *Prov. Panama*: *Dtto. Panama*, Parque Nacional Soberanía, Sendero Natural El Charco, 1-XII-2004, 70 m, Salazar Allen, Chung, De Gracia 20992, 20994, 20995, 20997 (PMA). PANAMA. *Prov. Panama*: *Dtto. Panama* Parque Nacional Soberanía, La Cascada, 1-XII-2004, 76 m, Salazar Allen, Chung, De Gracia 20999 (PMA). PANAMA. *Prov. Panama: Dtto. Panama* Parque Nacional Chagres, Campo Chagres, 1-XII-2004, ± 110 m. Salazar Allen, Chung, De Gracia 21002 (PMA). PANAMA. *Prov. Panama: Dtto. Panama*, en taludes a lo largo de la carretera después del mirador de la Represa Madden, 1-XII-2004, ±118 m, Salazar Allen, Chung, De Gracia 21003, 21004 (PMA). PANAMA. *Prov. Coclé*: *Dtto. Antón*, El Valle de Antón, río Las Mozas, 21-VII-2004, 580 m, Salazar Allen, Chung, De Gracia & Ramírez 20943, 20947 (PMA). PANAMA. *Prov. Coclé*: Dtto. Antón, El Valle de Antón, río Las Mozas, 27-VIII-2004, 580 m, Salazar Allen, Chung, De Gracia & Ramírez 20946, 20950 (PMA).

Cyathodium foetidissimum. Tipo: COSTA RICA. *Prov. Cartago*, *Cantón de Paraíso*, Parque Nacional Tapantí, Valle del Río Grande de Orosi, II-2002, 1200 m, Salazar Allen, Lépiz, Villarreal, Carranza & Lizano 20618 (*Paratipo* (PMA!), 20619 (PMA, USJ).

Leiosporoceros dussii. PANAMA. *Prov. Coclé: Dtto. Antón*, El Valle de Antón, Monumento Natural Cerro Gaital, en laderas del río El Guayabo, 580 m, Salazar Allen, Chung, De Gracia & Ramírez 75, 77, 76, 80, 20940, 20942 (PMA). PANAMA. *Prov. Coclé: Dtto. Antón*, El Valle de Antón, en suelo, al borde de la carretera hacia La Mesa, 828 m, Salazar Allen, Chung, De Gracia & Ramírez 20932 (PMA).

Results

Compounds identified in the five species studied and their corresponding retention times (minutes) are listed in Table 1.

In Table 2, the results of secondary metabolites identified in samples of *Cyathodium spruceanum* at different growth stages and collected from different sites are presented. Germacrene D and bicyclogermacrene were present in all samples of *C. spruceanum* studied. In the female sample with sporophytes (FS) from Campo Chagres, nine compounds were identified representing a total of 73,3%. The principal components were 12-*nor*cyercene B (70,2%), germacrene D (1,6%) and longifolenaldehyde (1,4%). In the senescent male sample (SM) collected from rocks, 68,4% of the total compounds could be detected. The major components were cyercene (63,2%) and germacrene D (1,6%), while 11 compounds representing a total of 69,5% were identified in the female sample (FS) collected from Madden, a site close to Campo Chagres. This sample also contained cyercene (65,8%) and germacrene D (1,6%) (Table 2). At the Cascada (Parque Nacional Soberanía), the female sample (FS) was found to contain 14 compounds (67,8%), of which the major compounds were: 4-(3,4-dimethoxybenzylidene)-1-(4-nitrophenyl)-3-phenyl-2-pyrazolin-5-one (61,0%) and germacrene D (1,6%). In the senescent male sample (SM) collected from the nature trail El Charco we identified 9 compounds (totaling 76,6%). The major components were cyercene (74,6%) and germacrene D (1,2%). From the female sample (FS) from the same site, 9 compounds (81,1%) were also detected, of which the major components were cyercene (51,0%), 12-*nor*cyercene B (21,7%), phytol isomer (3,5%) and germacrene D (3,2%). In the young male sample (YM) collected at Las Mozas, we identified 14 compounds (79,0%), of which the principal components were: 4-(3,4-dimethoxybenzylidene-1-(4-nitrophenyl)-3-phenyl-2-pyrazolin-5-one (43,8%), cyercene (20,3%), (1R,3S,6S,7R)-11-methylene-2-oxa-4,4,7-trimethyltricyclo[6.3.1.0(1,6)]dodecane (6,7%), 1S-*cis*-calamenene (2,5%), germacrene D (1,7%) and (+)-clavukerin A (1,2%). From the female sample (FS) collected also at Las Mozas nine compounds (77,6%) were identified, of which the principal components were 4-(3,4-dimethoxybenzylidene)-1-(4-nitrophenyl)-3-phenyl-2-pyrazolin-5-one (66,9%) and (1R,3S,6S,7R)-11-methylene-2-oxa-4,4,7-trimethyltricyclo[6.3.1.0 (1,6)]dodecane (6,8%).

In *Cyathodium bischlerianum*, 12 compounds were identified representing a total of 70,3%, of which the main compounds were nerolidol (26,7%), γ-terpinene (15,7%) and limonene (7,2%) (Table 3). (+)-Nerolidol is present in *Gymnocolea inflata* (Huds.) Dumort. and *Lophocolea heterophylla*

Table 1. Compounds identified in four species of *Cyathodium* and in *Leiosporoceros dussii*. Retention times are reported in minutes.

Retention time (in minutes)	Compound	*Cyathodium. spruceanum*	*Cyathodium bischlerianum*	*Cyathodium cavernarum*	*Cyathodium foetidissimum*	*Leiosporoceros dussii*
2.44	β-pinene		X			
2.77	d-(l)-limonene		X			
3	γ-terpinene		X			
3.25	α-terpinolene		X			
3.73	1-ethenyl-4-methoxybenzene				X	
5.5	camphene	X				
5.49	bicycloelemene	X				
5.58	3,4-dimethoxystyrene				X	
5.78	3-methyl-1H-indol				X	
5.95	β-bourbonene	X				
5.98	β-elemene		X			
6.16	α-gurjunene	X				
6.17	neoisolongifolene	X				
6.21	β-gurjunene	X				
6.31	β-cubebene	X				
6.62	trans-caryophyllene			X		
6.87	cadina-1,4-diene	X				
6.99	4,4-dimethyl-8-methylene-2-propyl-1-oxaspiro[2.5]octane			X		
7.03	germacrene-D	X	X			
7.17	bicyclogermacrene	X	X			
7.24	ionole	X		X		
7.34	β-bourbonen-13-ol	X				
7.36	shyobunol	X				
7.38	1,1,4,4-tetramethyl.-2-tetralone	X				
7.38	calamene	X				
7.39	nerolidol		X			
7.57	(-)-spathulenol	X				
7.63	2-methoxy-9-methylanthracene	X				
7.75	cyercene	X				
7.76	12-norcyercene-B	X				
7.76	(+)- clavukerin A	X				
7.88	2-propenoic acid, 3-(4-methoxyphenyl)-, methyl ester			X		
7.98	cadina-1(10),6,8-triene	X				
8.05	1,2,4,6,7,7a-hexahydro-7a-methyl-3-(2-methyl-1-oxopropyl)-5H-Inden-5-one	X				

Retention time (in minutes)	Compound	*Cyathodium. spruceanum*	*Cyathodium bischlerianum*	*Cyathodium cavernarum*	*Cyathodium foetidissimum*	*Leiosporoceros dussii*
8.07	1,4.dimethyl-3-(2-methyl-1-propene-1-yl)-4-vinyl-1-cycloheptene		X			
8.18	unknown					X
8.23	dehydroaromadendrene	X				
8.22	1(5)-3-aromadenedraidene	X				
	1-deoxycapsidiol	X				
8.52	2-propenoic acid, 3-(4-methoxyphenyl)-, methyl ester			X		
8.52	1S-cis-calamenene	X				
8.82	2-methoxy-6-allyloxy-7-methyl-7H-purine	X				
8.89	(1,3-dimethyl-2,4,6-trioxohexahydropyrimidine)-5-spiro-2’-(4’-methyl-3’,6’-dihydro-2’H-pyran)	X				
8.89	(1R,·3S, 6S,7R)-11-methylene-2-oxa-4,4,7-trimethyltricyclo[6.3.1.0(1,6)] dodecane	X				
8.94	heptadecane-3-methyl	X			X	
9.02	cadina-1,3,5-triene	X				
9.36	4-(3,4-dimethoxybenzylidene)-1-(4-nitrophenyl)-3-phenyl-2-pyrazolin-5-one	X				
9.58	longifolenaldehyde	X				
9.79	Methyl-3,4-dimethoxycinnamate		X			
9.92	methyl-3,4,5-trimethoxycinnamate		X			
10.65	unknown					X
10.7	4-isopropyl-2,5-dimethoxybenzyl acetate		X			
10.77	phytol isomer	X				
11	kaur-16-ene					X
11.86	alnulin	X				
11.87	unknown					X
12.42	4-hydroxy-3,3’,4-trimethoxystilbene					X

(Shrad.) Dumort. The same alcohol has been isolated from the essential oils of *Plagiochila ovalifolia* Mitt. and *Wiesnerella denudata* (Mitt.) Stephani (Asakawa, 1995).

In *C. cavernarum*, collected from El Valle de Antón, two compounds were identified (representing 99.94%), and the major component was 4,4-dimethyl-8-methylene-2-propyl-1-oxaspiro [2.5-]octane (70,6%) (Table 4).

In axenically grown samples of *Cyathodium foetidissimum* of Costa Rica, six compounds were identified representing a total of 87,9 %. The main compounds found were: 3-methylindole (44,7%) and 1-ethenyl-4-methoxy-benzene (30,9%) (Table

Table 2. Percentages of major volatile compounds in *Cyathodium spruceanum* from plants of different sites (A: El Charco, B: La Cascada, C: Campo Chagres, D: Madden, E: Las Mozas) and various sexual condition (FS: female plants with sporophytes, YM: Young males, SM: senescence males).

Collection Number	Site					Sexual Condition			Altitude (m)					Substrate		Percentage of Major Compounds
	A	B	C	D	E	FS	YM	SM	70	76	110	118	580	Soil	Rock	
20995, 20997	X							X	X					X		cyercene (74,6%) germacrene D (1,2%) bicyclogermacrene (0.2%)
20992, 20994	X					X			X					X		cyercene (51,0%) 12-*nor*cyercene-B (21,7%) phytol isomer (3,5%) germacrene D (3,2%) bicyclogermacrene (0,8%)
20999		X				X				X					X	4-(3,4-dimethoxybenzylidene)-1-(4-nitrophenyl)-3-phenyl-2-pyrazolin-5-one (61,0%) germacrene D (1,6%) 1(5), 3-aromadinedraine (1,2%)
21002			X			X					X			X		12-*nor*cyercene-B (70,2%) germacrene D (1,6%) longifolenaldehyde (1,4%) bicyclogermacrene (0,6%)
21003				X		X						X			X	cyercene (65.8%) germacrene D (1,6%) bicyclogermacrene (0,7%)
21004				X				X				X			X	cyercene (63.2%) germacrene D (1,6%) bicyclogermacrene (0,7%) spathulenol (0,7%)
20943, 20947					X		X						X		X	4-(3,4-Dimethoxybenzylidene)-1-(4-nitrophenyl)-3-phenyl-2-pyrazolin-5-one (43,8%) cyercene (20,3%) (1R,3S,6S,7R)-11-methylene-2-oxa-4,4,7-trimethyltricyclo[6.3.1.0(1,6)] dodecane (6,7%) 1S-cis-calamenene (2,5%) germacrene D (1,7%) (+)-clavukerin A (1,2%) bicyclogermacrene (0,8%)

Collection Number	Site					Sexual Condition			Altitude (m)					Substrate		Percentage of Major Compounds
	A	B	C	D	E	FS	YM	SM	70	76	110	118	580	Soil	Rock	
																4-(3,4-dimethoxybenzylidene)-1-(4-nitrophenyl)-3-phenyl-2-pyrazolin-5-one (66.9%)
20946, 20950					X	X							X			(1R,3S,6S,7R)-11-methylene-2-oxa-4,4,7-trimethyltricyclo[6.3.1.0(1,6)] dodecane (6,8%)
																1S-cis-calamenene (2,5%)
																germacrene D (1,7%)
																(+)-clavukerin A (1,2%)
																bicyclogermacrene (0,8%)

5). This is the only species in this genus that has been previously studied chemically (Ludwiczuk *et al.,* 2009). Skatole or 3-methylindole is a mildly toxic white crystalline organic compound belonging to the indole family. It occurs naturally in feces (it is produced from tryptophan in the mammalian digestive tract) and coal tar and has a strong fecal odor. However, at low concentrations, it has a floral odour and is present in several flowers and essential oils, including those of orange blossoms, jasmine, and *Ziziphus mauritiana* Lam. It is used as a fragrance and fixative in many perfumes and as an aroma compound. Its name is derived from the Greek root skato-meaning

Table 3. Percentages of major volatile compounds in *Cyathodium bischlerianum* collected in Sendero El Charco (Soberanía National Park), Province of Panama.

Collection Number	Sexual Condition	Altitude (m)	Substrate	Percentage of Major Compounds
20993	Plants with sporophytes	70	On cortex of *Anacardium excelsum (Bertero & Balb. ex Kunth) Skeels*	nerolidol (26,7%)
				γ-terpinene (15,7%)
				d-(l)-limonene (7,2%)
				4-isopropil-2,5-dimethoxybenzyl acetate (4,2%)
				bicyclogermacrene (3,8%)
				α-terpinolene (3,5%)
				β-pinene (2,8%)
				1,4-dimethyl-3-(2-methyl-1-propene-1-yl)-4-vinyl- 1-cycloheptene (2,3%)
				germacrene D (1,2%)

Table 4. Percentages of major volatile compounds from various samples of *Cyathodium cavernarum* collected in two sites in El Valle de Antón, Province of Coclé.

Collection Number	Site		Sexual Condition	Altitude (m)		Substrate		Percentage of Major Compounds
	El Guayabo	El Mirador		624	736	Soil	Cement	
20926		X	Young plants		X	X		4,4-dimethyl-8-methylene-2-propyl-1-oxaspiro[2.5-] octane (70,6%)
								trans-caryophyllene (1,0%)
20927		X	Young plants		X		X	4,4-dimethyl-8-methylene-2-propyl-1-oxaspiro[2.5-] octane (98,3)
								trans-caryophyllene (1,6%)
20939	X		Young plants	X			X	4,4-dimethyl-8-methylene-2-propyl-1-oxaspiro[2.5-] octane (96,9%)
								trans-caryophyllene (3,0%)

"dung". Skatole was discovered in 1877 by the German physician Ludwig Brieger (1849 – 1919). It is used by the U.S. military in its non-lethal weaponry arsenal; specifically as malodorants (Patent 6,386,11).

Four compounds were identified in the hornwort *Leiosporoceros dussii*. The major compounds were 4-hydroxy-3,3',4-trimethoxystilbene (56,5%) and kaur-16-ene (31,4%). The sample in decomposition had kaur-16-ene (64.9%) and an unidentified compound. The sterile samples contained 4-hydroxy-3,3',4-trimethoxystilbene (57,0%), kaur-16-one (28,6%) and an unidentified compound. All of the male samples also produced 4-hydroxy-3,3',4-trimethoxystilbene (57.6%) and kaur-16-ene (36,3%) as major compounds (Table 6).

Discussion

There is a clear distinction between the four neotropical *Cyathodium* species studied according to their chemical composition. The chemistry of these species supports previous morphological and genetic studies (Salazar Allen, 2005; Salazar Allen & Korpelainen, 2006). According to these studies each species is clearly distinct based on gametophytic characters [frequency of pores, oil bodies in idioblasts (*C. bischlerianum*, *C. cavernarum* and *C. foetidissimum*) or with chloroplasts in the same cell (*C. spruceanum*)], type of rhizoids; sexual condition (monoecious or dioecious) position and size of male receptacles and rhizoid morphology among some and, also according to sporophytic

Table 5. Percentages of major volatile compounds in *Cyathodium foetidissimum* of Costa Rica grown in axenic cultures.

Collection Number	Site	Sexual condition	Altitude (m)	Substrate	Percentage of Major Compounds
20618, 20619	Valle del Río Grande de Orosi	Sterile from axenic cultures	1200	Sandy rock	Skatole: 3-methylindole (44,7%)
					1-ethenyl-4-methoxy-benzene (30,9%)

Table 6. Percentages of major volatile compounds in the hornwort *Leiosporoceros dussii* according to its sexual condition and of a decomposing thallus from samples collected in El Valle de Antón, Province of Coclé.

<table>
<tr><th rowspan="2">Collection Number</th><th colspan="2">Site</th><th rowspan="2">Sexual Condition</th><th colspan="2">Altitude (m)</th><th>Substrate</th><th rowspan="2">Percentage of major compounds</th></tr>
<tr><th>El Guayabo</th><th>La Mesa</th><th>580</th><th>828</th><th>Soil</th></tr>
<tr><td rowspan="2">20932</td><td rowspan="2"></td><td rowspan="2">X</td><td rowspan="2">Female</td><td rowspan="2"></td><td rowspan="2">X</td><td rowspan="2">X</td><td>4-hydroxy-3-3´,4-trimetoxystilbene (56,5%)</td></tr>
<tr><td>kaur-16-eno (31,4%)</td></tr>
<tr><td rowspan="2">20940, 20942</td><td rowspan="2">X</td><td rowspan="2"></td><td rowspan="2">Female</td><td rowspan="2">X</td><td rowspan="2"></td><td rowspan="2">X</td><td>4-hydroxy-3-3´,4-trimetoxystilbene (56,5%)</td></tr>
<tr><td>kaur-16-ene (31,4%)</td></tr>
<tr><td rowspan="2">Decomposing</td><td rowspan="2">X</td><td rowspan="2"></td><td rowspan="2">Unknown</td><td rowspan="2">X</td><td rowspan="2"></td><td rowspan="2">X</td><td>kaur-16-ene (64,9%)</td></tr>
<tr><td>unidentified compound</td></tr>
<tr><td rowspan="3">75, 77, sterile plants</td><td rowspan="3">X</td><td rowspan="3"></td><td rowspan="3">Sterile</td><td rowspan="3">X</td><td rowspan="3"></td><td rowspan="3">X</td><td>4-hydroxy-3,3’,4-trimethoxystilbene(57,0%)</td></tr>
<tr><td>kaur-16-ene (28,6%)</td></tr>
<tr><td>unidentified compound</td></tr>
<tr><td>76, 80, male plants</td><td>X</td><td></td><td>Male</td><td>X</td><td></td><td>X</td><td>4-hydroxy-3,3’,4-trimethoxystilbene (57.6%)
kaur-16-ene (36,3%)</td></tr>
</table>

characters (presence or absence of an operculum, ornamentation of the upper cells of the sporophyte and spore ornamentation). According to genetic studies (Salazar Allen & Korpelainen, 2006), each species is also genetically distinct but no differences between male and female plants of *C. spruceanum* were genetically studied. Nevertheless, although male and female plants of this species were found to be similar regarding their chemistry, three compounds, 12-*nor*cyercene-B, longifolenaldehyde and 1(5)-3-aromadenedraidene were found only in female plants. These differences point to the need to pursue genetic studies in this dioecious species to determine if the chemical differences observed can be genetically determined.

Although *C. bischlerianum* was not included in the genetic studies of Salazar Allen & Korpelainen (2006), this species shares with *C. spruceanum* the sesquiterpenes germacrene D and bicyclogermacrene, although they were present in low percentages. The presence of these sesquiterpenes suggests a genetic affinity between *C. bischlerianum* and *C. spruceanum*, the latter considered the most plesiomorphic of all neotropical *Cyathodium* species. Additional work is needed, which should include samples from other sites in the Neotropics, to determine if this chemical similarity is characteristic for both species. Both sesquiterpenes have been reported in other foliose and thalloid liverworts (Asakawa *et al.,* 2013).

Cyathodium bischlerianum unlike the other species contains mainly aromatic monoterpenes with nerolidol as the main compound (Table 1). (*E*)–nerolidol has a floral and woody fragance (Padalia *et al.*, 2015). It is used in fine fragrances, cosmetics, shampoo, soaps, detergents and cleaning products. The world consumption of the compound is ca. 10-100 tons/year (Queiroga *et al.*, 2014). It has also been approved by the Food and Drug Administration of the United States as a flavoring agent for food**.** γ-Terpinene, identified in this species, is one of the major components of the oil of the African and Iranian “ajowan” or Bishop’s weed seed oil (*Carum copticum* Benth, Apiaceae) (Boskabadi *et al.,* 2017).

It is noteworthy that this species is the smallest in size of the neotropical species and occurs as an epiphyte on the bark of trees near creeks, or in soil and humus accumulated in the forks or concavities of trees, and on rocks in humid places (Fig. 1).

The chemistry of *C. spruceanum* and *C. cavernarum* coincides with morphogenetic results previously reported (Salazar Allen and Korpelainen, 2006) where samples from close geographical areas were genetically more closely related than those from geographically distant samples (*e.g.*, plants from El Valle de Antón all share the same compounds). Chemical differences in the same species growing in different sites have been reported for other liverworts, *e.g.*, *Bryopteris filicina* (Sw.) Nees, a foliose liverwort collected in Costa Rica that produced two germacrenes that were not found in the same species collected in Panama (Asakawa *et al.,* 2013). It would be interesting to compare the chemical composition of *Cyathodium* species from Panama with those from Costa Rica.

Chemically, *C. foetidissimum* is very distinct from the three *Cyathodium* species analyzed in this study. It does not share any of the major compounds found in the other species. The presence of indol compounds in *C. foetidissimum* from Costa Rica and Tahiti suggests that these compounds may be potential chemosystematic markers for the species. Skatol responsible for the fecal odor of the plants, was found in plants from Tahiti as well as from axenically grown plants from Costa Rica. It will be useful to have samples from other areas where the species have been reported, *e.g.*, Java, Sumatra, Nukahiva (Srivastava & Dixit, 1996) and Cameroon (Wigginton & Grolle, 1996; Wigginton, 2002) to compare their chemical composition.

The hornwort *Leiosporoceros dussii* contains a great number of compounds that could not be identified. Nevertheless, the diterpene found (in low quantities) is known for *Anthoceros caucasicus* Steph. (Sonwa & Köning, 2003). It is important to grow *Leiosporoceros* in axenic cultures to determine if the secondary metabolites it produces are, at least in part, the result of its association with its *Nostoc* endosymbiont.

There is a need to continue the study of these plants by collecting samples during different seasons and from different substrates throughout the year, to determine if there are variations in the type of compounds produced or in their concentrations according to the substrate on which they occur, their growth and sexual states. Also, extracts from axenically grown plants will be helpful in clarifying the role of endosymbionts (*e.g.*, in *Leiosporoceros*) in the production of these chemicals. Another interesting line of research is the study of the bioactivity of these compounds.

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