ANA CARLA FEIO DOS SANTOS

CROTON SECT. CYCLOSTIGMA (EUPHORBIACEAE): NOVIDADES ANATÔMICAS E TAXONÔMICAS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Botânica, para obtenção do título de Doctor Scientiae.

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APROVADA: 26 de Janeiro de 2016.

_____	_____
Aristéa Alves Azevedo	Ana Paula Gonçalves
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Renata Maria Strozi Alves Meira
(Orientadora)

DEDICO

À minha família: mami, mana e manos, além dos lindos sobrinhos que mesmo consumindo minhas energias só me fortalecem. Além da minha grande amiga e parceira de vidas passadas (é a única explicação para tanto amor) Ana Cristina. (Aninha).

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Leon Tolstoi (modificado)

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SANTOS, Ana Carla Feio dos, D. Sc., Universidade Federal de Viçosa, janeiro de 2016. **Croton sect. Cyclostigma (Euphorbiaceae): novidades anatômicas e taxonômicas**. Orientadora: Renata Maria Strozi Alves Meira. Coorientadora: Ricarda Riina.

Croton L. é um gênero megadiverso, amplamente distribuído, monofilético e que, até o momento, possui como única sinapomorfia os estames encurvados no botão floral. Já em nível infragenérico, não há sinapomorfias morfológicas que distingam a maioria das seções. Dentre os caracteres utilizados nas descrições taxonômicas de Croton, destacam-se as estruturas secretoras mostrando-se promissoras como subsídio para a taxonomia e filogenia, pois exibem diversidade morfológica e produzem compostos de natureza química complexa. No entanto, imprecisões em caracterizações anatômicas e descrições taxonômicas que não combinam parâmetros como a natureza química, a estrutura e período de atividade das estruturas secretoras, vêm causando equívocos principalmente quanto ao reconhecimento de coléteres e nectários extraflorais (NEF). Tais estruturas podem ocupar posição equivalente e possuir semelhanças morfológicas. O presente estudo tem como foco Croton seção Cyclostigma sensu stricto, plantas arborescentes e exclusivamente Neotropicais. As espécies desta seção são popularmente conhecidas como sangue de dragão devido à exsudação de látex avermelhado quando os troncos sofrem injúrias. A filogenia molecular de Croton seção Cyclostigma demonstrou uma evolução reticulada, com pouca resolução entre as diferentes linhagens. Neste estudo pretende-se ampliar e

gerar uma base de dados mais robusta incluindo caracteres anatômicos foliares. Adicionalmente, a natureza química da secreção produzida pelas estruturas secretoras foliares e florais de C. echinocarpus e C. urucurana foi investigada visando esclarecer a classificação destas estruturas e apontar novas interpretações para o gênero. As amostras foram preparadas conforme metodologia usual para análises em microscopia de luz e eletrônica de varredura. Após descrição da anatomia foliar, foi construída uma matriz binária, a partir da qual foi calculada a distância, gerando um dendrograma de similaridade. Folhas e flores de Croton echinocarpus e C. urucurana apresentaram cinco tipos de estruturas secretoras, que foram similares em ambas as espécies. Os idioblastos secretam compostos de natureza mista, os laticíferos são não-articulados ramificados e coléteres e nectários produzem secreção hidrofílica. As glândulas marginais são coléteres do tipo padrão e as flores também apresentaram este tipo de coléter, uma novidade para o gênero. A análise conjunta da estrutura anatômica, histoquímica e período de atividade secretora foi essencial para permitir uma classificação precisa e assim discutir possíveis homologias das estruturas secretoras no gênero. A presença de diferentes tipos de tricomas estrelados, laticíferos não-articulados, além da ausência de NEF nas margens e tricomas lepidoto e fasciculado foram as principais características anatômicas que possibilitaram agrupar os representantes da seção Cyclostigma. Os caracteres anatômicos agregaram dados na descrição de um novo táxon. A partir da amostragem examinada, não foi confirmada a hipótese sobre a homologia entre idioblastos secretores na epiderme e tricomas secretores, assim como, sobre a generalização de laticíferos articulados em Crotonoideae. Embora não tenha um caracter anatômico único para a seção Cyclostigma, a combinação das características examinadas em diferentes níveis de agrupamento foi útil para demonstrar as semelhanças entre espécimes, estabelecendo a identidade taxonômica específica.

ABSTRACT

SANTOS, Ana Carla Feio dos, D. Sc., Universidade Federal de Viçosa, January, 2016. **Croton sect. Cyclostigma (Euphorbiaceae): anatomical and taxonomic novelties**. Adviser: Renata Maria Strozi Alves Meira. Co-Adviser: Ricarda Riina.

Croton L. is a megadiverse genus, widely distributed species, monophyletic and that, up to now, possesses the curved stamens in flower buds as the only synapomorphy, and at the infrageneric level there are no morphological synapomorphies that distinguish most sections. Among the characters used in taxonomic descriptions of Croton, the secretory structures are particularly important as they have been promising on the suport of taxonomic and phylogenetic studies, taken in to account they exhibit morphological diversity and produce compounds of complex chemical nature. However, imprecisions in anatomical and taxonomic descriptions that do not match parameters as chemical nature, structure and activity period of secretory structures, have been causing mistakes especially for recognition of colleters and extrafloral nectaries (EFN). These structures may occupy an equivalent position and possess morphological similarities. The present study focuses in Croton section Cyclostigma s.s., arborescent plants and exclusively Neotropical. The species of this section are popularly known as dragon's blood due to the reddish latex exudates on trunks upon injuries. The molecular phylogeny of Croton section Cyclostigma showed a reticulate evolution with little resolution among the different lineages of Croton species investigated. This study aims to expand and produce a database including leaf anatomical characters. Additionally, the chemical nature of the

secretion produced by leaf and floral secretory structures of C. echinocarpus and C. urucurana was investigated aiming to clarify the classification of these structures and to point new interpretations to the genus. Samples were prepared according to the methodology for analysis under light and scanning electron microscopy. A binary matrix was constructed based on the leaf anatomical description, after that the distance was calculated and a similarity dendrogram was generated. Leaves and flowers of Croton echinocarpus and C. urucurana present idioblasts that secrete mixed nature compounds, laticifers are non-articulated branched and colleters and nectaries produce hydrophilic secretion. The marginal glands are standard type colleters, which are also present in flowers that represents a novelty for the genus. The combined analysis of anatomical structure, histochemistry and secretory activity period was essential to accurate classification and thus discuss possible homologies of secretory structures in the genus. The presence of different types of stellate trichomes, non-articulated laticifers, besides the absence of EFN on the leaf margin and lepidote and fasciculate trichomes were the main anatomical features that made it possible to cluster the representatives of section Cyclostigma. The anatomical characters aggregated data in the description of a new taxon. From the examined sampling was not confirmed the hypothesis about the homology between secretory idioblasts in the epidermis and secretory trichomes, as well as about the generalization of articulated laticifers in Crotonoideae. Although has not a unique anatomical pattern for section Cyclostigma, the combination of features examined on different levels of clustering was useful to demonstrate the similarities between specimens, and to establish taxonomic identities.

INTRODUÇÃO GERAL

De todas as famílias da ordem Malpighiales, Euphorbiaceae é insuperável em riqueza de espécies, diversidade morfológica e fitoquímica e ainda em importância econômica (Wurdack et al., 2005). A família, na circunscrição atual, é composta por 340 gêneros e aproximadamente 5.800 espécies (Wurdack et al., 2004; Xi et al., 2012), com o principal centro de diversidade os trópicos (Judd, 2009). No Brasil ocorrem cerca de 63 gêneros e 922 espécies (Cordeiro et al., 2014), em diferentes tipos de vegetação e com diversas formas de vida, excetuando-se epífitas (Barroso, 1991; Souza & Lorenzi, 2008).

Croton L. destaca-se na família Euphorbiaceae como um gênero gigante, com aproximadamente 1.300 espécies, de ampla distribuição geográfica (pantropical e subtropical) e por isso é considerado taxonomicamente complexo (Riina et al., 2009). Croton tornou-se um grupo monofilético após a inclusão de Crotonopsis Michx., Cubacroton Alain, Eremocarpus Benth., Julocroton Mart. e Moacroton Croizat, bem como a exclusão da seção Astraea (Klotzsch) Baill., a qual foi elevada à categoria de gênero Astraea Klotzsch. (Berry et al., 2005). Até o momento, Croton tem uma única sinapomorfia identificada que são os estames encurvados no botão floral (Berry et al., 2005; Lima & Pirani, 2008).

Estudos clássicos sobre as delimitações infragenéricas foram desenvolvidos por Baillon (1858, 1864), Müller (1865, 1866, 1873) e Webster (1993), este último, apontou Croton como sendo um grupo natural. Embora Webster (1993, 2001) tenha reconhecido 40 seções e 5 subseções para Croton, os trabalhos de filogenia

molecular (Berry et al., 2005; van Ee et al., 2008; Riina et al., 2009; van Ee et al., 2011) têm demonstrado que estas seções e subseções são polifiléticas. Adicionalmente, não foram identificadas sinapomorfias morfológicas que distinguissem satisfatoriamente as seções de Croton (Riina et al., 2009; van Ee et al., 2011). O estudo mais abrangente de filogenia molecular que amostrou as seções de Croton tomou como base dados moleculares, morfológicos e biogeográficos (van Ee et al., 2011). Estes autores organizaram as espécies em 31 seções, distribuídas em quatro subgêneros: Quadrilobi, Adenophylli, Geiseleira - espécies do Novo Mundo - , e Croton (espécies do Velho Mundo).

Cyclostigma Griseb. está inserida no subgênero Adenophylli e destaca-se por ser uma seção Neotropical de grande representatividade nos Andes e no Sudeste do Brasil, com algumas espécies estendendo-se pela Amazônia e alcançando a América Central e o México (Riina et al., 2009; van Ee et al., 2011). Na circunscrição de Webster (1993, 2001) Croton seção Cyclostigma Griseb. incluia 63 espécies em quatro subseções: Cyclostigma, Sampatik, Palanostigma e Xalapenses. Entretanto, Riina et al. (2009) realizaram um estudo filogenético utilizando marcadores moleculares (trnL-F e ITS) e verificaram que a seção Cyclostigma sensu Webster era polifilética. Neste estudo, um clado formado por 41 espécies foi denominado como Cyclostigma sensu stricto ou clado Cyclostigma (por incluir a espécie tipo da seção) o qual foi confirmado na atualização da classificação infragenérica de Croton (van Ee et al., 2011). As demais espécies de Cyclostigma sensu Webster (1993, 2001) ficaram dispersas nos demais clados de Croton (Riina et al., 2009).

Grande parte das espécies de Cyclostigma possui características como: presença de látex vermelho nos troncos, címulas bissexuais basais com flores pistiladas pediceladas e apétalas, estiletes bífidos a quadrífidos, flores estaminadas (a maioria com ≥16 estames, poucas com mais de 150), folhas grandes palmatinérveas, geralmente com base cordada, duas ou mais glândulas peciolares, estípulas bem desenvolvidas e tricomas estrelados. Estas características não estão uniformemente distribuídas entre os táxons da seção, sendo a maioria dos caracteres homoplásicos no gênero (Riina et al., 2009; van Ee et al., 2011).

A dificuldade em encontrar caracteres morfológicos que auxiliem a interpretação sobre as relações de parentesco entre as espécies de Cyclostigma, bem como que ratifiquem a organização a partir da filogenia molecular, foi reafirmada no estudo de Riina et al. (2009), no qual também foi evidenciada uma evolução reticulada para esta seção, com origem em um evento antigo de hibridação.

Adicionalmente, não foi possível reconhecer o grupo irmão de Cyclostigma pois, os dados nucleares (ITS) apontaram Adenophylli, enquanto os dados plastidiais (trnL-F) sugeriram a seção monotípica Cupreati. Morfologicamente, a sect. Adenophylli possui semelhanças com Cyclostigma, como os tricomas estrelados (em C. cupreatus são metálico lepidotos) e número de estames ≥16 (inferior a 10 em C. cupreatus). Por outro lado, C. cupreatus tem hábito arbóreo e címulas bissexuais com flores pistiladas pediceladas, semelhante à Cyclostigma, características ausentes em Adenophylli. Tais discordâncias demonstram o quão complexa é a seção Cyclostigma e evidenciam a necessidade de dados adicionais que incrementem a base de dados, como a anatomia foliar, que possam contribuir para o esclarecimento das relações filogenéticas e sustentar as hipóteses filogenéticas do grupo (Riina et al., 2009).

Caracteres anatômicos vêm sendo enfatizados como parâmetros úteis de diagnóstico em diversas famílias botânicas, como: o padrão de venação, a organização do mesofilo, a presença, a posição e a diversidade de tricomas e de estruturas secretoras (Solereder, 1908; Metcalfe & Chalk, 1983; Dickison, 2000). Para espécies de Croton, muitos destes caracteres vêm sendo confirmados como taxonomicamente importantes, tais como: os tricomas foliares de C. seção Barhamia (Gordillo & Matías, 2005), de diversas seções de Croton (Lucena & Sales, 2006) e de espécies de Croton da China (Liu et al., 2013), a estrutura anatômica foliar de Croton, Brasiliocroton e Astraea (Sá-Haiad et al., 2009) e a presença de laticíferos no xilema secundário de Croton sect. Cyclostigma (Wiedenhoeft et al., 2009).

Estudos anatômicos vêm contribuindo para taxonomia de Croton, seja adicionando dados às filogenias e/ou ratificando resultados advindos destas, analisando táxons ainda não amostrados do ponto de vista anatômico (Soares 2013; Vitarelli et al., 2015).

Dentre os caracteres anatômicos foliares, as estruturas secretoras se destacam em Croton tanto nas descrições taxonômicas, quanto nas caracterizações anatômicas. Tais estruturas podem estar presentes ou ausentes, e quando presentes, podem exibir distribuição variada. Na literatura percebe-se que os dados sobre as estruturas secretoras têm se mostrado promissores nos estudos de taxonomia e filogenia pela diversidade tipológica e da natureza química do exsudado (Fahn, 1979; Elias, 1983; Bernadello, 2007). Para Croton já foram relatados nectários florais (NFs) e extraflorais (NEFs) (Metcalfe & Chalk, 1950; Fahn, 1979; Elias, 1983; Freitas et al., 2001, Vitarelli et al., 2015), nectários pós-florais (Freitas et al., 2001); tricomas glandulares (Metcalfe & Chalk, 1950; Webster et al., 1996, Vitarelli et al., 2015),

coléteres foliares (Soares, 2013; Vitarelli et al., 2015), idioblastos com secreção lipofílica (Metcalfe & Chalk, 1950; Webster et al., 1996; Sá-Haiad et al., 2009; Vitarelli et al., 2015) e laticíferos (Metcalfe & Chalk, 1983; Rudall, 1989, 1994; Farías et al., 2009; Wiedenhoeft et al., 2009, Vitarelli et al., 2015).

Para a seção Cyclostigma, Wiedenhoeft et al. (2009) adicionaram a presença de laticíferos ou células especializadas na produção de látex no xilema secundário como sinapomorfia. Ressalta-se que os laticíferos possuem origem polifilética estando amplamente distribuídos em Croton e também em mais de 20 famílias não relacionadas de Angiospermas, contudo, podem ser um indicador morfológico de parentesco interespecífico (Farrell et al., 1991). Entretanto, o número de táxons amostrados até o presente ainda é pequeno quando comparado à riqueza de espécies do gênero, limitando a utilização e interpretação destes caracteres em diversos estudos (Caruzo et al., 2011).

A caracterização anatômica e histoquímica, muitas vezes é primordial para se classificar com segurança uma estrutura secretora, seja ela interna ou externa. Em Croton, por exemplo, as estruturas presentes na margem das folhas já foram descritas como nectários extraflorais (Freitas et al., 2001; Sá-Haiad et al., 2009) e como coléteres (Vitarelli et al., 2015). Embora coléteres e nectários extraflorais possam ocorrer em posições equivalentes, coléteres secretam mucilagem (Fahn, 1979; Thomas, 1991) ou uma mistura de mucilagem e substâncias lipofílicas (Fahn, 1979; Barreiro & Machado, 2007) enquanto nectários secretam néctar, uma solução adocicada que secreta principalmente açucar (Fahn, 1979). Logo, a análise da natureza química da secreção é necessária para a identificação correta de tais estruturas.

A presença de nectários foliares acropeciolares ou basilaminares unifica as espécies da seção Cleodora (Caruzo, 2010; Caruzo et al., 2011), enquanto a sua ausência é característica da seção Lamprocroton (van Ee et al., 2011). Este dado foi confirmado no estudo anatômico destes grupos que classificou estas estruturas como nectários extraflorais (Vitarelli et al., 2015).

Ao analisar o desenvolvimento floral de algumas espécies de Croton e Astraea, De-Paula et al. (2011) reforçaram a elevação da seção Astraea à gênero (Berry et al., 2005), pois a presença de nectários florais foi registrada como uma sinapomorfia entre os dois gêneros, entretanto, em Croton os nectários são vascularizados, já em Astraea este estado de caráter está ausente. Adicionalmente, está destacado como caráter diferencial entre os dois gêneros, a presença de coléteres

somente nas flores de Astraea. Contudo, novas evidências indicam que o registro da ausência de coléteres pode ser devido a problemas de amostragem, já que tais estruturas podem ser caducas e não detectadas na maturidade do órgão. Tal situação foi observada por Soares (2013) e Vitarelli et al. (2015) que trouxeram como primeiro registro a presença de coléteres nas folhas de Astraea e Croton, enquanto Machado et al. (2015) detectaram pela primeira vez coléteres no eixo da inflorescência de Croton glandulosus.

A denominação popular de sangue de dragão para as espécies da seção Cyclostigma se deve ao látex avermelhado que exsuda quando os troncos sofrem injúrias (Farías et al., 2009; Riina et al., 2009). Este látex é utilizado por populações locais para fins medicinais (Meza, 1999a, 1999b; Jones, 2003). Devido as propriedades biológicas, este produto vem interessando e sendo foco da indústria farmacêutica (Ubillas et al., 1994; Borges & King, 2000; Salatino et al., 2007). O látex é utilizado, principalmente, para impedir infecções provenientes de ferimentos e para acelerar a cicatrização (Ubillas et al., 1994; Salatino et al., 2007). As espécies desta seção também são indicadas para restauração de áreas degradadas, por apresentarem crescimento rápido (como C. urucurana Baill.), em projetos de paisagismo pelo hábito arbustivo (como C. bogotanus Cuatrec.) e pelas propriedades da madeira que é utilizada na construção civil e naval (Lorenzi, 1992; Carrenho et al., 1997). Na Costa Rica, Croton draco Schltdl. & Cham., é usado industrialmente com uma fonte de taninos condensados, o que indica que este recurso possui utilidade e grande potencial para a indústria, especialmente em países onde esta e outras espécies de sangre de drago não ocorrem naturalmente (Castro et al., 1999).

Entre os diversos metabólitos já encontrados no látex de sangre de dragão, Salatino et al. (2007) chamam a atenção para os alcalóides, como a taspina, para os taninos, diversos diterpenos e um grande número de óleos voláteis. Como propriedades biológicas destes compostos, apenas dois usos medicinais foram cientificamente comprovados para o látex da casca: a neutralização da hemorragia produzida pela picada de cobra Bothrops asper e a atividade imunomoduladora em células sanguíneas in vitro (Castro et al., 1999; Tsacheva et al., 2004). Contudo, existem muitos usos na medicina popular, essencialmente, nas Américas que podem ser vistos na tabela 1 para a espécie Croton draco.

Tabela 1 – Principais usos de Croton draco na medicina tradicional nas Américas. Fonte: Castro et al. (1999)

Usos	País
Medicina: Feridas, infecções, inflamação (Tsacheva et al., 2004)	Brasil
Medicina: Ferimento, doenças do estômago, inflamação, hipertensão, câncer (Murillo, 2004)	Colômbia
Medicina: Sapinho, acne, febre, úlceras, hemorragias (anti-hemorrágico) Outros usos: fonte de tanino, verniz, substituto detergente (Castro et al., 1999; González, 2006)	Costa Rica
Medicina: Inflamação, gripe, tosse, diarreia, úlceras, herpes, germicida após a extração do dente (Murillo, 2004)	Equador
Medicina: Adstringente, febre, goma, tratamento anti-hemorrágico, ferida (Standley and Steyermark, 1958)	Guatemala
Medicina: Dentes (fortalecer, limpar), úlceras, antimalárico (González, 2006)	Honduras
Medicina: Febre, adstringente, fortalecer os dentes, infecções de garganta, pé de atleta, espinhas, feridas, tuberculose, diarreia, cólera, antirreumáticos, tumores, tosse, úlceras, herpes, antisséptico para a extração do dente, dor de dente (Gupta et al., 2008; Salatino et al., 2007). Outros usos: madeira, árvore de sombra, gabinete, lenha, verniz, construção casa (pólos em forma de V, cercas) (García & García, 2008)	México
Medicina: Gripe, tosse, diarreia, úlceras, herpes, a extração de dente (germicida), fertilidade, a secreção vaginal, acne, diabetes, hemorroidas, hepatite, ajuda a perda de peso (Murillo et al., 2001; De Restrepo et al., 2005)	Peru
Medicina: Feridas, inflamações e infecções (Tsacheva et al., 2004)	Venezuela

Diante da complexidade taxonômica e importância econômica que possui a seção Cyclostigma, há a necessidade de estudos que ampliem a base de dados para auxiliar na resolução dos problemas taxonômicos, bem como compreender e classificar de forma mais consistente as estruturas secretoras que ocorrem em representantes deste grupo.

A partir de análises independentes e integradas da base de dados anatômicos obtidos na presente tese pretende-se responder as seguintes perguntas:

- Há caracteres anatômicos que diferenciam Croton seção Cyclostigma s.s. das seções irmãs?
- Os caracteres anatômicos aqui levantados são úteis para resolver casos problemáticos de delimitação de espécies, a qual não tem sido possível até agora com os caracteres morfológicos tradicionais, dentro da seção Cyclostigma s.s.?
- Qual a natureza química dos exsudados produzidos pelas estruturas secretoras presentes em representantes da seção Cyclostigma s.s.?

ORGANIZAÇÃO DA TESE

O presente trabalho encontra-se organizado sob a forma de artigos científicos, como disposto nas normas de redação de teses da Universidade Federal de Viçosa. Cada artigo segue as normas do periódico que ou foi publicado, ou está aceito ou será submetido.

Artigo I:

A new species of dragon’s blood Croton (Euphorbiaceae) from South America with singular inflorescences

Publicado no periódico Webbia: Journal of Plant Taxonomy and Geography.

Artigo II:

Secretory structures in leaves and flowers of two dragon's blood Croton (Euphorbiaceae): new evidence and interpretations

Aceito no periódico International Journal of Plant Sciences.

Artigo III:

Leaf Anatomy and its contributions to the systematics of the dragon's blood Croton sect. Cyclostigma (Euphorbiaceae)

À ser enviado ao periódico Botanical Journal of the Linnean Society.

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ARTIGO I

A new species of dragon's blood Croton (Euphorbiaceae) from South America with singular inflorescences

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A new species of dragon's blood Croton (Euphorbiaceae) from South America with singular inflorescences

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Croton amentiformis, a new species of Euphorbiaceae from western South America, is described and illustrated. Morphological evidence and data from leaf anatomy indicate that the new species belongs to the dragon's blood group (Croton section Cyclostigma). The dense and congested inflorescence is unusual within the genus. This singular dragon's blood species is dedicated to the Florence Tropical Herbarium (FT) on the occasion of its 100th Anniversary celebrated in 2014.

Keywords: Croton, Crotoneae, section Cyclostigma, Ecuador, Euphorbiaceae, Peru, Taxonomy.

Introduction

Recent studies in Neotropical Croton L. continue to recognize taxonomic novelties in different sections of the genus (e.g., Caruzo et al. 2008, 2010; Carneiro et al. 2011; Secco et al. 2012; Berry and Galdames 2013; Silva and Sodré 2014). In this paper we describe the morphology and leaf anatomy of a new arborescent species, Croton amentiformis, occurring in a remote area near the border between Ecuador and Peru. The German botanist Hermann Karsten made the first collection of this plant in 1854, when he traveled from Colombia to Ecuador (Diaz-Piedrahita 1996), but the species remained undescribed for more than 160 years. The new species is dedicated to the Florence Tropical Herbarium (FT) on the occasion of its 100th Anniversary celebrated in 2014.

Material and Methods

Morphology and Taxonomy

Comparative morphological study of the new species was conducted using collections from the following herbaria: COL, CPUN, F, GUAY, HA, HUTPL, LOJA, MA, MICH, MO, NY, Q, QAP, QCA, QCNE, and W. The protologue and type specimens of similar arborescent Croton from Ecuador and Peru were studied

and compared with the new species. Morphological characters used in recent species treatments of arborescent Croton (Riina et al. 2010, 2014) were examined and described. Trichome terminology followed Webster et al. (1996). Information about habit, habitat, and distribution was taken from specimen labels and recent field observations. The conservation status was evaluated using IUCN criteria (IUCN 2001).

Leaf Anatomy

Leaf samples from Croton amentiformis (Jorgensen 97; Cumbicus 1925, 2072, 2074) were prepared for light and scanning electron microscopy examination following the methods described in similar studies (Meira and Martins 2003; Vitarelli et al. in review; Bozzola and Russel 1992).

Results

Croton amentiformis Riina, **sp. nov.** (Figures 1, 2)

Diagnosis

Monoecious trees, 6–12 m high; young branches with a dense to sparse indumentum of stellate porrect trichomes; older apical branches smooth and shiny; petiole scars prominent; latex clear or light orange to reddish. Stipules subulate, 6–7.3 mm long, covered by sparse stellate trichomes, with colleters on upper margin and apex. Leaf blade lanceolate to ovate-lanceolate, discolorous, 9–18 × 2.8–6 cm; base rounded to slightly cuneate (cordate in young plants), sometimes slightly asymmetrical; apex acute; margin entire, slightly revolute, old colleters present on the margin; venation pinnate, brochidodromous; primary and secondary veins raised on the abaxial side; adaxial leaf side almost glabrous, with a few scattered stellate trichomes mostly along the midrib (densely hairy in young leaves); abaxial side densely covered with stellate porrect trichomes; petiolar glands 3–6, stipitate, apex convex, attached to the petiole on the adaxial side; petiole 3–6 cm long, with a dense to scattered indumentum of stellate trichomes. Inflorescences terminal, erect when young, usually pendant in full anthesis, 5–10 cm long; cymules densely congested along the inflorescence axis with pistillate flowers at the base and the staminate ones on the mid and upper part of the axis; bracts on the female portion of the axis 3.8–5 × 1.8–2.5 mm, lanceolate to spatulate, margin sometimes irregularly dissected; bracts on

the male portion of the axis 2–4 × 1–2.2 mm, lanceolate acute to rhomboid with apex sometimes incurved, margin entire. Staminate flowers sessile, sepals 5, ovate with acute apex, 3–3.4 × 1.3–1.5 mm, partially fused at the base, indumentum of simple trichomes present only on the apical margin; petals spatulate with acute apex, 3–3.2 × 0.2–1.2 mm, with simple trichomes along the margin, the apical portion with a denser indument; stamens 13–15, filaments 1.7–2 mm long, slightly pilose, anthers 0.6–0.8 × 0.3–0.4 mm. Pistillate flowers sessile, sepals 5, valvate or slightly imbricate, narrowly ovate to elliptic, 4.8–5.2 × 1.5–1.8 mm, abaxial surface with sparse to dense stellate trichomes, adaxial surface glabrous; glandular filaments present in the position of the petals, ovary slightly pyriform, 2.7–3.2 mm long, densely covered by stellate porrect trichomes; immature fruit 3.5–4.5 mm long, with sparse stellate trichomes; one of the three locules reduced in size probably by ovule abortion; styles 3, 3–4-bifid, united at the base, with sparse stellate trichomes. Mature fruits and seeds unknown. Common name: "Palo blanco" (Peru, Sánchez Vega 5304).

Type: Ecuador: Loja, Cerro Célica, Célica-Guachanamá, km 2.7, 04º05'46''S, 79º56'45''W, 2250 m, 12 April 1994, P. Jørgensen, C. Ulloa, H. Vargas and G. Abendaño 97 [holotype QCA; isotypes LOJA, MA, MICH, MO, QCNE].

Phenology

From the specimens examined, the species is known to flower from June to January.

Distribution and Habitat

The species occurs as a medium-sized tree in southwestern Ecuador (Loja) and northwestern Peru (Piura). It grows in more or less dense populations along roads, streamsides and forest edges in montane cloud forest, at elevations of 2200–2750 m.

Conservation Status

The geographical range of Croton amentiformis covers a relatively large area between southern Ecuador and northern Peru; however, forest vegetation in this region is fragmented due to human activities. As with many Croton species, C. amentiformis behaves as an early successional species, so forest perturbations promote its expansion. In some of the recently sampled localities in southern Ecuador, we found numerous individuals, including juveniles. For these reasons, we

consider this species as LC (“least concern”) according to the IUCN red list criteria (IUCN 2001).

Etymology

The specific epithet refers to the dense and compact inflorescences, resembling aments, which become pendant in anthesis (Figs. 2B, 2C).

Additional material examined (paratypes)

Ecuador. Loja: Cantón Célica, Parroquia Célica, Cerro Wayra Pungo, 04º04’29’’S, 79º55’44’’W, 2450 m, 28 June 2014, C.E. Cerón 74212 (QAP, QCNE, QCA, Q, COL), 74214 (QAP, QCNE, COL), 74219 (QAP, QCNE, QCA, Q, LOJA, GUAY, COL); Cantón Paltas, Parroquia Guanchanmá, Cerro Pucará, 04º03’09’’S, 79º52’08’’W, 2600 m, 29 June 2014, C.E. Cerón 74255 (QAP, QCNE, QCA, Q, HA, COL); via Célica, Cerro Guachanamá, 17615966-9547676, 2255 m, 22 November 2013, N. Cumbicus, F. Tinitana, O. Cabrera and C. Naranjo 1925, 2074 (HUTPL, MA), 24 January 2014, N. Cumbicus 2072 (HUTPL, MA); s.d., H. Karsten 23 (W); Llano del Amazonas, s.d., H. Karsten s.n. (LE). Peru. Piura: Prov. Huancabamba, entre Huancabamba y el Cuello del Indio (11 km), 2550 m, 4 May 1990, I. Sánchez Vega 5304 (CPUN, F); Prov. Morropón, Chalaco, carretera hacia Las Pircas, 2200-2750 m, 17 October 1988, C. Díaz and R. Vásquez 2984 (F, MO, NY).

Leaf Anatomy

The hypostomatic leaf blade presents abaxial epidermis with sinuous anticlinal walls and adaxial epidermis with straight walls (Figure 3A). Non-glandular, stipitate-stellate trichomes with a longer porrect central ray are also present (Figure 3B), predominating on the abaxial side along with paracytic stomata (Figure 3A). The petiole has a circular outline, with unistratified epidermis followed by 2-3 layers of angular-annular collenchyma and the collateral vascular system arranged as a ring with two smaller accessory bundles on the adaxial side (Figure 3C). The biconvex midrib presents 5-10 layers of angular-annular collenchyma interrupted by palisade parenchyma, and the vascular system is composed of collateral bundles (Figure 3D). The mesophyll is dorsiventral with palisade parenchyma thicker than the spongy parenchyma (Figure 3E). The epidermis is unistratified with a thickened cuticle and a single-layered hypodermis present on the adaxial side (Figure 3E). The margin is

revolute, with discontinuous palisade parenchyma (Figure 3F). Extrafloral nectaries (EFNs) are observed both in basilaminar and acropetiolar positions (Figure 3G, H) and standard type colleters occur on the teeth along the margin (Figure 3I, J). The stipitate EFNs present a convex surface with the secretory palisade-like epidermis covered by a thin cuticle and the subepidermal parenchyma portion vascularized mainly by phloem. Druse crystals (Figure 3K) and secretory idioblasts (Figure 3L) as well as non-articulated ramified laticifers (associated with phloem and colleters) (Figure 3M) occur both in apical meristems and mature leaves.

Discussion

Morphology and Taxonomy

This species is unique within Croton because of its peculiar pendant inflorescence with congested flowers and dimorphic bracts. The species is placed in section Cyclostigma (Riina et al. 2009; van Ee et al. 2011) based on morphology and leaf anatomy. Besides the anatomical features discussed below, Croton amentiformis shares several morphological characters with members of this section, i.e. arborescent habit, presence of orange to reddish latex, stellate indumentum, well developed stipules, and conspicuous petiolar glands or EFNs. Croton amentiformis is similar to C. rimbachii Croizat, which has similar habitat (cloud forest) and geographic distribution (Bolivia, Ecuador, and Peru). However, they are easily distinguished by the inflorescence morphology (densely flowered and pendant vs. sparsely flowered and erect), flower pedicels (sessile vs. long-pedicellate), and number of styles branches (multifid vs. bifid).

Leaf Anatomy

Croton amentiformis presents leaf anatomical features that are similar to the pattern reported for some species in the genus (Solereder 1908; Metcalfe and Chalk 1983; Webster et al. 1996; Sá-Haiad et al. 2009; Freitas et al. 2001; Vitarelli et al. in review), and more specifically for members of section Cyclostigma (Feio et al. in prep.). A distinctive character was the presence of single-layered hypodermis, which has never been described for Croton (Metcalfe and Chalk 1983), and was observed in some species of section Cyclostigma, namely C. bogotanus Cuatrec., C. coriaceus Kunth, C. floccosus B.A.Sm. and C. rimbachii Croizat (Feio et al. in prep.). The hypodermis is considered a common structure for the storage of water, also playing

an important role in heat economy (Madison 1977). Espírito Santo and Pugialli (1998) attribute its presence to a greater exposure of the leaves to light. Croton amentiformis occurs along mountain creeks, cloud forest edges, and along roads. Despite the high humidity present in montane cloud forests, the species is exposed to high luminosity, which can also explain the presence of the thickened cuticle, besides the well developed palisade parenchyma as xeromorphic features (Fahn 1990). The occurrence of only colleters along the leaf margin distinguishes the species of this section from Croton groups where such structures alternate with EFNs along the leaf margin, as in section Cupreati (Feio et al. in prep.) and section Cuneati (Riina et al. 2010, their fig. 3F). The colleters are active in young leaves and apical meristems, while they may be persistent (old and inactive) or absent (caducous) in mature leaves (Vitarelli et al. in review; Feio et al. in prep.).

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Figures legends

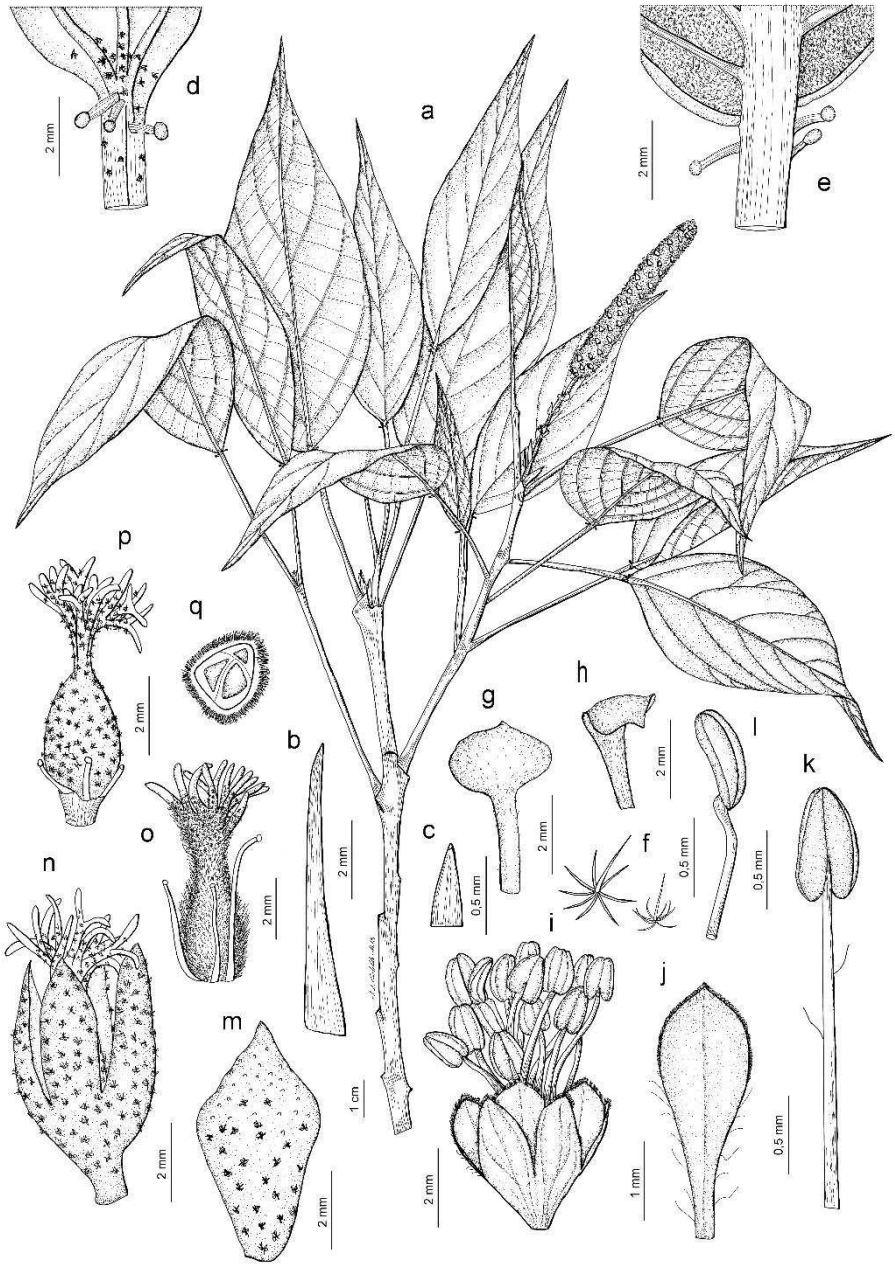

Figure 1. Croton amentiformis: (**A**) Flowering branch. (**B**) Stipule. (**C**) Detail of the stipule apex. (**D**) Leaf base, adaxial side. (**E**) Leaf base, abaxial side. (**F**) Detail of the leaf indumentum. (**G**) Male bract. (**H**) Male bract with incurved apex. (**I**) Staminate flower. (**J**) Petal of the staminate flower. (**K**) Stamen, abaxial side. (**L**) Anther insertion, lateral view. (**M**) Female bract. (**N**) Pistillate flower. (**O**) Young pistillate flower with glandular filaments instead of petals, calyx removed. (**P**) Pistillate flower, calyx removed. (**Q**) Cross section of ovary. Based on Cumbicus 2072 (MA).

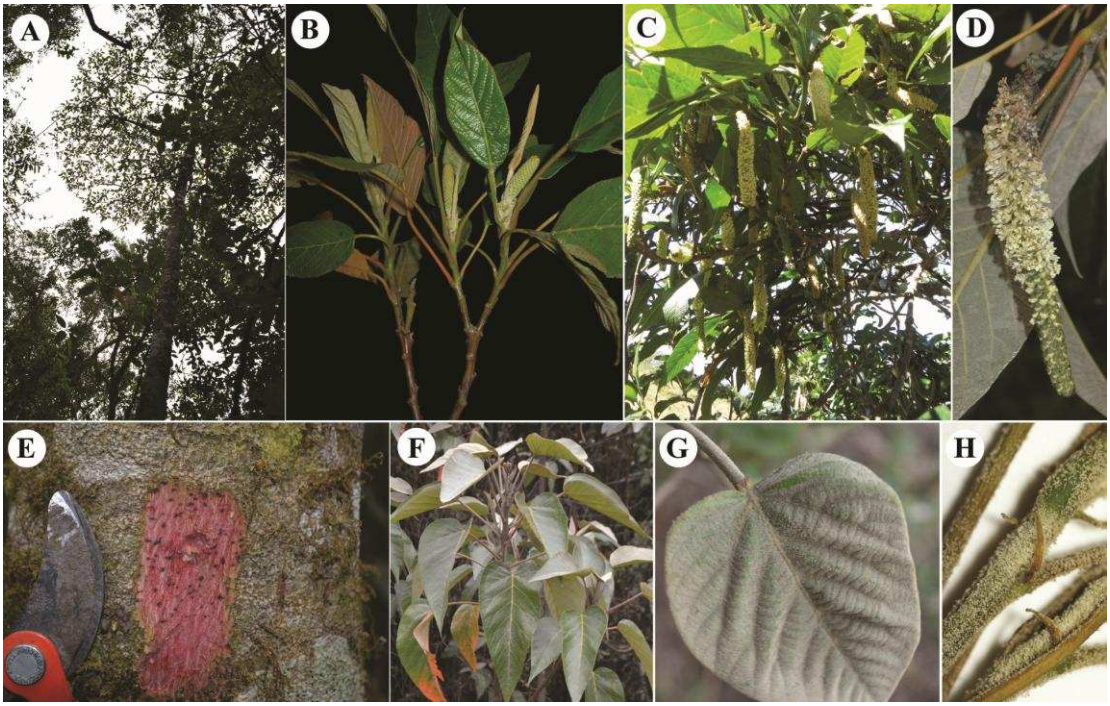

Figure 2. Croton amentiformis: (**A**) Adult individual in its natural habitat. (**B**) Flowering branch with young inflorescences. (**C**) Flowering branches with mature, pendant inflorescences. (**D**) Detail of inflorescence. (**E**) Part of trunk with a bark cut. (**F**) Young individual with deeply cordate leaves. (**G**) Young leaf of juvenile plant showing dense indumentum on the adaxial side, and petiolar glands. (**H**) Young branches showing indumentum and stipules. Photographs by CER, NC, and RR.

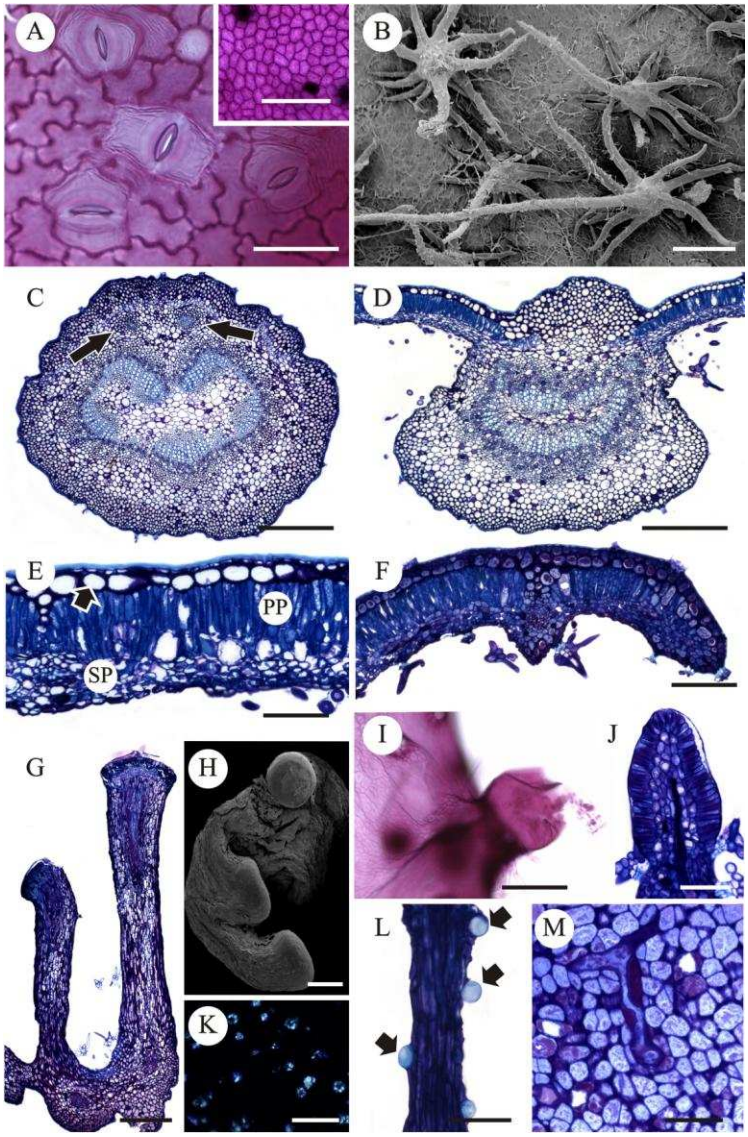

Figure 3. Leaf anatomy of Croton amentiformis: (**A**, **B**, **H**) Details of the leaf surface. (**C**-**F**) Cross sections. (**G**, **J**, **L**, **M**) Longitudinal sections. (**A**) Abaxial epidermis with sinuous anticlinal walls and paracytic stomata. The inset shows the adaxial epidermis with straight walls. (**B**) Scanning electron micrograph showing the non-glandular stipitate-stellate trichomes with a longer porrect central ray. (**C**) Arrangement of vascular system with two smaller accessory bundles on the adaxial side (arrows). (**D**) General view of the midrib. (**E**) Details of mesophyll showing single-layered hypodermis on the adaxial side (arrow), palisade parenchyma (PP), and spongy parenchyma (SP). (**F**) General view of the revolute margin. (**G**, **H**) Stipitate EFNs showing their convex apical surface. (**I**) Marginal colleter, inactive stage, on diaphanized mature leaf; (**J**) Marginal colleter, active stage, on apical meristem, note the presence of laticifers intensely stained in the central axis of parenchyma cells. (**K**) Polarized-light image of druse idioblasts. (**L**) Portion of EFN stipite showing secretory idioblasts (arrows) above the epidermal cells. (**M**) Non-articulated ramified laticifer in the apical meristem. Bars: (**A**) = 70µm; (**inset**) and (**H**) = 200µm; (**B**) and (**K**) = 100µm; (**C**), (**D**) and (**G**) = 900µm; (**E**), (**J**), (**L**) and (**M**) = 150µm; (**F**) and (**I**) = 300µm.

ARTIGO II

Secretory structures in leaves and flowers of two dragon's blood Croton (Euphorbiaceae): new evidence and interpretations

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SECRETORY STRUCTURES IN LEAVES AND FLOWERS OF TWO DRAGON'S BLOOD CROTON (EUPHORBIACEAE): NEW EVIDENCE AND INTERPRETATIONS

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Short title: Secretory structures of dragon's blood Croton

ABSTRACT

Premise of the Research. Previous studies of secretory structures in species of the Neotropical dragon's blood Croton (section Cyclostigma) show inconsistencies in their classification. An accurate assessment of the identity and homology of such structures is essential for taxonomic and evolutionary studies.

Methodology. Field-collected material of leaves, stipules, and flowers at different developmental stages were sampled. The material was subjected to standard anatomical study by light microscopy and SEM, and secretion was evaluated by histochemical analyses.

Pivotal Results. Leaves and flowers of Croton echinocarpus and C. urucurana present five secretory structures (idioblasts, laticifers, colleters, extrafloral nectaries and floral nectaries) with high similarity between the two species. Idioblasts secrete compounds of a mixed nature; laticifers are branched non-articulated; and colleters and nectaries present hydrophilic secretion. The leaf marginal glands previously described as extrafloral nectaries are actually colleters of the standard type. We found colleters in staminate and pistillate flowers. The histochemical tests detected proteins in the secretions of all structures.

Conclusions. The classes of secondary metabolites detected support the biological activities of secretion described in the literature. The correct identification of colleters in flowers establishes a new register of these structures in flowers of this genus. We show that an approach integrating anatomic structure, histochemistry, and period of secretion activity, allow for a more accurate classification and homology assessment of secretory elements in this genus, which is exceptionally rich in this type of structures.

Keywords: anatomy, colleters, Crotoneae, histochemistry, nectaries, stipules.

INTRODUCTION

Croton echinocarpus Müll. Arg. and C. urucurana Baill. are members of section Cyclostigma, a group of tree species popularly known as dragon's blood because of the exudation of abundant red latex (van Ee et al. 2011; Riina et al. 2009) that is used for medicinal purposes across the Neotropics. Besides laticifers, four other secretory structures (colleters, idioblasts, extrafloral nectaries, and secretory trichomes) have been identified in the leaves of several Croton lineages different than section Cyclostigma (Vitarelli et al. 2015). Previous anatomical surveys on members of this section included the two species studied here and described secretory structures from both leaves and flowers (Sá-Haiad et al. 2009, De-Paula et al. 2011), however classification and homology assessment of such structures remain unclear and require further investigation.

Extrafloral nectaries have been recorded to occur on the margin, base and blade of leaves of Croton echinocarpus and C. urucurana (Sá-Haiad et al. 2009). However, similar marginal glands have been described as colleters of the standard type in different sections of Croton and other Crotoneae genera (Astraea Klotzsch and Brasiliocroton P.E. Berry & Cordeiro) (Riina et al. 2014, 2015; Vitarelli et al. 2015). De-Paula et al. (2011), in a study of the floral morphology and anatomy of several Crotoneae species, including C. urucurana, found glands alternate to floral nectaries that they described as filamentous structures. These authors also reported glands along the surface of sepals as secretory trichomes, although they noticed their similarity to colleters (De-Paula et al. 2011).

For an accurate classification of a secretory structure, its anatomical characterization must be accompanied by an analysis of the chemical nature of its exudate and an evaluation of the period of secretory activity. Descriptions based solely on morphology are not sufficient to assign a role to a structure (Lersten and Curtis 1996) and to determine the importance of the exudate for the plant. Additionally, morphologically similar structures may play different functional roles. Extrafloral nectaries can be confused with other secretory structures such as colleters because the latter are also external structures that can have similar anatomy and topology. However, the period of activity and the composition of the secretion, show that the activity of colleters is earlier than that of nectaries, and the colleters'

secretion is characterized by the presence of mucilage, lipids, proteins, and the absence of sugar (Fahn 1979; Thomas 1991; Cruz et al. 2002; Klein et al. 2004; Barreiro and Machado 2007). Nectaries, on the other hand, secrete nectar, a sugary substance consisting mainly of glucose, fructose and sucrose (Fahn 1979; Bentley and Elias 1983).

Due to inconsistencies distinguishing between nectaries and colleters in previous studies of Croton and the importance of an accurate classification of such structures for taxonomy and evolutionary studies requiring accurate homology assessment, we analyze the structure and histochemistry of leaf and floral secretory structures of Croton echinocarpus and C. urucurana. We also discuss the relationship between structure, secretion composition, and function, as well as the chemical nature of the secretions and its connections to the phytochemistry findings and medicinal uses reported for both species in the literature.

MATERIAL AND METHODS

Croton echinocarpus is endemic to the Atlantic Forest of southeastern Brazil (Caruzo and dos Santos 2015), while C. urucurana is more widely distributed in southern South America (Brazil, Argentina, Paraguay and Bolivia), occurring as a pioneer species in riparian forest (Caruzo and Cordeiro 2007; Cordeiro et al. 2015). Both species occur spontaneously in Minas Gerais state, as well as around the Campus da Universidade Federal de Viçosa (20°45'10.109" S, 42°52'16.167"W) and along the BR-356 (20°54'3.939" S42°39'12.857"W), where field work for this study was conducted. Samples were collected from vegetative and reproductive branches from both species. Herbarium vouchers were deposited at the VIC herbarium (Coutinho et al. 337, 338). Leaves at different developmental stages (shoot apex, leaf primordium and mature leaves), stipules and staminate and pistillate flowers in different stages of development were sampled (from bud to early fruit).

Three fixative treatments were used for three different sets of samples. The first set was fixed in FAA (Johansen 1940) for 24 h (formalin, acetic acid, ethanol 50%, 1:1:18, by vol) to carry out a structural characterization using light microscopy,

and to detect hydrophilic compounds. The second was fixed in neutral buffered formalin for 48 h (NBF; Lillie 1965) to detection lipophilic compounds and ferrous sulfate in formalin (FSF; Johansen 1940), a fixative utilized for demonstrations of total phenolic compounds. Finally, the third group of samples was fixed in formaldehyde-glutaraldehyde 2.5% (phosphate buffer 0.1M pH 7.3; Karnovsky 1965) for structural and histochemical complementary analysis.

For the description of surface characters, entire leaves were cleared by using a solution of 10% sodium hydroxide and 20% sodium hypochlorite, interspersed with successive washes in distilled water (Shobe and Lersten 1967). The materials were stained with basic fuchsin (0.5% alcoholic solution) and slides were mounted with glycerinated gelatin (Keiser 1880).

Part of the samples was dehydrated in an ethanol series and embedded in methacrylate (Historesin, Leica Instruments, prepared according to the manufacturer instructions). The samples were cross and longitudinally sectioned at 4 to 6 µm in an automatic rotary microtome (model RM2265, Leica® Biosystems, Nussloch GmbH) using glass knives (Leica®, Biosystems, Nussloch GmbH). Sections were stained with toluidine blue pH 4.6 (O'Brien et al. 1965) and slides were mounted with synthetic resin (Permount®, New Jersey, USA).

Part of the samples was also dehydrated through a tert-butanol series (Johansen 1940), embedded in histological paraffin with DMSO (Histosec®, ©Merck KGaA, Darmstadt, Germany) and serially sectioned at 10 to 12 µm thickness on a rotary microtome (Spencer® 820, ©American Optical, Buffalo, New York). For structural characterization, longitudinal and cross sections were stained with Astra blue 1% (Kropp 1972) and Safranin O 1% (Bukatsch 1972, adapted) and mounted with synthetic resin. For structural characterization and histochemical tests, floral samples were dehydrated through tert-butanol series and embedded in histological paraffin with DMSO.

For detection of the main classes of secondary compounds the following histochemical tests were carried out: PAS (Periodic-Acid-Schiff's reagent) for total polysaccharides (McManus 1948), ruthenium red for acidic mucilage (Gregory and Baas 1989), tannic acid/ferric chloride for neutral mucilage (Pizzolato and Lillie 1973), Sudan black B (Pearse 1985) and neutral red (Kirk Jr. 1970) for total lipids in visible and under UV light, respectively, NADI reagent for terpenoids (David and Carde 1964), copper acetate/rubeanic acid for fatty acids (Ganter and Jollés 1969,

1970), Nile blue for neutral and acidic lipids (Cain 1947), vanillin/hydrochloric acid for tannins (Mace and Howell 1974), Wagner's reagent for alkaloids (Furr and Mahlberg 1981), xilidine Ponceau (O'Brien and McCully 1981) and ninhydrin/Schiff's reagent for protein (Pearse 1985). Standard control procedures were carried out simultaneously as required for each test and the slides were mounted in glycerinated gelatin (Kaiser 1880). To verify the occurrence of glucose in the secretion, Glicofita Plus® (Accu-Chek Active® - F. Hoffmann-La Roche Ltd.©) was used directly on the secretion.

Observations and photographic documentation were performed with a light microscope (Model AX70TRF, Olympus Optical, Tokyo, Japan) equipped with a U-Photo system and digital camera (AxioCam HRc; Carl Zeiss, Gottingen, Germany) and an epifluorescence HBO 50W mercury vapour lamp and a filter block A (exciter filter BP 340–380, dichroic mirror 450, barrier filter LP-430). Macro images were obtained using a stereomicroscope (Stemi 2000-C®, ©Carl Zeiss Microscopy GmbH, Jena, Germany) coupled with digital camera (AxioCam ERc5s®, ©Carl Zeiss Microscopy GmbH, Jena, Germany).

For studies in scanning electron microscopy (SEM), the samples previously fixed in FAA or formaldehyde-glutaraldehyde 2.5%, were dehydrated in ethanol and critical point dried with CO2 in a 020 CPD dryer (Bal-Tec; Balzers, Liechtenstein). The samples were mounted onto stubs and coated with gold using a FDU 010 sputter coater (Bal-Tec). Examinations and image captures were conducted using a Leo 1430VP SEM (Zeiss, Cambridge, United Kingdom) at the Centro de Microscopia e Microanálises at Universidade Federal de Viçosa.

For the classification of the laticifers was followed De Bary (1884) and the colleters were classified according to Thomas (1991).

RESULTS

Field observations of morphological features and secretions

Flowers in the studied species are relatively small (< 1 cm), unisexual and arranged in thyrsoid inflorescences (fig. 1). Floral nectaries (FNs) in pistillate and staminate flowers, and glands along the leaf margin are minute and at the time of fieldwork we did not observe any secretion with the naked eye. For this reason we were unable to perform the Glicofita Plus® test on their secretions.

The leaves of both species present two types of glands. The acropetiolar/basilaminar glands, located at the junction of the petiole with the lamina, are easily noticed by their conspicuous size (fig. 2-3). The translucent secretion of these glands is produced centrally on the surface of each gland (fig. 2). The glands are globular and sessile in Croton echinocarpus (fig. 3) and patelliform and stipitate in C. urucurana (fig. 2, 4). Ovoid glandular structures, much smaller than the acropetiolar/basilaminar glands, are evident along the margin of young leaves and leaf primordia in both species (fig. 5). In mature leaves, these glands are persistent and with a brown coloration in C. echinocarpus, or deciduous and leaving a scar in C. urucurana. Ants were only observed visiting (fig. 6) and colleting secretion (fig.7) of the acropetiolar/basilaminar glands of both species.

The application of Glicofita Plus® on the secretion of acropetiolar/basilaminar glands detected significant glucose concentration (263 mg/dL; fig. 8), confirming that these glandular structures are best interpreted to be extrafloral nectaries (EFN). Unfortunately, the secretion of the glands along the leaf margin could not be tested in the field due to the minute size of such glands.

In relation to the latex, we observed a sticky secretion when young branches were cut or damaged. When fresh, the latex was green in C. urucurana (fig. 9) and light brown C. echinocarpus (fig. 10), and it quickly turned reddish by oxidation of its compounds in both species (fig. 11). The same sticky secretion was observed when making cuts directly on the trunk bark.

Internal Secretory Structures

Secretory idioblasts are dispersed in ground tissues and epidermis. Leaf epidermal idioblasts, in all developmental stages, are larger than ordinary epidermal

cells and are projected outside the surface (fig. 12). In few cases, idioblasts are present at the base of non-glandular trichomes (fig. 13). In pistillate and staminate flowers, secretory idioblasts in different development stages are dispersed in the ground tissues of all floral whorls. Regardless of the region and development phase, idioblasts are fully differentiated, with heavily stained content in flowers and leaves.

Non-articulated branched laticifers (fig. 14) are dispersed in the ground tissues of both leaves and flowers. Our observations of shoot meristems show these laticifers with a Y-shaped branching pattern (inset fig. 14) and with evident secretory activity.

External Secretory Structures

Colleters occur on the margin of leaves (fig. 15), on the base, margins and apex of stipules (fig. 16, inset fig. 16), and in pistillate (fig. 17, 19) and staminate (fig. 18) flowers where they alternate to FNs. Colleters are fully developed and active in young organs, and they are present in shoot meristems, developing leaves (fig. 15), flower buds (fig. 17-19), and flowers in pre-anthesis (fig. 20). The structure of these colleters is of the standard type (fig. 21, inset fig. 16), since they are composed of secretory palisade epidermis covered with a thick cuticle, arranged radially to a non-secretory and non-vascularized parenchymal central axis. Secretory idioblasts, laticifers, and cells with druse crystals are common among the cells of the central axis of colleters (fig. 21).

Before maturity, leaves exhibit conduplicate ptyxis, and are sealed along the margins by both intertwining stellate trichomes and the sticky secretion produced by the marginal colleters (fig. 15). Likewise, the sticky secretion produced by colleters of floral buds overflows and helps to keep the buds closed. When the leaves fully expand and flowers become anthetic, the colleters senesce (fig. 22, 23) and turn brown (C. echinocarpus leaves) or are deciduous, each leaving a scar (fig. 25; C. urucurana leaves, and flowers of both species).

Although the two species present morphologically distinct EFNs: globular and sessile in Croton echinocarpus (fig. 26-28) and patelliform and stipitate in C. urucurana (fig. 29-31), they are anatomically similar. They have a convex surface consisting of a uniseriate palisade secretory epidermis (fig. 32), with dense protoplast, covered by a smooth and thick cuticle (fig. 28, 31, 32). Underlying the

secretory epidermis, there are 1-2 layers of sclerenchyma (fig. 32, 33), and 10-12 layers of nectariferous parenchyma, of isodiametric cells with dense cytoplasmic contents (fig. 32-35). This parenchyma contains druse crystals, secretory idioblasts and laticifers (fig. 32-36). EFNs are vascularized by bundles originating from the lamina and/or the petiole. These bundles reach the entire nectariferous parenchyma and consist of both xylem and phloem but with the latter being more abundant (fig. 31, 34). At the base of EFNs, non-glandular trichomes are also present (fig. 26, 27, 29).

The EFNs present synchronism with leaf development. Before leaf lamina expansion, EFNs are inactive and the surface of each has an entire, thick cuticle (fig. 26). Upon leaf expansion, the nectaries become active and accumulate secretion beneath the cuticle of each EFN. This process promotes the distension of the cuticle, mainly towards the center of each EFN (fig. 27, 29), and also its subsequent rupture during the secretory phase (figs. 30, 36). When EFNs become senescent, a necrotic parenchyma can be observed sometimes (fig. 37).

Floral nectaries of pistillate and staminate flowers do not differ anatomically between the two species. Each flower has a five-lobed FN (fig. 38) located in the region between the outer wall of the ovary and the adaxial side of the sepals (fig. 39, 40). In longitudinal sections of flowers, the lobes are horn-like (fig. 39, 40), whereas in cross sections they present convex surface with a slight concavity in the central part. The epidermis consists of cuboidal secretory cells, covered by a thin cuticle (fig. 41, 42) with few inactive stomata, through which the secretion of nectar occurs (inset, fig. 40). Longitudinal sections of flowers under fluorescent light show a 1-3 layers of columnar sclerenchyma underlying the secretory epidermis of FN lobes (fig. 43, 44), with an arrangement similar to that in EFNs. The secretory parenchyma is well developed consisting of vacuolated cells, and secretory idioblasts with acidophile content heavily stained with safranin, druse crystals and laticifers (fig. 41, 42). The vascularization is dense, derived mainly from the vascular system of the receptacle, with 1-2 branches of vascular bundles that reach the secretory parenchyma (fig. 45); some vascular bundles are surrounded by secretory idioblasts (fig. 41).

Histochemical analysis

The results of histochemical tests are summarized in Table 1 and figures 46-72. In laticifers, fatty acids were detected only in Croton urucurana. The other metabolites (i.e. total lipids, acidic lipids, fatty acids, phenolic compounds, alkaloids and proteins) were also detected in idioblasts of both species, except carbohydrates. EFNs, FNs and colleters produce exclusively hydrophilic secretions, rich in carbohydrates and proteins.

DISCUSSION

New evidences regarding idioblasts and laticifers

For species of tribe Crotoneae, secretory idioblasts have been described as containing lipophilic substances (Webster et al. 1996; Freitas et al. 2001; Sá-Haiad et al. 2009), and Vitarelli et al. (2015) indicated these structures as sites of lipophilic compounds, however the above mentioned studies only tested for total lipids and total polysaccharides. Our results show that in Croton echinocarpus and C. urucurana, idioblasts secrete compounds of a mixed nature, including both lipophilic (fatty acids) and hydrophilic substances (Table 1). We detected alkaloids in both idioblasts and laticifers in all tissues and external secretory structures. In fact, it is possible that part of the synthesis and storage of precursors of indolic, tropanic and nicotinic alkaloids occur in the vacuole of secretory idioblasts, as observed in Apocynaceae (DeLuca and Cutler 1987; DeLuca and St-Pierre 2000), so these structures act as transition sites.

The type of laticifer in the two studied species, i.e. branched non-articulated, agrees with the pattern described in the literature for section Cyclostigma (Rudall 1987, 1989; Wiedenhoeft et al. 2009). In contrast, in Croton sections Alabamenses, Lamprocroton and Cleodora, laticifer type varies (between articulated and non-articulated), even within the same section (Vitarelli et al. 2015). These authors suggested that articulated laticifers might be the most common and widespread in the

entire tribe Crotoneae, however their taxon sampling was limited considering the size of Croton, and section Cyclostigma was among the unsampled clades.

The latex and the cork of dragon's blood Croton are used for medicinal purposes throughout the Neotropics (Meza 1999a, 1999b; Jones 2003; Salatino et al. 2007). Phytochemical studies have reported bactericidal and antifungal (Peres et al. 1997; Gurgel et al. 2005) activities for Croton urucurana, and both activities are related to the presence of phenolic compounds, alkaloids and diterpenes. Although diterpens have not been detected for Croton echinocarpus, this species also showed bactericidal activity, as well as antioxidant and anti-HIV action (Athayde 2013).

Distinguishing colleters from nectaries

Colleters and nectaries (EFNs and FNs) in C. echinocarpus and C. urucurana present hydrophilic secretion, but sugar was only investigated in nectaries. Even if both colleters and nectaries have similar histochemical results, the other two criteria, anatomical characterization and period of secretory activity, allow us to make a distinction between the two structures. When nectaries are still differentiating and unable to produce secretion, colleters are fully developed and active in the shoot meristems. On the other hand, when nectaries become active, colleters become senescent or fall off, showing the asynchronous development and activity of these structures only in the early stages of development. This asynchrony between colleters and nectaries was also observed by Riina et al. (2015) and Vitarelli et al. (2015) for other species of Croton. Although we did not conduct systematic measurements of colleters and nectaries at maturity, our field observations suggest that size and external shape might be useful characters to distinguish these two structures. In fact the reason why we could not conduct the Glicofita Plus® test on the secretion of colleters (marginal glands) was because colleters were minute and smaller than nectaries. Future studies should look at size and external shape of colleters versus nectaries using an overall sampling of the genus to corroborate these observations.

Sá-Haiad et al. (2009) studied several species of tribe Crotoneae, including Croton echinocarpus and C. urucurana, however the leaf marginal glands described by these authors as EFNs, are actually colleters of the standard type (Riina et al. 2014, 2015; Vitarelli et al. 2015). Our results show that colleters are also present in the reproductive organs of C. echinocarpus and C. urucurana where they alternate with the lobes of the FN, and a recent study documented colleters along the inflorescence axis of C. glandulosus L. (Machado et al. 2015).

In the vegetative organs of Croton echinocarpus and C. urucurana, colleters occur only along the leaf margin, and at the base and margin of stipules, which is similar to what was found for C. amentiformis Riina, another species of section Cyclostigma (Riina et al. 2015). These leaf marginal colleters distinguish species of section Cyclostigma from other Croton groups where such structures alternate with EFNs along the leaf margin, as in section Cupreati (Feio et al. in prep.) and section Cuneati (Riina et al. 2010, their fig. 3F). In taxonomic description of Croton, leaf marginal colleters have been called ovoid glands (Riina et al. 2010) or simply glands (e.g. Caruzo and Cordeiro 2013). None of them are incorrect, however, gland is a generic term used just to recognize a structure usually protruding and secretory.

De-Paula et al. (2011) observed secretory structures in flowers of Croton (including C. urucurana) with the same position of the colleters found here, however, they described them as filamentous structures instead of colleters. Likewise, structures on the surface of sepals were described as secretory trichomes instead of colleters, even if the above authors recognized their similarity with colleters. To date, the absence of colleters in the flowers of Croton was one important difference separating this genus from its close relative genus Astraea (De-Paula et al. 2011), however, our results suggest that colleters in flowers appear not to be exclusive of Astraea and could be widespread in Croton, given the relatively conserved floral morphology across the genus (van Ee et al. 2011).

We found sclerenchyma cells forming a continuous layer in the active EFNs and senescent FNs, underneath the secretory epidermis, which was also observed by Freitas and Paoli (1999) in EFNs of Croton urucurana. Sclerenchyma cells in nectaries are considered a specialized feature that may be related to the protection of the parenchematic secretory tissue (Belin-Depoux 1989). Another characteristic regarded as highly specialized is the vascularization of nectaries (Elias 1983), as observed in Croton echinocarpus and C. urucurana. The vascularized nectaries found here support the findings of Vitarelli et al. (2015) regarding EFNs, and De-Paula et al. (2011) for FNs.

The role of FNs in Croton is still unclear and needs further investigation with a better taxon sampling across the genus. The few pollination studies of Croton available report entomophilous and anemophilous species, such as C. urucurana (Pires et al. 2004) and C. sarcopetalus Müll. Arg. (Freitas et al. 2001), respectively, as well as several ambophilous species (Bullock 1994; Webster 1994). On the other

hand, Narbona and Dirzo (2010) described FNs that possess the functions extranuptial and nuptial in Croton suberosus Kunth. In Croton urucurana, it has been hypothesized that the FNs are not essential for pollination because of the amount of nectar produced and the morphology of the flowers, which fit better the anemophilous syndrome (Pires et al. 2004). The anemophilous syndrom was also suggested for C. floribundus Spreng. and C. priscus Croizat (Passos 1995).

Histochemical evidences

The histochemical tests detected proteins in the secretions of EFNs, FNs, colleters, laticifers and idioblasts. The presence of proteins in laticifers can be due to the fact that the latex blends with the cytoplasmic contents of the laticifer cell, where proteins are usually found (Demarco et al. 2006). On the other hand, the proteins present in nectaries can be related to the energetic demands for nitrogen of flower visitors and pollinators (Nicolson and Thornburg 2007) since many insects have deficiencies in the production of proteins (Baker 1977).

In the case of colleters, which primary function is of lubrification and protection against desiccation of organs in early development (Fahn 1979), proteins are also important for the protection against herbivores and pathogens (Klein et al. 2004; Miguel et al. 2006). Additionally, the presence of mucilage in these structures also indicates their classification as colleters. Mucilage is important for the protection of developing organs, for water retention, and defense against herbivores (Fahn 1979; Gregory and Baas 1989).

The classes of secondary metabolites found in this study (Table 1) support the biological activities described in the literature for Croton urucurana and C. echinocarpus, especially antifungal, anti-inflammatory, and antioxidant activities (Gurgel et al. 2005; Salatino et al. 2007; Simionatto et al. 2007). These properties are related to substances primarily derived from phenolic compounds such as catechin, terpenes like the acetyl aleuritic acid (Gurgel et al. 2005), and alkaloids such as taspine (Salatino et al. 2007).

CONCLUSION

The great similarity in morpho-anatomy and position of secretory structures between Croton echinocarpus and C. urucurana is in agreement with their phylogenetic proximity (Riina et al. 2009) and habitat affinities. We detected the presence of colleters in Croton flowers, a structure that was overlooked or misinterpreted in previous studies dealing with floral anatomy of Croton, and which may be a unifying character between this genus and the closely related genus Astraea. The diversity of secretory structures and the chemical compounds detected confirm the potential of these species for bioprospection.

Despite having similar histochemical composition, nectaries and colleters can be distinguished based on their structure, function, and period of activity. Our results show that the use in combination of the three criteria applied here (anatomical structure, histochemistry, and period of activity) allow a more accurate classification and homology assessment of secretory elements in a genus exceptionally rich in this type of structures.

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FIGURES AND LEGENDS

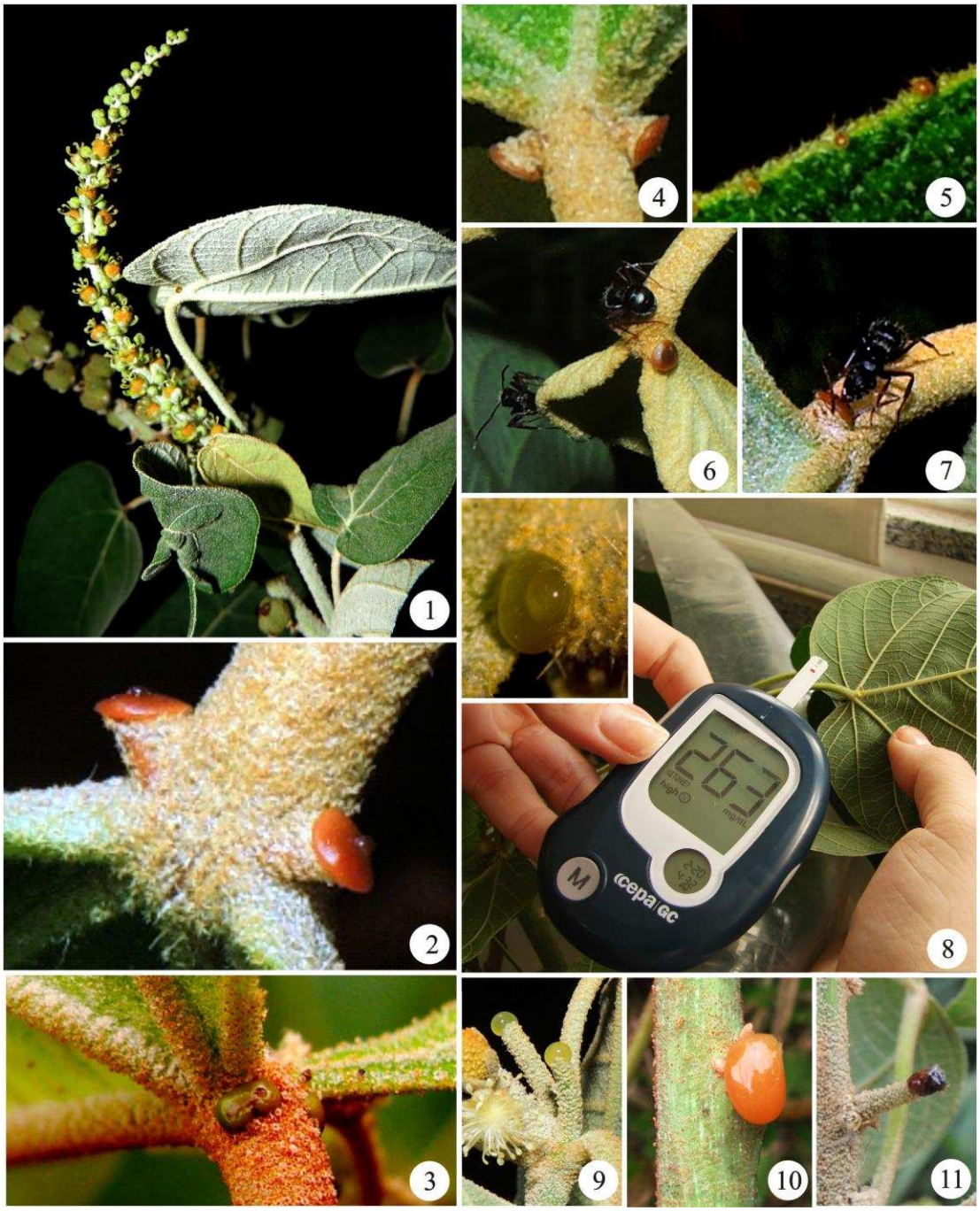

Fig. 1-11. Croton urucurana (1, 2, 4, 6-9, 11) and C. echinocarpus (3, 5, 10) in the field. 1. Unisexual flowers arranged in thyrsoid inflorescence; 2-4. Acropetiolar/basilaminar extrafloral nectaries (EFNs), 2. Translucent secretion on the central portion of the EFN; 3. Globular and sessile EFN; 4. Pateliform and stipitate EFN; 5. Marginal colleters; 6-7. Ants visiting the EFNs; 8. Application of glicofita showing the glucose concentration; 9-11. Secretion in different colorations of latex exuding from wounded petiole; 9. Fresh secretion; 10. Fresh secretion, 11. Dry and oxidized secretion (reddish); Photos: Coutinho, I.A.C.

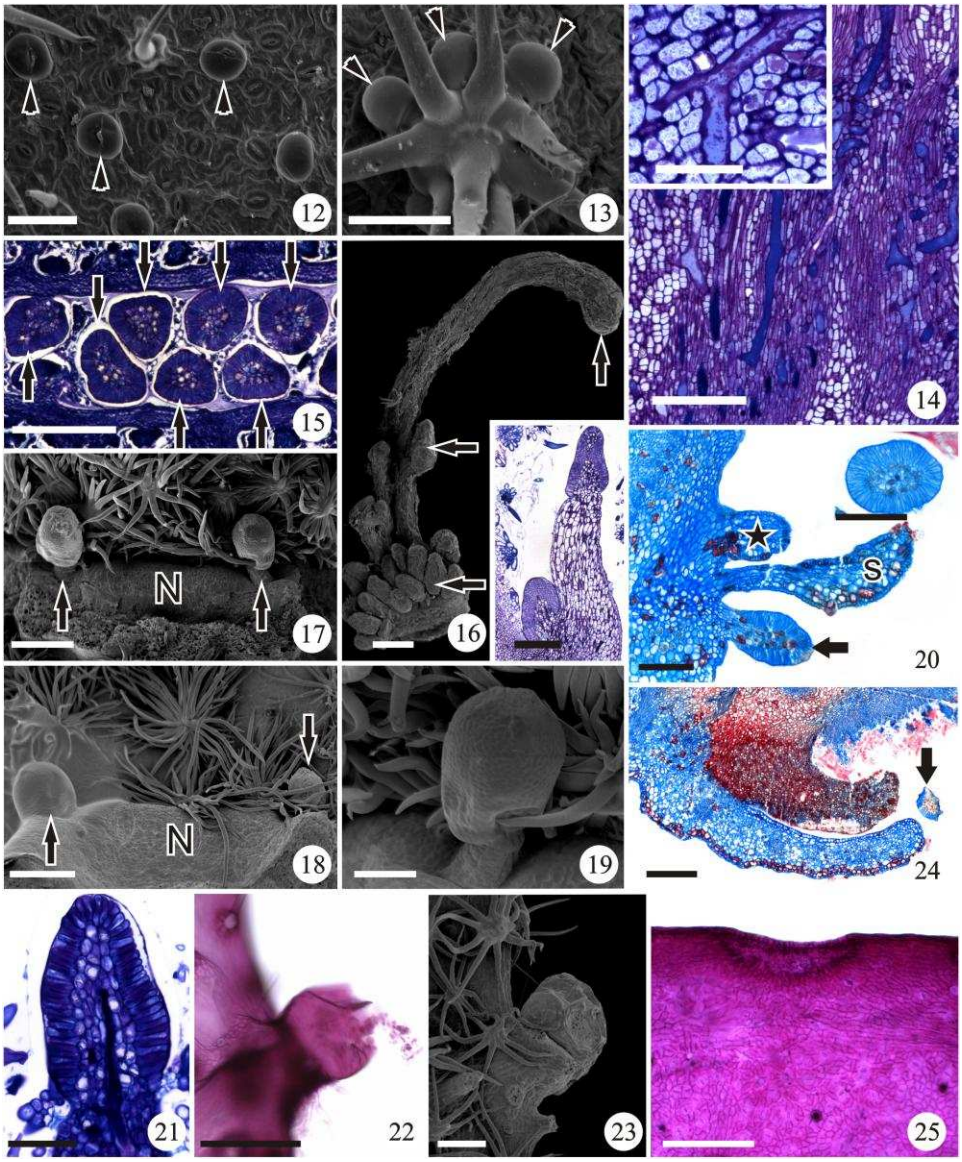

Fig. 12-25. Idioblasts, laticifers and colleters in C. echinocarpus (12, 15, 18, 22, 24) and C. urucurana (13, 14, 16, 17, 19-21, 23, 25). 12. Idioblasts above the level of the other epidermal cells (arrowhead); 13. Idioblasts at the base of non-glandular trichome (arrowhead); 14. Laticifers dispersed in the ground tissues, inset with a non-articulated laticifer showing a "Y" branching pattern; 15. Colleters (arrows) along the margin of a closed young leaf in a longitudinal section of the stem apex; 16. Colleters at the base, margins and apex of stipules (arrows), inset showing the longitudinal section of the stipule; 17. Colleters (arrows) in pistillate flower, alternate to FN lobes (N); 18. Colleters (arrows) in staminate flower, alternate to FN lobes (N); 19. Detail of colleter; 20. Staminate flower in longitudinal section showing the arrangement of floral nectary lobe (star), sepal (S), colleter (arrow), note inset with the cross section of the colleter; 21. Colleter of the standard type showing the parenchymal central axis; 22. Rupture of colleter; 23, 24. Colleter in senescence stage; 23. Colleter on leaf margin; 24. Colleter on pistillate flower in anthesis (arrow); 25. Scar of leaf marginal colleter. Bars: 50 µm = (19); 100 µm = (12, 13, 23); 150 µm = (21); 200 µm = (inset in the figs. 14, 16, 17, 18); 300 µm = (14); 400 µm = (22); 500 µm = (15, 20, inset in the figs. 16, 20, 24); 600 µm = (25).

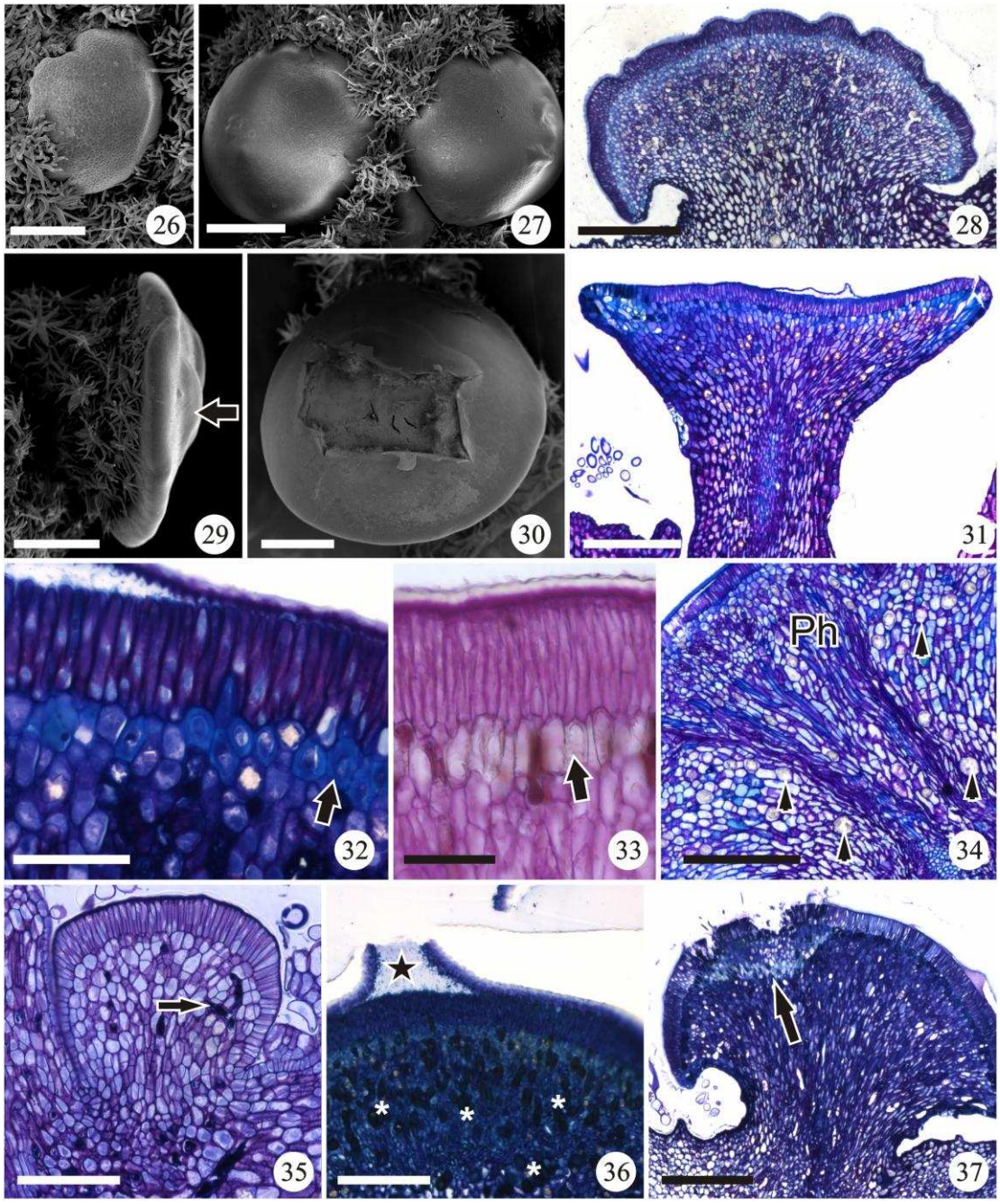

Fig. 26-37. Extrafloral nectaries of C. echinocarpus (26-28, 32, 34-37) and C. urucurana (29-31, 33). 26-28. Globular and sessile; 29-31. Patelliform and stipitate, note the distension of the cuticle in 29 (arrow); 32, 33. Layers of sclerenchyma (arrow) between the secretory epidermis and nectariferous parenchyma; 34-36. Nectariferous parenchyma vascularized mainly by phloem (Ph), with secretory idioblasts (asterisks), laticifers (arrow) and druse crystals (arrowhead) in different stages, note in fig. 36 the cuticle breaks during the secretory stage, yet with a little secretion (star); 37. EFN in senescence stage and with necrotic parenchyma (arrow). Bars: 100 µm = (36); 200 µm = (26, 32, 33, 37); 400 µm = (27, 29, 30); 500 µm = (28, 31, 35); 600 µm = (34).

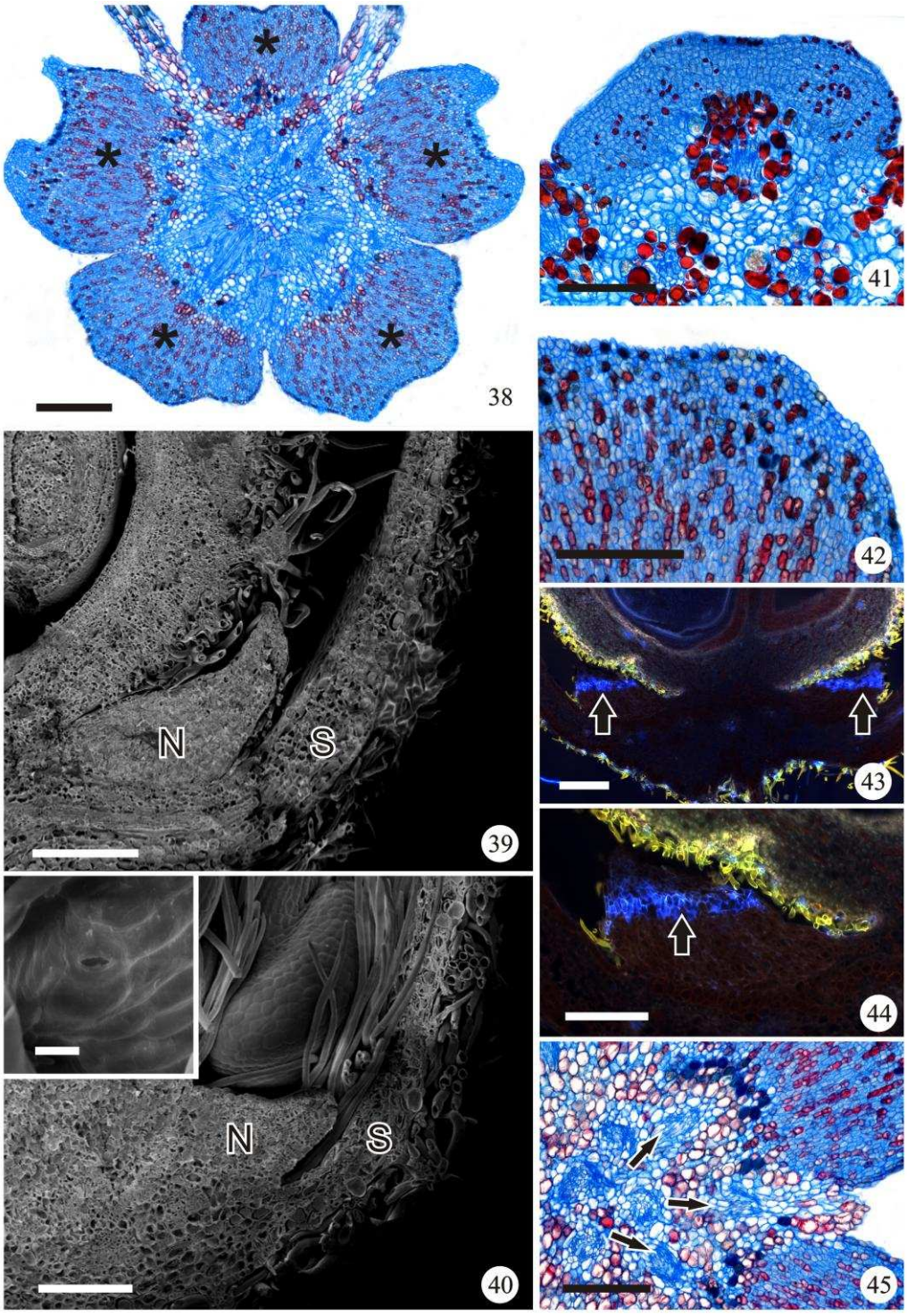

Fig. 38-45. Floral nectaries of C. urucurana (38-40, 42, 45) and C. echinocarpus (41, 43, 44). 38. Pistillate flower in cross section showing the five-lobed FN (asterisks); 39, 40. Horn-like FN lobe (N) opposite to sepal (S); 41. Convex surface of FN lobe; 42. Detail of FN lobe with epidermis not differentiated and thin cuticle; 43. Fluorescent micrograph of FN exhibiting layers of sclerenchyma in blue (arrow), see detail in 44 (arrow); 45. General aspect of vascularization (arrows) derived from the receptacle. Bars: 50 µm = (inset in 40); 100 µm = (40); 200 µm = (39, 41); 300 µm = (42); 400 µm = (44, 45); 500 = (38); 600 µm = (43).

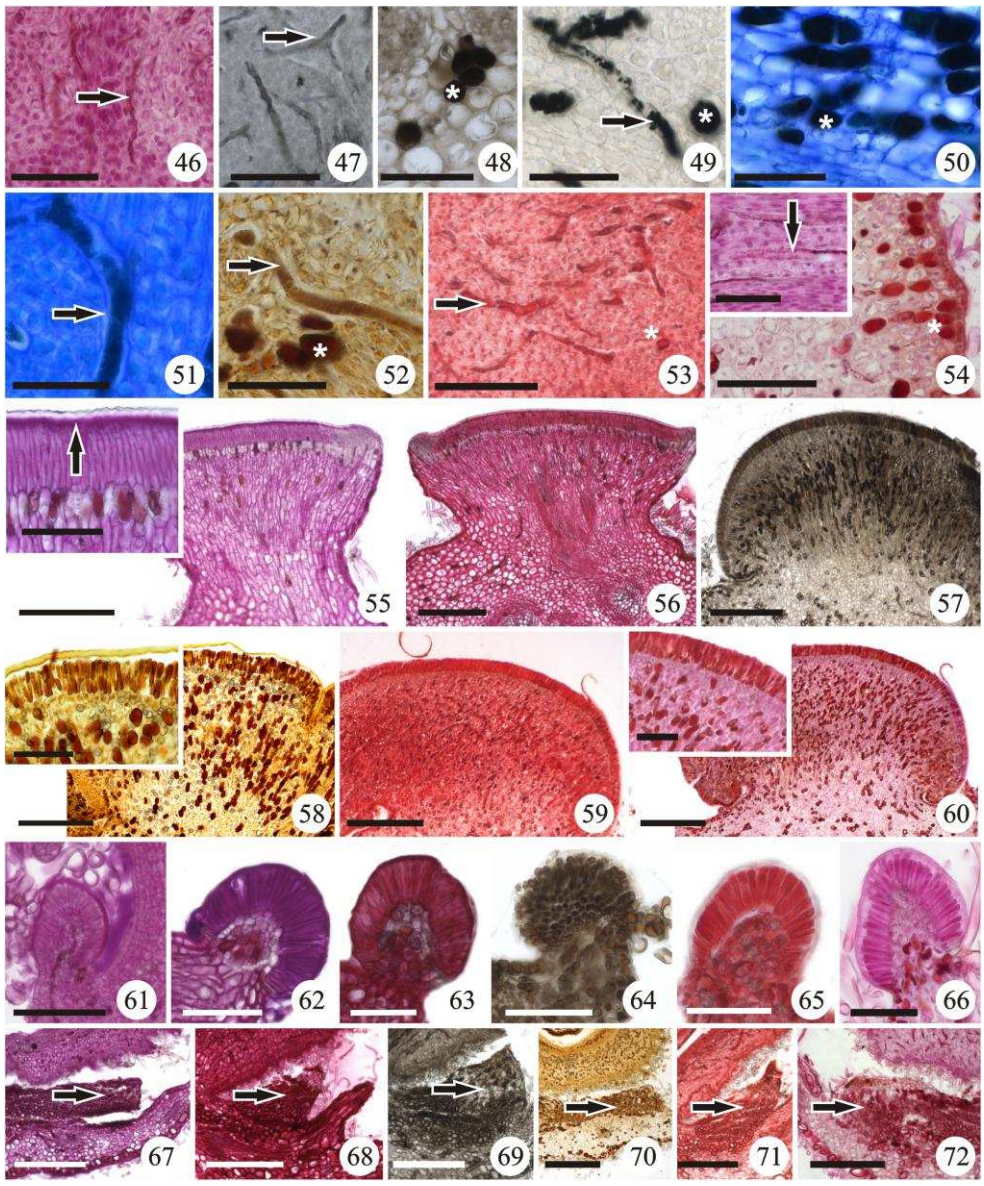

Fig. 46-72. Positive histochemical results on the secretion of the secretory structures in C. echinocarpus (46, 48-50, 53, 54, 57, 59, 60, 62, 64, 65, 69, 71) and C. urucurana (47, 49, 51, 52, 55, 56, 58, 61, 63, 66-68, 70, 72). 46-54. Idioblasts (white asterisk) and laticifers (arrow); 55-60. Extrafloral nectaries; 61-66. Colleters showing the reaction in the secretory epidermis; 67-72. Floral nectaries (arrow); 67, 70, 72. Pistillate flower; 68, 69, 71. Staminate flower; 46, 55, 61, 62, 67. Periodic acid–Schiff reaction showing total polysaccharides, note the inset showing strong reaction below cuticle (arrow) in 55; 56, 63, 68. Ruthenium red, acidic mucilage; 57, 64, 69. Tannic acid/ferric chloride, neutral mucilage; 47. Sudan black B, total lipids; 48. Copper acetate/rubeanic acid, fatty acids; 49. Ferrous sulfate in formalin, phenolic compounds; 50, 51. Nile blue, acidic lipids; 52, 58, 70. Wagner's reagent, alkaloids, note the inset in 58 showing reaction on secretory epidermis and idioblasts; 53, 59, 65, 71. Xilidine Ponceau, total proteins; 54, 60, 66, 72. Ninhydrin/Schiff's reagent, proteins, note the inset in 60 showing secretory epidermis and idioblasts. Bars: 100 µm = (61-66, 54, inset in the fig. 54, 47, 48, 46, 50, 51, 49, 52); 150 µm = (inset in the fig. 55); 200 µm = (inset in the figs. 60 and 58); 250 µm = (53); 500 µm = (58); 600 µm = (67-69, 71, 72); 800 µm = (55-57, 59, 60, 70).

Table 1. Results of histochemical tests applied to the secretion of different structures present in Croton echinocarpus and C. urucurana.

Tests	Species/Secretory structures									
	C. echinocarpus					C. urucurana				
	Col	Id	EFN	FN	Lat	Col	Id	EFN	FN	Lat
Carbohydrates										
PAS	+	-	+	+	+	+	-	+	+	+
Ruthenium red	+	-	+	+	+	+	-	+	+	+
Tannic acid/ferric chloride	+	-	+	+	-	+	-	+	+	-
Lipids/Terpenoids										
Sudan black B	-	+	-	-	+	-	+	-	-	+
Neutral red	-	+	-	-	+	-	+	-	-	+
NADI	-	-	-	-	-	-	-	-	-	-
Copper acetate/rubeanic acid	-	+	-	-	-	-	+	-	-	+
Nile blue	-	+	-	-	+	-	+	-	-	+
Phenolic compounds										
FSF	-	+	-	-	+	-	+	-	-	+
Vanillin/hydrochloric acid	-	-	-	-	-	-	-	-	-	-
Alkaloids										
Wagner's reagent	-	+	+	+	+	-	+	+	+	+
Proteins										
Xylidine ponceau	+	+	+	+	+	+	+	+	+	+
Ninhydrin/Schiff's reagent	+	+	+	+	+	+	+	+	+	+

Notes: **(**+**)**: positive result; **(-)**: negative result; Col.: colleter; Id.: idioblast; EFN: extrafloral nectary; FN: floral nectary; Lat.: laticifer.

ARTIGO III

Leaf anatomy and systematics of dragon's blood Croton section Cyclostigma (Euphorbiaceae)

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Running head: Anatomy of dragon's blood Croton

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Leaf anatomy and systematics of dragon's blood Croton section Cyclostigma (Euphorbiaceae)

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ABSTRACT

The dragon's blood trees (Croton section Cyclostigma) is one of the most diverse Neotropical groups within the giant genus Croton. Establishing species limits within this section using characters from external morphology and common molecular markers has proven cumbersome. Given this scenario, we seek to explore and identify anatomical characters that could be useful to tease apart species or groups of species within this section. We analyzed 104 specimens belonging to section Cyclostigma and related groups, described anatomical characters, and recorded 52 qualitative characters from leaf and stem. These characters were assemblaged in a matrix and analyzed using clustering techniques based on anatomical similarity in order to evaluate their utility in determining and/or confirming the taxonomic identity of specimens. Our results show that trichomes constitute one of the most diverse and variable anatomical features among the studied specimens. Although we did not detect a single anatomical character uniting section Cyclostigma, combinations of characters were useful in many cases to establish species limits and taxonomic identities.

ADDITIONAL KEYWORDS: colleters – hypodermis – idioblasts – nectaries – non-articulated laticifers – non-glandular trichomes.

INTRODUCTION

The medicinally important dragon's blood trees (Croton section Cyclostigma Griseb., Euphorbiaceae) are one of the most diverse Neotropical groups within the genus Croton (van Ee et al., 2011). According to ongoing taxonomic studies, this section consists of about 50 species distributed from Mexico to northern Argentina. All species in this section produce abundant colored latex ranging from reddish to orange/yellowish. The latex is widely used in Latin American countries to treat

several ailments, but especially for skin wounds, including burns, and stomach ulcers to prevent infection and accelerate cicatrization (Meza, 1999; Borges & King, 2000; Jones, 2003; Salatino et al., 2007).

Despite advances in the understanding of Croton's taxonomy and phylogenetics, species relationships and species limits within most of its sections remain poorly known. The only phylogenetic study dealing with section Cyclostigma (Riina et al., 2009) delimited the group based on analyses of nuclear ITS and plastid trnL-F sequences, and established a monophyletic section, however resolution at the species level was low and many species remained to be sampled. Riina et al. (2009) also detected a possible reticulate origin of section Cyclostigma, whose position was strongly incongruent between the plastid and nuclear phylogenies with the monotypic section Cupreati Riina and section Adenophylli Griseb. (section Cascarilla in Riina et al., 2009) as sister groups in the trnL-F and ITS phylogenies, respectively.

Although molecular data have contributed to redefine a monophyletic section Cyclostigma, external morphological synapomorphies supporting this clade have not been identified. The only apparent unifying character for this clade was identified in a study of wood anatomy that revealed the presence of secondary xylem rays containing laticifers only in section Cyclostigma among all Croton sections sampled (Wiedenhoeft et al., 2009). In addition to this wood feature and the abundant colored latex, the section is morphologically characterized by a suite of traits including arborescent habit, stellate indumentum, conspicuous stipules, acropetiolar glands, usually long thyrsoid inflorescences with bisexual cymules predominantely at the base, and staminate flowers with numerous stamens (> 16). Species in this group grow predominantly in moist forests, including riverine, lowland and montane forests, however a few species occur in dry forests.

Previous and ongoing systematic studies have been instrumental to disentangling the difficult taxonomy of the dragon's blood group as well as to identify unknown species (Riina et al., 2007, 2009, 2014, 2015), most of which are in the process to be described. However, establishing species limits using molecular data and external morphology has proven cumbersome because of the prevalent homoplasy in most of the morphological characters traditionally used in Croton taxonomy and the lack of resolution at the species level with the molecular markers used so far (Riina et al., 2009; van Ee et al., 2011). Given this scenario, we conduct a

comparative analysis of leaf anatomical features using a broad sampling of section Cyclostigma, a selection of species from its sister clades (sections Adenophylli and Cupreati), and a representative of a distant clade section Sampatik (G.L. Webster) Riina aiming to identify characters that can help to tease apart species or species groups within section Cyclostigma, and to expand the database of characters available for studies of character evolution in Euphorbiaceae.

MATERIAL AND METHODS

Taxon sampling

We sampled 104 specimens, of which 90 belong to Croton section Cyclostigma or have morphological affinities with that section, 11 specimens were from Croton section Adenophylli, one from the monotypic section Cupreati, and two from section Sampatik. Specimens were identified to section based on their morphological affinities according to van Ee et al. (2011). Plant material for anatomical studies was obtained from herbarium specimens (Appendix 1). Herbarium acronyms follow those of Thiers (2016, continuously updated).

Analysis using Light Microscopy

Samples of leaf and shoot apex were subjected to the herborization reversion process (Smith & Smith, 1942), dehydrated in ethanol series and stored in 70% ethanol. Subsequently the samples were dehydrated up to 95% ethanol and embedded in methacrylate for sectioning (Historesin Leica®, solutions prepared according to manufacturer's instructions) following Meira & Martins (2003). Fragments from the middle portion of the leaf blade (midrib and margin) and petiole, were cross- and longitudinally sectioned (3-7 µm) with an automatic rotary microtome (model RM 2265, Leica® Biosystems, Nussloch GmbH) using disposable glass knives (Leica®, Biosystems, Nussloch GmbH). Due to the presence of basilaminar and acropetiolar glands, the leaf base and distal portion of the petiole

were also sampled. Sections were stained with toluidine blue at pH 4.6 (O'Brien et al., 1965) and slides were mounted in resin (Permount®, Fisher Scientific, New Jersey, USA).

Part of the samples were also subjected to diaphanization (Shobe & Lersten, 1967). They were cleared with 5% sodium hydroxide and 20% hypochlorite solutions, stained with 50% ethanol-diluted fuchsin and mounted in glycerinated gelatin (Keiser, 1880).

Observations and photographic documentation were performed with a light microscope (Model AX70TRF, Olympus Optical, Tokyo, Japan) equipped with a U-Photo system and digital camera (AxioCam HRc; Carl Zeiss, Gottingen, Germany) Macro images were obtained using a stereomicroscope (Stemi 2000-C®, ©Carl Zeiss Microscopy GmbH, Jena, Germany) with a coupled digital camera (AxioCam ERc5s®, ©Carl Zeiss Microscopy GmbH, Jena, Germany).

Scanning Electron Microscopy (SEM)

Samples were stored in alcohol 70%, dehydrated in ethanol and critical point dried with CO2 (Bozzola & Russel 1992) in a 020 CPD dryer (Bal-Tec; Balzers, Liechtenstein). They were mounted onto stubs and coated with gold using a FDU 010 sputter coater (Bal-Tec). Examination of specimens and image capture were conducted using a Leo 1430VP SEM (Zeiss, Cambridge, United Kingdom) at the Centro de Microscopia e Microanálises at the Universidade Federal de Viçosa.

Description and analysis of anatomical characters

Anatomical descriptions were performed according Metcalfe & Chalk (1983, 1979) and Evert (2006). Trichome terminology followed the classification of Webster et al. (1996). Fifty two qualitative characters from leaf and stem apex were recorded (table 1). A matrix of specimens by characters was built, where character states were coded as binary (Appendix 2). A distance matrix was calculated using the Dice-Sorensen and Jaccard coefficients and similarity dendrogram were generated using the software PAST3 vs. 3.06 (Hammer et al., 2001). Dendrogram were further edited in FigTree vs.1.4 (Rambaut, 2012) and CorelDRAW X3®.

Identity of specimens was either confirmed or modified by integrating information from the clustering pattern based on leaf anatomical data, morphology from taxonomic descriptions, field observations, type specimens, habitat and distribution.

RESULTS

Description of leaf anatomy

The matrix of anatomical characters was almost complete with only 1.3% of missing data coded as "?" (Table 2). There were 17 specimens, listed in Table 2, for which we could not evaluate some characters (1-11) because the original herbarium material was too old or poorly preserved. Within section Cyclostigma the most critical specimens regarding missing data were Croton bogotanus Cuatrec. (Riina-1591) and C. mutisianus Kunth (Barkley-3768), both with the maximum percentage of missing characters (21%) (Table 2).

We identified 10 types of non-glandular trichomes: lepidote (Fig. 1A, 1B), dendritic (Fig. 1C), rosulate (Fig. 1D, 1E), stellate-cushion (Fig. 1F, 1G), stipitate-stellate (Fig. 1H), stipitate-stellate porrect (Fig. 1I, 1J), appressed-stellate porrect (Fig. 1K, 1L), appressed-stellate (Fig. 1M), multiradiate (Fig. 1N), and simple (Fig. 1O) trichomes. The rosulate, stipitate-stellate porrect, appressed-stellate porrect and simple types are more typical for section Cyclostigma. Lepidote and fasciculate trichomes are absent from all known section Cyclostigma species, with the exception of lepidote trichomes that were present in the group of C. urucurana-1 and are registered here for the first time for section Cyclostigma. Some trichome types are present only in a few specimens, such as lepidote (C. urucurana-1, C. ruizianus Müll. Arg. and C. cupreatus Croizat), dendritic (C. vulnerarius-2, C. draco-1, and C. celtidifolius-1) and stellate-cushion trichomes (C. huberi Steyerm., C. speciosus Müll. Arg., medusae-1, medusae-4 and C. coriaceus-3). Of all the surveyed characters, trichome type is one of the most diverse and variable among species (Fig.

1), however there is not trichome type exclusive to any of the four Croton sections sampled.

All specimens present hypostomatic leaves, with parasitic stomata (Fig. 2A, 2B, 2C), epidermis with sinuous anticlinal walls on the abaxial side (Fig. 2A) and straight to slightly sinuous walls on the adaxial side (Fig. 2C), with a slightly striated cuticle covering the epidermis (Fig. 2B).

All specimens present unistratified epidermis covered by a thin cuticle, and stomata, which are situated at the same level of the ordinary epidermal cells (Fig. 2D, 2E).

In some specimens of section Cyclostigma (C. amentiformis Riina, C. bogotanus, Riina-1403 (C. coriaceus-3), Riina-1417 (C. coriaceus-2), C. floccosus B.A. Sm., C. rimbachii Croizat, Riina-1416) and in section Cupreati, we observed a single-layered hypodermis underlying the adaxial epidermis (Fig. 2D).

The mesophyll is dorsiventral in all specimens, having from 1-2 layers of palisade parenchyma and 4-5 layers of spongy parenchyma (Fig. 2D, 2E). The vascular system is formed by collateral bundles (Fig. 2E), which possess bundle-sheath extension only in sections Cyclostigma and Cupreati (Fig. 2D).

Under polarized light, we detected abundant druse crystals in all the studied specimens, but they are found mostly in the fundamental tissue of different regions of the leaf (Fig. 2F).

Leaf margins are predominantly revolute (Fig. 2G, inset), however, in section Cyclostigma there are specimens with slightly involute margins (Fig. 2H, inset) as in: C. bogotanus, C. draco-2, C. echinocarpus Müll. Arg., C. erythrochilus Müll. Arg., C. erythrochyloides Croizat, C. floccosus, and Riina-1416. Leaf margins can present either continuous (Fig. 2G, 2H) or discontinuous (Fig. 2I) palisade parenchyma.

The midrib is biconvex (Fig. 2J) in sections Cyclostigma, Sampatik, Cupreati, C. abutilifolius Croizat and C. gracilipes Baill. (section Adenophylli), and with adaxial side flat to slightly concave in C. conduplicatus Kunth and C. ruizianus (section Adenophylli) (Fig. 2K). Sections Cyclostigma and Adenophylli possess midrib with five to ten layers of angular-annular collenchyma (Fig. 2J, 2L, 2M), and a continuous palisade parenchyma (Fig. 2K, 2M). In most species the vascular system consists of collateral bundles arranged in an open arch and 1-5 associated bundles; all bundles are surrounded by fibers (Fig. 2J, 2L). Croton aequatoris

Croizat and C. conduplicatus (section Adenophylli) are the only species where associated bundles are absent (Fig. 2K, 2M).

In all specimens, petiole cross section is rounded with a slight depression in the central region of the adaxial side (Fig. 2N). The epidermis is unistratified with tiny cells and covered by a thin cuticle (Fig. 2O). In the cortical region, 9-10 layers of angular collenchyma are present underlying the epidermis, followed by 7-10 layers of parenchyma. The collateral vascular system is ring-shaped with fibers in the perivascular region, and with the same arrangement described for the midrib (Fig. 2O). In the adaxial face, adjacent to the main vascular bundle, there are two accessory bundles surrounded by fibers (Fig. 2P).

Secretory structures

We identified four types of secretory structures with different topologies and structural organization, distributed in all studied specimens: idioblasts, laticifers, colleters and extrafloral nectaries (EFNs).

Secretory idioblasts were common in all specimens, and consist of large cells, sometimes with dense cytoplasm. They varied in topology, being located in the abaxial epidermis (Fig. 3A) and scattered in the mesophyll (Fig. 3B) in most specimens; in the abaxial epidermis and palisade parenchyma in C. cupreatus (Fig. 3C), and in the palisade parenchyma (Fig. 3D) in C. pseudopopulus Baill., C. floccosus, Riina-1416, and C. urucurana-1. Some idioblasts were situated at the stipite of trichomes (Fig. 3E, 3F), but this character state was not included in the matrix because it was not informative for taxonomy.

Non-articulated laticifers are dispersed in ground tissues of leaves in all specimens. They exhibit a "Y" branching pattern (Fig. 3G) in their early development in shoot meristems, where they show evident secretory activity.

Colleters are of the standard type (Fig. 3H), non-vascularized, with epidermis in palisade covered by a thin cuticle, forming a 1-layered palisade radially arranged. They have a slightly constricted base and a central axis composed of fundamental parenchyma where secretory idioblasts, druse crystals and laticifers can be present. In all specimens analyzed, colleters occur exclusively along the leaf margin.

Extrafloral nectaries, even if present in all specimens, show variations in topology, occurring at the base of the leaf blade (basilaminar) (Fig. 3I), at the distal

portion of the petiole (acropetiolar) (Fig. 3J, 3K), dispersed on the blade (Fig. 3L) or along the leaf margin (Fig. 3M, inset). In some specimens, both basilaminar and acropetiolar EFNs (Fig. 3N, 3O) can be present. EFNs varied from sessile (Fig. 3K, 3M, 3N) to stipitate (Fig. 3I, 3J, 3O), and their surface can be concave (Fig. 3P, 3Q), convex (Fig. 3R) or flat (Fig. 3S). The basilaminar and acropetiolar types are uniformly distributed in most species, however, in C. piptocalyx Müll. Arg. the EFNs occur scattered on the blade (Fig. 3L), and along the leaf margin alternating with colleters.

Cluster analysis of anatomical characters

The clustering pattern generated using the Dice-Sorensen or Jaccard similarity indices did not present differences in their branching patterns, so we will use the dendrogram from the analysis using Jaccard's similarity index (Fig. 4) for describing and discussing our results below. The cluster analysis performed on the 52 characters and specimens examined in this study produced a dendrogram (Fig. 4) with cophenetic correlation coefficient of 0.78. The successive aggregations of the 104 specimens studied, yielded 44 groups. These groups were established arbitrarily based on similarity values greater or equal to 0.8 ($J \geq 0.8$) based on their anatomical similarities (a value of 1 indicates 100% similarity among specimens). We assigned the more appropriate specific taxonomic identity integrating the clustering based on anatomical similarity with information on external morphology, ecological knowledge and geographic data. Thus, of the 44 determined and/or confirmed species, 36 belong to section Cyclostigma (in black), six to section Adenophylli (in red), one to section Sampatik (in blue) and one to section Cupreati (in green).

Thirteen specimens marked with a red start (Fig. 4) could not be assigned to any known species, however, they all have morphological affinities to members of section Cyclostigma. Some of these undetermined specimens were assigned to an informal taxonomic group (the "medusae" group) based on external morphology. This group appears as four different separate branches in the dendrogram (Fig. 4).

There were about 10 groups of specimens with 100% similarity regarding anatomical features (e.g., C. floccosus, C. pseudopopulus, C. perspeciosus Croizat, etc., Fig. 4), however there were also specimens identified using external morphology for which the clustering pattering was unexpected, such as C. coriaceus

Kunth (-1, -2, -3), C. draco Schltdl. (-1, -2), C. urucurana (-1, -2), C. mutisianus (-1, -2), C. celtidifolius Baill. (-1, -2, -3), and the medusae group (-1, -2, -3, -4). These species appear scattered across the dendrogram forming two up to four separate groups identified by the species name followed by the a group number (-1, -2, -3, -4) as indicated above (Fig. 4).

To better explain the overall similarity pattern among specimens, we divided the dendrogram from its base to the tips in three major levels (L1, L2, L3) indicated by the dotted vertical lines (Fig. 4). These levels correspond to 0.57, 0.60 and 0.66 similarity values, respectively.

The subgroups at L1 are g1 with two members (C. conduplicatus and C. bonplandianus Baill.) of section Adenophylli, g2 with the second part of section Adenophylli including C. aequatoris and C. ruizianus, and finally g3 including the rest of the taxa, i.e. sections Sampatik (C. piptocalyx) Adenophylli (C. abutilifolius and C. gracilipes), Cyclostigma and Cupreati (C. cupreatus). At L2 the dendrogram shows two groups previously described (g1, g2), then g4 with C. piptocalyx (section Sampatik), and g5 with all specimens of sections Cyclostigma, Cupreati, and Adenophylli (C. abutilifolius and C. gracilipes). Finally, at L3 the dendrogram shows the following groups of sections Adenophylli: g6 (C. conduplicatus), g7 (C. bonplandianus), g8 (C. aequatoris), and g9 (C. ruizianus); group g10 of section Sampatik, and the largest group, g11, with most species of section Cyclostigma along with Adenophylli (C. abutilifolius and C. gracilipes); group g12 is a small and isolated group of section Cyclostigma consisting of C. urucurana-1 and C. pseudopopulus; g13 includes three groups of specimens (C. floccosus, Riina-1416, and C. mutisianus-1) of section Cyclostigma with low similarity among them and with the rest of Cyclostigma; finally, the isolated group g14 formed by C. cupreatus of the monotypic section Cupreati.

DISCUSSION

Significance of leaf anatomical features for systematics

Anatomical characters were useful for the identification of 36 species of section Cyclostigma. Some characters states were common to all sampled species of Croton, such as the presence of hypostomatic leaves, paracitic stomata, dorsiventral mesophyll, collateral bundles, druse crystals, basilaminar/acropetiolar extrafloral nectaries, colleters, non-articulated laticifers, and stellate trichomes. Among the character states only present in some species of section Cyclostigma and useful to separate species within the section were the presence of hypodermis, dendritic trichomes, and the absence of nectaries along the margin and on the leaf blade.

Trichome morphology is very plastic among the studied species, sometimes showing variation even within the same individual. Previous studies about comparative anatomy of Croton (Webster et al., 1996; Senakun & Chantaranothai, 2010; Liu et al., 2013) or Crotoneae (Sá-Haiad et al., 2009; Vitarelli et al., 2015) showed that trichome types are useful for taxonomy, mostly at the species level, however they could vary within species/individuals and are highly homoplastic across the genus.

We identified 10 types of non-glandular trichomes. Section Cyclostigma can be characterized by the absence of fasciculate trichomes, which were not observed in any of the Cyclostigma specimens studied. On the other hand, most species of this section lack lepidote trichomes with the only exception being the two specimens of C. urucurana-1. The absence of lepidote trichomes has been used as character state to easily distinguish section Cyclostigma from other primary lepidote Croton clades such as sections Cupreati, Lasiogyne (Klotzsch) Baill., and Lamprocroton (Müll. Arg.) Pax in Engl. & Prantl (van Ee et al., 2011). This new finding of lepidote trichomes in species of section Cyclostigma, needs to be confirmed with additional specimens of C. urucurana. This species has a large distribution range and we could only sample three individuals, which came out as two separated groups in our cluster analysis. In addition, the two specimens of C. urucurana-1 are among the ones with missing data in our anatomical matrix, however, the absence of lepidote trichomes in C. urucurana-2 justify its separation from C. urucurana-1 in the dendrogram. Given

these results and the wide distribution of C. urucurana in southern South America, additional sampling is required to investigate how common lepidote trichomes are in this taxon, or whether the presence of these trichomes in C. urucurana-1 is just a variation related to local of occurrence. Both specimens of C. urucurana-1 come from the State of São Paulo, around Campinas and Piracicaba, whereas the stellate specimen (C. urucurana-2) comes from Distrito Federal, near Brasilia. It will be important for a better delimitation and characterization of this species to investigate the anatomy of samples of C. urucurana from other Brazilian states, where the species occurs and also from northern Argentina and Paraguay.

Simple trichomes also showed variation in their distribution on the plant, however, the main variation was related to development, because different developmental phases can be present in the same sample, thus the simple trichome could be regarded as an early phase of other, more structurally complex, trichome types. This is in disagreement with Webster et al. (1996) who took the hypothesis of L´eonard (1962, apud Webster et al., 1996) and Borhidi & Orosz-Kovacs (1991, apud Webster et al., 1996) and discussed the possibility that the simple trichome type is the most primitive one, correlating ontogenetics with phylogenetics. Given our observations so far, we recommend not using the simple trichome character state for taxonomic purposes in Croton.

The presence of the hypodermis in six out of the 36 species of Cyclostigma and in C. cupreatus is an interesting finding, and it is a new report for Croton. Cyclostigma species occur along mountain creeks, edges and gaps of forests, river sides, and along roads. Despite the high humidity present in some of its habitats (e.g. cloud forest), these Croton species are exposed to high luminosity and having a hypodermis is clearly advantageous. In fact, the seven species that present hypodermis occur all in montane forest at high elevation in the Andes, however, they are not the only species in the section occupying this kind of habitats.

Although secretory idioblasts are common in Croton, their location was variable and the chemical nature of their secretion was complex in representatives of C. sect. Cyclostigma (Feio et al., in press). Most studies report idioblasts having a lipophilic secretion (e.g. Sá-Haiad et al., 2009; Vitarelli et al., 2015), however, in a detailed study of two species of section Cyclostigma, Feio et al. (in press) detected polysaccharides, phenolic compounds, alkaloids, and proteins in idioblasts, suggesting a richer and more complex nature of their secretion. In the specimens

analyzed here, secretory idioblasts occurred in various parts of the leaf. Glandular trichomes are absent in all species studied, corroborating the findings of Webster et al. (1996). However, Vitarelli et al. (2015) studying individuals of sections Lamprocroton, Cleodora (Klotzsch) Baill., and Alabamensis B.W. van Ee, found secretory idioblasts and glandular trichomes on abaxial surface of leaves, and suggest that such structures could be homologous.

All specimens studied possess non-articulated branched laticifers distributed from the shoot meristem to mature leaves. Vitarelli et al. (2015) described articulated laticifers in most of the Croton species they analyzed and suggested that the articulated type might be widespread in subfamily Crotonoideae. However, they recognized that an in-depth analysis including representatives from all Croton sections and other Crotoneae genera was needed to confirm this hypothesis. Our results do not support their hypothesis because section Cyclostigma and representatives of other three sections, present only non-articulated laticifers, which was also found by Rudall (1994) for C. antisyphiliticus Mart. ex Müll. Arg., C. conduplicatus, C. heteropleurus Urb., C. megalobotrys Müll. Arg., C. occidentalis Müll. Arg., C. sagreanus Müll. Arg., C. sylvaticus Hochst., and four undetermined Croton species.

The absence of vascularization in the parenchymal axis of colleters may be associated with the ephemeral nature and small size of these structures, considering that they are not constantly nourished. Carlquist (1969) pointed out that the presence of vascularization is directly related to the size of a structure and not necessarily to its state of development. On the other hand, Thomas (1991) suggested that the vascularization of colleters was more related to the derivation or not of vascular bundles from the organs in which colleters are attached, regardless the size of the structure (Arekal & Ramakrishna, 1980; Appezzato-da-Glória & Estelita, 2000).

Structural variation and location of extrafloral nectaries were useful to distinguish group of species at different levels in the cluster analysis. For example, the species of section Adenophylli were separated in three subgroups: one group with sessile EFNs and convex surface (g1), a second group with stipitate EFNs and concave surface (g2), and a third group (C. abutilifolius, C. gracilipes), which although it was more similar to members of section Cyclostigma, can be separated from Cyclostigma using characters from external morphology and molecular evidence (Riina et al., 2009). The presence of nectaries scattered on the blade and

along the leaf margin (alternating with colleters) confirmed the classification of Riina et al. (2009), which removed C. piptocalyx from section Cyclostigma, a former member of the section sensu Webster (1993).

Delimitation of groups and taxonomic implications

Our study provides additional characters that can contribute to clarify the taxonomic affinities of species within section Cyclostigma, and it gives support to the taxonomic position of the section adopted by Riina et al. (2009) and van Ee et al. (2011). As with external morphology (Riina et al., 2009), the section can also be characterized by a suite of anatomical characters, and so far only one anatomical wood character (secondary xylem rays containing non-articulated laticifers) has been pointed out as a possible synapomorphy for section Cyclostigma (Wiedenhoeft et al., 2009).

Specimens of sections Sampatik, Cupreati and the group of Adenophylli (C. abutilifolius, C. gracilipes) appear more similar anatomically to section Cyclostigma than specimens of Adenophylli (g1, g2). This gives an indication of the homoplastic nature of the anatomical characters used in this study, which is similar to the pattern found with external morphological characters. It is clear that morphoanatomical characters alone will not be enough to separate groups at the sectional level within the genus Croton, however, our results show that the use of anatomical characters could facilitate taxonomic delimitation at the species level within Croton sections. It is also possible that using anatomical and morphological characters in combination could be even more useful for establishing species limits.

Subgroups within Adenophylli (g1, g2) were further divided in four subgroups (g6, g7, g8 and g9) based on characteristics such as: acropeciolar EFNs (g6: C. conduplicatus), basilaminar EFNs and appressed-stellate trichome (g7: C. bonplandianus), appressed-stellate porrect trichomes and absence of associated bundles in the midrib (g8: C. aequatoris), and presence of 1-5 associated bundles in the midrib (g9: C. ruizianus). The other group of section Adenophylli (C. abutilifolius, C. gracilipes) along with most species of section Cyclostigma were grouped, essentially, by the presence of secretory idioblasts in the abaxial epidermis and stipitate-stellate porrect trichomes.

Specimens Soto-414 and Soto-430 are from the same locality. They are morphologically identical and were initially identified based on external morphology as C. aff. urucurana. These two specimens clustered with Riina-1498, which was initially identified as C. tyndaridum. It is possible that these three specimens belong to the same species given their high similarity (100%) regarding anatomical characters. Riina-1498 is problematic because it comes from a cultivated plant in Lima (Peru) and there were no records of its provenance, so the identification as C. tyndaridum was always questionable, since C. tyndaridum is only known from the type locality. The two specimens of Soto are from Bolivia. Because Soto-414 and Soto-430 did not group with any of the C. urucurana groups, and the uncertainty regarding the identity and origin of Riina-1498, we did not assign any name to this group and consider it as an unknown and probably undescribed species of section Cyclostigma.

Croton draco-1 (Berry-7595) despite that it was originally identified as C. draco based on morphology, did not group with C. draco-2 (Marquez-558 and Martínez-6054). Croton draco-1 presents bundle-sheath extension, dendritic trichomes and revolute margin, which are features absent in C. draco-2. Croton draco is another widespread and variable species occurring from Mexico to Panama, and given its wide geographic range it would have been desirable to have a better sampling of it. Our results show that there might be at least two morphotypes that could be easily separated by anatomical characters. This group obviously merits further investigation using anatomy, morphology and molecular data.

Croton mutisianus-1 and C. mutisianus-2 did not cluster together as expected. Instead the latter clustered with C. quadrisetosus. It is possible that this grouping is an artifact since C. mutisianus-2 has the highest percentage of missing data (21%) of our matrix. In fact, C. mutisianus-2 (Barkley-3768) was a poor sample and it was not possible to collect information about EFN and stem apex to characterize the type of laticifers. External morphology does not support this grouping either, since C. mutisianus-2 and C. quadrisetosus have many differences in floral morphology (e.g. flowers longely pedicellate, and stigmas bifid and terete in C. mutisianus vs. flowers sessile to subsessile and stigmas multifid with expanded and flattened tips in C. quadrisetosus). In addition, Croton mutisianus is widespread in montane forests of Colombia, and C quadrisetosus is only known montane forests of Peru.

Unexpected grouping patterns, similar to the ones shown by specimens of C. urucurana, C. draco, C. mutisianus, and the medusae group, previously discussed above, were found for specimens of C. celtidifolius, C. vulnerarius, and C. coriaceus. These specimens show relatively low levels of similarity in the anatomical characters examinated appearing in different branches of the dendrogram. It is possible that in some cases the species are indeed more polymorphic in relation to anatomical characters than to characters from external morphology, however the limited sampling prevent us about using anatomical data along to further divide these species, especially because the external morphology does not support the observed clustering pattern. All these taxa need further investigation using a broader sampling across their range, and integrating both anatomy and morphology in the evaluation of species limits.

CONCLUSIONS

Although we did not find a unique anatomical character uniting section Cyclostigma, the use of suite of characters was useful to identify similarities between specimens and to confirm and/or to determine the specific taxonomic identity.

The presence of different types of stellate trichomes, non-articulated laticifers, the absence of marginal EFN, and the lack of lepidote and fasciculate trichomes can be used in combination to define section Cyclostigma anatomically. Our results did not support the hypothesis about the widespread occurrence of articulated laticifers in Crotonoideae.

Knowing that non-glandular trichomes are significant features taxonomically, and they have been recorded in previous studies of Croton, there is a necessity of a broader survey for other clades in the genus and other genera within the Crotoneae tribe.

Our results highlight the utility of anatomical characters at the species level within sections, as well as their limitations, due to rampant homoplasy, at higher taxonomic levels such as sections and probably genera within Crotoneae and Crotonoideae.

Future studies integrating characters from external morphology and anatomical features are promising to disentangle the complex taxonomy of Croton groups and species.

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FIGURES AND LEGENDS:

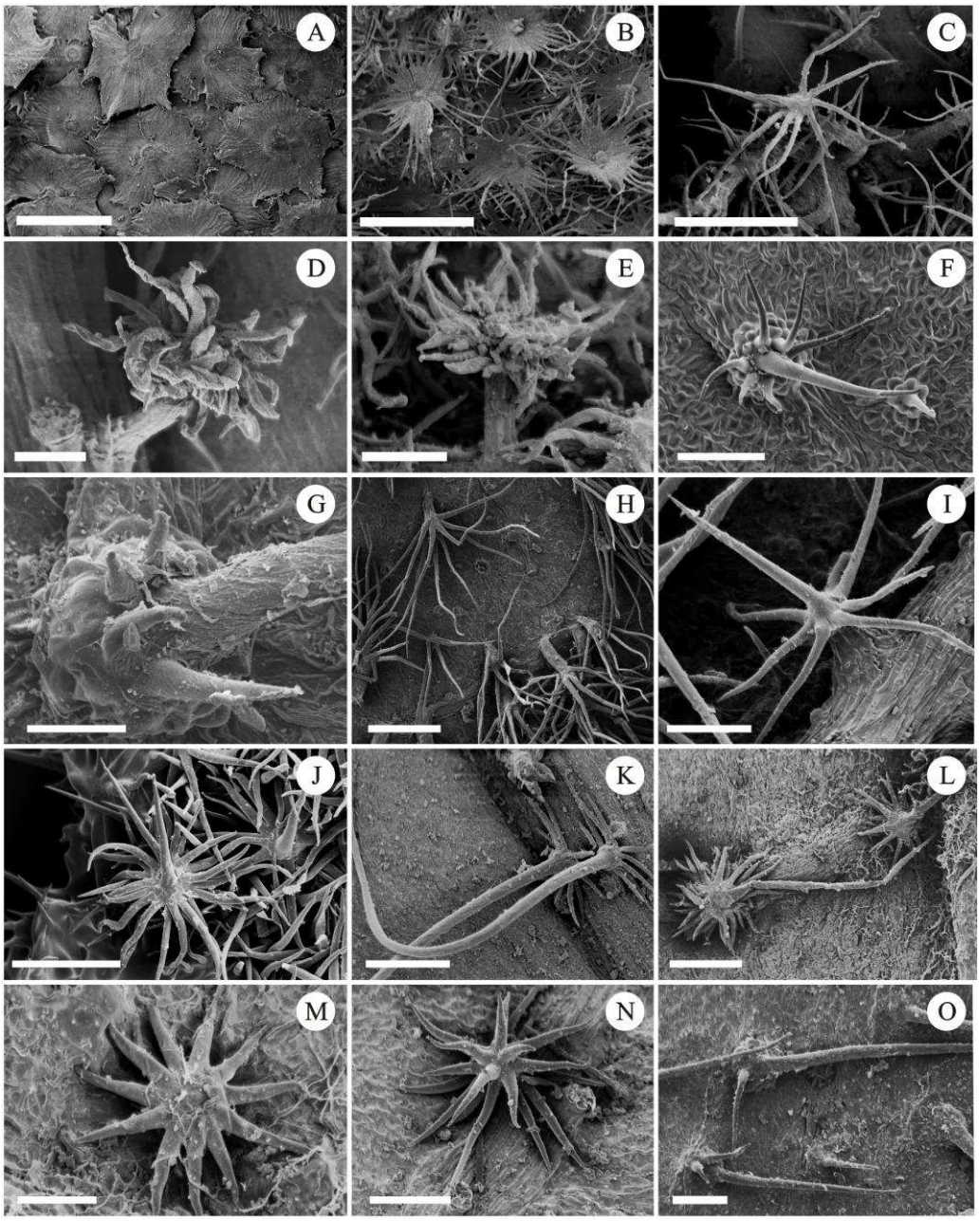

Figure 1. Diversity of non-glandular trichomes in Croton. **A**, **B**. lepidote trichomes, **A**. C. cupreatus, **B**. C. sp5; **C**. dendritic trichomes in C. vulnerarius; **D**, **E**. rosulate trichomes in C. floccosus; **F**, **G**. stellate-cushion in C. speciosus; **H**. Stipitate-stellate trichomes in C. alchorneicarpus; **I**, **J**. Stipitate-stellate porrect trichomes, **I**. C. macrobothrys, **J**. C. bonplandianus; **K**, **L**. Appressed-stellate porrect trichomes, **K**. C. redolens, **L**. C. aequatoris; **M**. Appressed-stellate trichomes in C. piptocalyx; **N**. Multiradiate trichomes in C. hibiscifolius; **O**. simple trichomes in C. coriaceus. Bars: 60 µm (G, M); 100 µm (D-F, I, N, O); 200 µm (H, J-L); 300 µm (A-C).

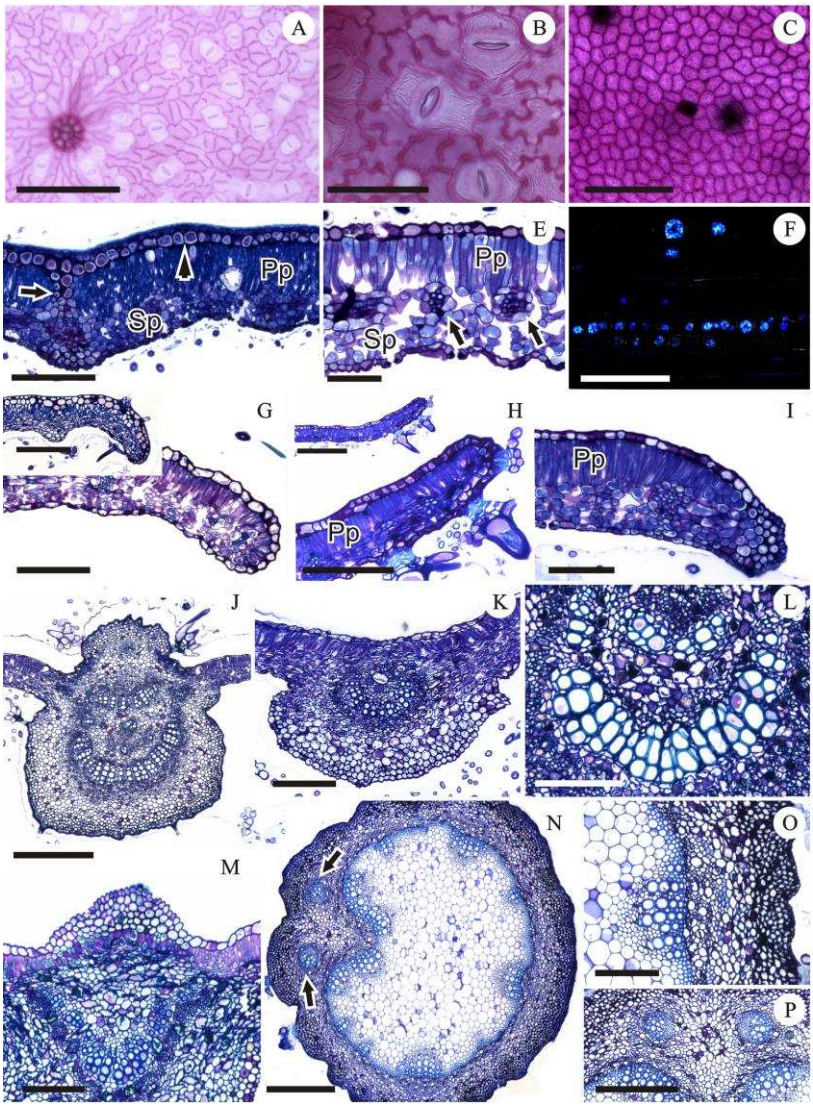

Figure 2. General anatomical features. **A**-**C**. frontal view; **D**-**P**. cross-sections. **A**. abaxial epidermis with sinuous anticlinal walls in C. celtidifolius, **B**. detail of abaxial epidermis, showing paracitic stomata and cuticle with attenuated stretch in C. macrobothrys, **C**. adaxial epidermis with straight to slightly wavy in C. macrobothrys; **D**. dorsiventral mesophyll with unistratified epidermis in C. amentiformis, note: hypodermis (arrowhead), bundle-shealth extension (arrow); **E**: dorsiventral mesophyll with collateral bundles (arrow) in C. ruizianus; **F**. druse crystals under polarized light in C. alchorneicarpus; **G**, **H**, **I**. leaf margin, **G**. revolute in C. conduplicatus, note inset in C. amentiformis, **H**. involute in C. bogotanus, Arrowhead: continuous palisade parenchyma, **I**. discontinuous palisade parenchyma in C. lechleri; **J**. biconvex midrib in C. lechleri; **K**. midrib with flat to slightly concave adaxial side in C. conduplicatus; **L**. vascular system of midrib with bundles associated in C. vulnerarius; **M**. vascular system of midrib with bundles associated absent C. aequatoris; **N**, **O**, **P**. petiole of C. charaguesis; **N**. general view of petiole with spherical shape and a slight depression and two accessory bundles (arrow); **O**. detail of petiole showing epidermis, cortical region and part of vascular system; **P**. detail of adaxial side of petiole with two accessory bundles. Pp: palisade parenchyma, Sp: spongy parenchyma, Bars: 100 µm (B); 150 µm (D, E, F, I); 200 µm (A, C); 250 µm (M); 300 µm (G, H, K, L, O); 600 µm (insets Fig. G and H, P); 700 µm (J); 800 µm (N).

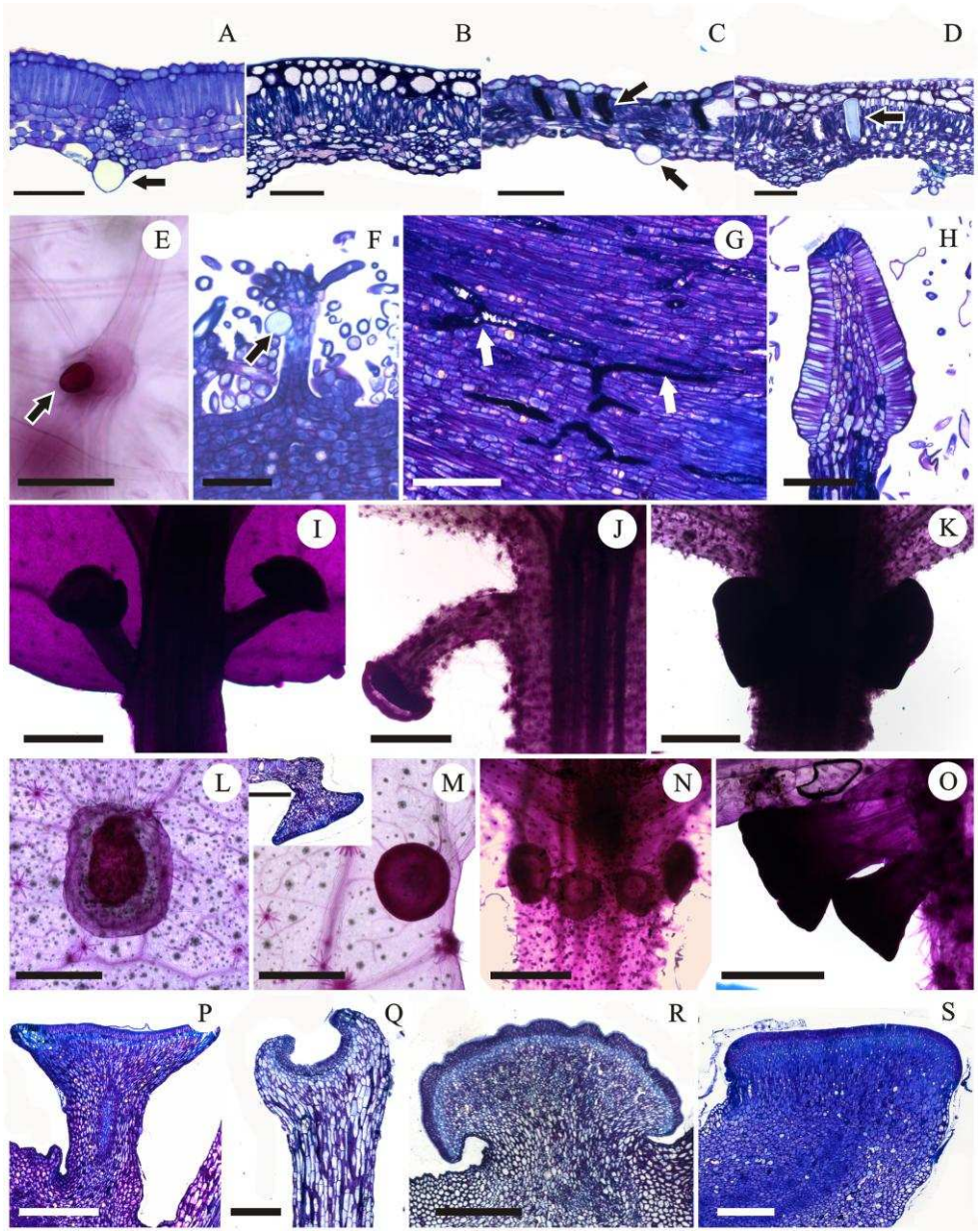

Figure 3. Diversity of secretory structures in Croton. **A**-**F**. secretory idioblasts (arrow); **G**. laticifers (white asterisk); **H**. colleters; **I**-**S**. extrafloral nectaries (EFN). **A**. idioblasts in the abaxial epidermis; B. idioblasts scattered in the mesophyll in C. amentiformis; **C**. idioblasts in the epidermis and palisade parenchyma in C. cupreatus; **D**. idioblasts in the palisade parenchyma in C. pseudopopulus; **E**, **F**. idioblasts situated at the base of the trichomes in C. lechleri and C. alchorneicarpus, respectively; **G**. non-articulated branched laticifers in the shoot meristema in C. amentiformis; **H**. non-vascularized colleter of the standard type in C. bogotanus; **I**. stipitate EFN basilaminar in C. macrobothrys; **J**, **K**. EFN acropetiolar; **J**. stipitate EFN in C. celtidifolius, **K**. sessile EFN in Riina-1520; **L**. dispersed EFN in the blade in C. piptocalyx; **M**. sessile EFN on the leaf margin in C. piptocalyx; **N**, **O**. EFN in both regions, **N**. sessile EFN in C. redolens, **O**. stipitate EFN in C. pseudopopulus; **P**, **Q**. EFN with concave surface in C. Riina-1592 and C. aequatoris, respectively; **R**. EFN with convex surface in C. gossypifolius; **S**. EFN with flat surface in C. cupreatus. Bars: 150 µm (A-F); 250 µm (G, H); 300 µm (Q); 800 µm (P, R, S); 0,5 mm (L, M, O); 1 mm (I-K, N).

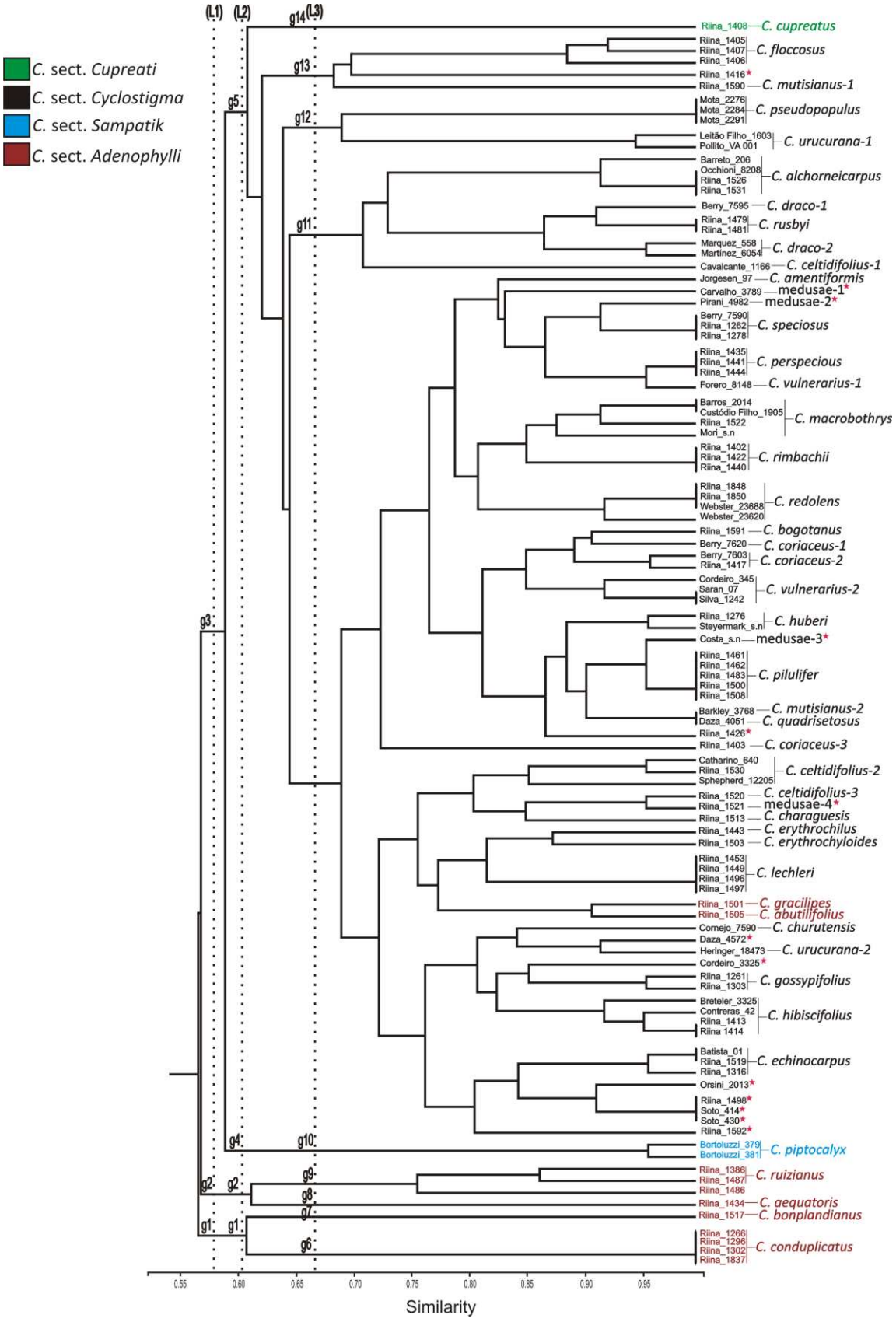

Figure 4. Dendrogram of the 104 specimens analyzed with Jaccard's coefficient. Right noted the taxonomic identity assigned to each group.

Table 1. Anatomical characters used in cluster analysis of Croton L.

No.	Anatomical character
1	Extrafloral nectaries - leaf: **(0)** absent; **(1)** present.
2	Non-glandular trichomes: **(0)** absent; **(1)** present.
3	Glandular trichomes: **(0)** absent; **(1)** present.
4	Secretory idioblasts: **(0)** absent; **(1)** present.
5	Druse crystal: **(0)** absent; **(1)** present.
6	Hypodermis: **(0)** absent; **(1)** present.
7	Continuous palisade parenchyma in the midrib: **(0)** absent; **(1)** present.
8	Bundle-sheath extension: **(0)** absent; **(1)** present.
9	Margin with continuous palisade parenchyma: **(0)** absent; **(1)** present.
10	Colleters on the leaf margin: **(0)** absent; **(1)** present.
11	Basilaminar/acropetiolar colleters: **(0)** absent; **(1)** present.
12	EFN basilaminar: **(0)** absent; **(1)** present.
13	EFN acropetiolar: **(0)** absent; **(1)** present.
14	EFN dispersed in the blade: **(0)** absent; **(1)** present.
15	EFN marginal: **(0)** absent; **(1)** present.
16	EFN sessile: **(0)** absent; **(1)** present.
17	EFN stipitate: **(0)** absent; **(1)** present.
18	EFN with concave surface: **(0)** absent; **(1)** present.
19	EFN with convex surface: **(0)** absent; **(1)** present.
20	EFN with flat surface: **(0)** absent; **(1)** present.
21	Lepidote trichomes: **(0)** absent; **(1)** present.
22	Rosulate trichomes: **(0)** absent; **(1)** present.
23	Dendritic trichomes: **(0)** absent; **(1)** present.
24	Stellate-cushion trichomes: **(0)** absent; **(1)** present.
25	Stipitate-stellate trichomes: **(0)** absent; **(1)** present.
26	Stipitate-stellate porrect trichomes: **(0)** absent; **(1)** present.
27	Appressed-stellate porrect trichomes: **(0)** absent; **(1)** present.
28	Appressed-stellate trichomes: **(0)** absent; **(1)** present.
29	Multiradiate trichomes: **(0)** absent; **(1)** present.
30	Fasciculate trichomes: **(0)** absent; **(1)** present.

31 Simple trichomes: **(0)** absent; **(1)** present.

32 Hypostomatic leaf: **(0)** absent; **(1)** present.

33 Amphistomatic leaf: **(0)** absent; **(1)** present.

34 Paracitic stomata: **(0)** absent; **(1)** present.

35 Anomocitic stomata: **(0)** absent; **(1)** present.

36 Secretory idioblasts in the abaxial epidermis: **(0)** absent; **(1)** present.

37 Secretory idioblasts in the adaxial epidermis: **(0)** absent; **(1)** present.

38 Secretory idioblasts in the abaxial epidermis and palisade parenchyma: **(0)** absent; **(1)** present.

39 Secretory idioblasts in the palisade parenchyma: **(0)** absent; **(1)** present.

40 Secretory idioblasts dispersed in the mesophyll: **(0)** absent; **(1)** present.

41 Non-articulated branched laticifers: **(0)** absent; **(1)** present.

42 Articulated laticifers: **(0)** absent; **(1)** present.

43 Unistratified epidermis: **(0)** absent; **(1)** present.

44 Dorsiventral mesophyll: **(0)** absent; **(1)** present.

45 Biconvex midrib: **(0)** absent; **(1)** present.

46 Midrib with flat to slightly concave adaxial face: **(0)** absent; **(1)** present.

47 Midrib with colateral bundles arranged in an open arch-like with one to five bundles associated: **(0)** absent; **(1)** present.

48 Midrib with colateral bundles arranged in an open arch-like without bundles associated: **(0)** absent; **(1)** present.

49 Colateral bundles in the midrib: **(0)** absent; **(1)** present.

50 Bicolateral bundles in the midrib: **(0)** absent; **(1)** present.

51 Revolute margin: **(0)** absent; **(1)** present.

52 Involute margin: **(0)** absent; **(1)** present.

Table 2. Specimens with missing data in the character matrix and their respective numbers and percentage. Total number of characters coded is 52, and total number of cells in the matrix is 5408 (52 characters x 104 specimens).

Species (Voucher)	# missing characters	% missing characters
C. bogotanus (Riina-1591)	11	21
C. mutisianus-2 (Barkley-3768)	11	21
C. bonpladianus (Riina-1517)	10	19
C. aequatoris (Riina-1434)	6	12
C. conduplicatus (Riina-1296)	6	12
C. urucurana-1 (Pollito-VA-001)	5	10
C. hibiscifolius (Breteler-3446)	4	8
C. rimbachii (Riina-1402)	3	6
C. urucurana-1 (Leitão Filho-1603)	3	6
C. hibiscifolius (Contreras-042)	2	4
C. piptocalyx (Bortoluzzi-379)	2	4
C. vulnerarius-1 (Forero-8148)	2	4
C. pseudopopulus (Mota-2276)	1	2
C. pseudopopulus (Mota-2284)	1	2
C. pseudopopulus (Mota-2291)	1	2
C. rusbyi (Riina-1479)	1	2
C. rusbyi (Riina-1481)	1	2
Total (entire matrix)	**70**	**1.3**

APPENDIX 1. List of species previously identified of Croton L. and voucher information. Herbarium acronyms in parentheses according to Thiers (2016).

Species	Collector and number (herbarium)
Croton sect. Cyclostigma Griseb.	
C. alchorneicarpus Croizat	**1**: Riina 1526 (MICH); **2**: Barreto 206 (MG); **3**: Riina 1531 (MICH); **4**: Occhioni 8208 (MBM)
C. amentiformis Riina	**5**: Jorgesen 97 (MO)
C. bogotanus Cuatrec.	**6**: Riina 1591(MICH)
C. aff. celtidifolius/pseudopopulus	**7**: Cavalcante 1166 (MG)
C. celtidifolius Baill.	**8**: Catharino 640 (MG); **9**: Riina 1520 (MICH); **10**: Riina 1530 (MICH); **11**: Sphepherd 12205 (MG)
C. charaguensis Standl.	**12**: Riina 1513 (MICH)
C. churutensis Riina & Cornejo	**13**: Cornejo 7590 (MICH)
C. coriaceus Kunth	**14**: Berry 7603 (MICH); **15**: Riina 1403 (MICH); **16**: Riina 1417 (MICH)
C. aff. coriaceus Kunth	**17**: Berry 7620 (MICH)
C. draco Schltdl.	**18**: Berry 7595 (MICH); **19**: Marquez 558 (MBM); **20**: Martínez 6054 (MBM)
C. echinocarpus Müll.Arg.	**21**: Riina 1316 (MICH); **22**: Riina 1519 (MICH); **23**: Batista 01 (VIC)
C. erythrochyloides Croizat	**24**: Riina 1503 (MICH)
C. fastuosus Baill.	**25**: Costa s/n 22265 (BHCB)
C. floccosus B.A. Sm.	**26**: Riina 1405 (MICH); **27**: Riina 1406 (MICH); **28**: Riina 1407 (MICH)
C. glaziovii Müll.Arg.	**29**: Riina 1521 (MICH)
C. gossypiifolius Vahl	**30**: Riina 1261 (MICH); **31**: Riina 1303 (MICH)
C. hibiscifolius Kunth ex Spreng.	**32**: Breteler 3446 (MG); **33**: Contreras 042 (MICH); **34**: Riina 1413 (MICH); **35**: Riina 1414 (MICH)
C. aff. hibiscifolius	**36**: Riina 1592 (MA); **37**: Orsini 2013-13 (MYF)
C. huberi Steyerm.	**38**: Riina 1276 (MICH); **39**: Steyermark s.n (MG)
C. lechleri Müll. Arg.	**40**: Riina 1443 (MICH); **41**: Riina 1449 (MICH); **42**: Riina 1496 (MICH); **43**: Riina 1497 (MICH)
C. aff. lechleri Müll. Arg.	**44**: Riina 1453 (MICH)
C. macrobothrys Baill.	**45**: Barros 2014 (MG); **46**: Custodio Filho 1905 (MG); **47**: Mori s.n (MG); **48**: Riina 1522 (MICH)
C. aff. medusea	**49**: Pirani 4982 (SPF)
C. aff. mutisianus Kunth	**50**: Riina 1590 (UEN, MA)
C. mutisianus Kunth	**51**: Barkley 3768 (MBM)

C. perspeciosus Croizat	**52**: Riina 1435 (MICH); **53**: Riina 1441 (MICH); **54**: Riina 1444 (MICH)
C. pilulifer Rusby	**55**: Riina 1500 (MICH); **56**: Riina 1508 (MICH)
C. aff. pilulifer Rusby	**57**: Riina 1461 (MICH); **58**: Riina 1462 (MICH); **59**: Riina 1483 (MICH)
C. plagiograptus Müll. Arg.	**60**: Carvalho 3789 (MBM)
C. pseudopopulus Baill.	**61**: Mota 2276 (VIC); **62**: Mota 2284 (VIC); **63**: Mota 2291 (VIC)
C. quadrisetosus Lam.	**64**: Daza 4051 (E)
C. redolens Pittier	**65**: Riina 1848 (MICH); **66**: Riina 1850 (MICH); **67**: Webster 23688 (MICH)
C. aff. redolens Pittier	**68**: Webster 23620 (MICH)
C. rimbachii Croizat	**69**: Riina 1402 (MICH); **70**: Riina 1422 (MICH); **71**: Riina 1440 (MICH)
C. rusbyi Britton ex Rusby	**72**: Riina 1479 (MICH); **73**: Riina 1481 (MICH)
C. sp1	**74**: Daza 4572 (E)
C. sp2	**75**: Riina 1426 (MICH)
C. sp3	**76**: Riina 1416 (MICH)
C. speciosus Müll. Arg.	**77**: Berry 7590 (MICH); **78**: Riina 1262 (MICH); **79**: Riina 1278 (MICH)
C. tyndaridum Croizat	**80**: Riina 1498 (MICH)
C. urucurana Baill.	**81**: Heringer 18473 (MG); **82**: Leitão Filho 1603 (MG); **83**: Pollito VA-001 (MG)
C. aff. urucurana Baill.	**84**: Cordeiro 3325 (SP); **85**: Soto 430 (USZ)
C. aff. urucurana/lechleri	**86**: Soto 414 (USZ)
C. vulnerarius Baill.	**87**: Cordeiro 345 (MG); **88**: Forero 8148 (MG); **89**: Saran 07 (MG); **90**: Silva 1242 (VIC)
Croton sect. Adenophylli Griseb.	
C. abutilifolius Croizat	**91**: Riina 1505 (MA)
C. aequatoris Croizat	**92**: Riina 1434 (MA)
C. bonplandianus Baill.	**93**: Riina 1517 (LPB)
C. conduplicatus Kunth	**94**: Riina 1266 (VEN); **95**: Riina 1302 (VEN); **96**: Riina 1296 (MICH); **97**: Riina 1837 (MA)
C. gracilipes Baill.	**98**: Riina 1501 (MA)
C. ruizianus Müll. Arg.	**99**: Riina 1386 (MICH); **100**: Riina 1486 (MICH); **101**: Riina 1487 (MICH)
Croton sect. Sampatik (G.L. Webster) Riina	
C. piptocalyx Müll. Arg.	**102**: Bortoluzzi 379 (VIC); **103**: Bortoluzzi 381 (VIC)
Croton sect. Cupreati Riina	
C. cupreatus Croizat	**104**: Riina 1408 (MA)

APPENDIX 2. Binary matrix with 104 specimens and 52 characters. Note: (0) absent (1) Present (?) unknown (-) inapplicable.

Specimen	Anatomical Characters																																																		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**
1	1	1	0	1	1	0	0	1	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1	0	1	0	0	0	1	1	0	1	1	1	0	1	0	1	0	1	0
2	1	1	0	1	1	0	0	1	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1	0	1	0	0	0	1	1	0	1	1	1	0	1	0	1	0	1	0
3	1	1	0	1	1	0	0	1	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1	0	1	0	0	0	1	1	0	1	1	1	0	1	0	1	0	1	0
4	1	1	0	1	1	0	0	1	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1	0	1	0	0	0	1	1	0	1	1	1	0	1	0	1	0	1	0
5	1	1	0	1	1	1	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	1	0	0	0	1	1	0	1	1	1	0	1	0	1	0	1	0
6	1	1	0	1	1	0	0	1	1	1	0	1	0	0	0	0	1	1	0	0	?	?	?	?	?	?	?	?	?	?	?	0	1	1	0	1	0	0	0	1	1	0	1	1	1	0	1	0	1	0	1	0
7	1	1	0	1	1	0	1	1	1	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	1	1	1	0	1	0	1	0	0	0
8	1	1	0	1	1	0	0	1	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	1	1	1	0	1	0	1	0	1	0
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19	1	1	0	1	1	0	0	0	1	1	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	1	1	0	1	0	0	0	1	1	0	1	1	1	0	1	0	1	0	0	1

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CONCLUSÕES GERAIS

A grande similaridade morfoanatômia e a equivalência na posição das estruturas secretoras de Croton echinocarpus e C. urucurana são resultados coerentes considerando a proximidade filogenética das espécies. Foi registrada pela primeira vez a presença de coléteres nas flores destas espécies, enfatizando que a observação desta característica pode ter sido negligenciada e/ou mal interpretada em estudos anteriores sobre anatomia floral de Croton. Assim, esta pode vir a ser uma característica unificadora entre este gênero e Astraea, gêneros proximamente relacionados. A diversidade de estruturas secretoras e os compostos químicos detectados confirmam o potencial dessas espécies para bioprospecção.

Apesar de apresentar composição histoquímica semelhante, nectários e coléteres puderam ser distinguidos com base em sua estrutura, função e período de atividade. Os nossos resultados mostraram que a partir da combinação destes três parâmetros é possível realizar uma avaliação e classificação mais precisa das estruturas secretoras.

A seção Cyclostigma apresenta características anatômicas que permitiram a delimitação de muitas espécies estudadas, principalmente pela presença de diferentes tipos de subtipos de tricomas estrelados, laticíferos não-articulados e pela ausência de nectários extraflorais nas margens foliares e tricomas fasciculados e lepidotos. Apesar de ainda não ter sido encontrado um padrão anatômico único para a seção, a combinação das características examinadas, em diferentes níveis de agrupamento analisados, foi importante para demonstrar as similaridades entre os espécimes amostrados e para confirmar e/ou determinar a identidade taxonômica específica de muitos táxons, além de agregar caracteres anatômicos na descrição de um novo táxon.